THE ISOLATION AND IMMUNOCHEMICAL CHARACTERISATION OF A CELL-WALL CARBOHYDRATE AND A MEMBRANE LIPOCARBOHYDRATE ANTIGEN OF GROUP B STREPTOCOCCUS, TYPE II

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SUMMARY. Two distinct carbohydrate antigens were isolated from the cell surface of group B streptococcus, type II. One antigen was extracted from SDS-purified cell walls by cold trichloroacetic acid and contained galactose, glucose, rhamnose, glucosamine and sialic acid in the approximate molar proportions 1·7:1·0:3·4:0·9:0·21 respectively. The serological activity of this polymer indicated that it is the group-specific antigen common to all group B streptococci. The second antigen was extracted by phenol from cell membranes and contained galactose, glucose, glucosamine, phosphorus and fatty acid in a molar ratio of 1·6:1·0:0·35:2·6:0·016 respectively. This antigen was shown to be specific for type II, group B streptococcus.

INTRODUCTION

Lancefield (1933) demonstrated that beta-haemolytic streptococci could be differentiated into several serological groups, labelled A–E, by the presence of a group-specific carbohydrate antigen or C-substance. Further studies by Lancefield (1934, 1938) divided group B streptococci (GBS) into four serological types by the use of acid extracts of type-specific surface or capsular polysaccharide antigens. These types were designated Ia, Ib, II and III, and later type Ic was identified by the detection of the type Ibc protein antigen on the surface of strains possessing a Ia polysaccharide antigen (Wilkinson and Eagon, 1971).

Early reports on the immunochemical character of GBS antigens indicated that the group-specific antigen is composed mainly of rhamnose (Slade and Slamp, 1962; Wittner and Hayashi, 1965). In addition, rhamnose was shown to be the principal immunodeterminant in the antigen, suggesting that it occupied a terminal position in the polysaccharide (Curtis and Krause, 1964). Recently, the chemical profile of the group-specific antigen from GBS Ib was determined (Cumming, Ross and Poxton, 1981).

In laboratory animals, GBS have been shown to elicit the production of multiple populations of protective antibodies, particularly against the surface type-specific antigens (Lancefield, McCarty and Everly, 1975). When extracted with hot HCl, these

antigens are partially degraded and immunologically incomplete (Baker, Kasper and David, 1976). Extraction of GBS type II polysaccharide antigen with cold trichloroacetic acid (TCA) gave a polymer containing two serological determinants, whereas a partial antigen with only one of the determinants was obtained by extraction with boiling HCl (Freimer, 1967). D-galactose was identified as the determinant of the HCl-extracted polysaccharide. The second determinant, present only in the TCA extract was not identified. Recent studies have identified sialic acid as the important second immunodeterminant (Kane and Karakawa, 1978; Tai, Gotschlich and Lancefield, 1979; Kasper *et al.*, 1979).

The numerous reports on the immunochemical nature of the cell surfaces of GBS have indicated a complexity of antigenic structure only partially understood. The present study investigated two distinct classes of antigen extracted from GBS type II. The purified cell-wall carbohydrate antigen was examined serologically and chemically, and the first investigation of the cell-membrane polymer (teichoic acid or analogue) was undertaken.

MATERIALS AND METHODS

Strains of group B streptococci. The following strains, representing the various serotypes, were obtained from the Streptococcus Reference Unit, Division of Hospital Infection, Central Public Health Laboratory, Colindale, London: 090R (type Ia), H36B (Ib), NCTC11078 (Ic), NCTC11079 (II), NCTC11080 (III).

Antisera. Specific antisera for GBS types Ia, Ib, II and III were raised in New Zealand white rabbits by intravenous injection of formalin-killed, trypsin-treated whole cells. Heat-killed (56°C for 30 min) whole cells of strain NCTC11078 were prepared to obtain antisera for type Ic GBS. The immunisation schedule has been described previously (Cumming *et al.*, 1980). Group B antiserum was purchased from Wellcome Reagents, Beckenham, Kent. Antisera were not absorbed and their specificity was checked against homologous and heterologous Lancefield extracts of GBS by double diffusion in agar gel.

Preparation of type II cell-wall extracts. Saline-washed bacteria from overnight cultures (10L) in Todd-Hewitt broth were suspended in ice-cold water and disrupted by passing through a French press (Aminco Inc., Silver Spring, MD., USA) at 48MPa (7000 lbf in⁻²). The suspension from the French press was centrifuged at 50 000 g for 15 min at 4°C. The supernate containing membranes was removed, freeze-dried and retained for subsequent extraction of antigen, while the upper layer of cell walls was carefully separated from the small pellet of unbroken cells. The suspension of cell walls in water was heated at 80°C for 3 min to destroy autolytic enzymes, then washed in water at 4°C. Non-covalently-linked protein and membranes were removed from the suspension by adding an equal volume of 5% (w/v) sodium dodecyl sulphate (SDS) and stirring for 4 h at room temperature. To remove SDS, the cell walls were washed by centrifugation at 50 000 g for 10 min in six successive changes of water at 20°C. Carbohydrate was extracted from the walls with 10% (w/v) trichloroacetic acid, as described previously by Cumming *et al.* (1981). Purification of carbohydrate was performed by ion-exchange chromatography. Samples in 2 ml of water were applied to a column (30 cm × 1.5 cm) of DEAE cellulose previously treated with 1M-pyridinium acetate (pH 5.3) then equilibrated with water and eluted with 50 ml of water and 50 ml of 1M pyridinium acetate (pH 5.3) to give a linear gradient from 0–1M concentration. Fifty fractions of 2 ml were collected and samples were analysed for carbohydrate.

Preparation of type II cell-membrane extracts. After French pressing, unbroken bacteria and cell walls were removed by centrifugation and the supernatant fluid containing cell membranes was freeze dried and weighed. Membranes (1 g) were de-fatted by stirring for 18 h at room temperature in 200 ml of chloroform-methanol (2:1 v/v). The membranes were filtered, rinsed with 200 ml chloroform-methanol mixture, and dried in air. Lipocarbohydrates were extracted from the membranes with cold 80% aqueous phenol as described by Coley, Duckworth

and Baddiley (1975). Nucleic acids were removed by incubation of the extract with RNase A and DNase I (Sigma Chemical Company, London) at concentrations of 10 µg/ml in 0.2 M sodium acetate-acetic acid buffer (pH 5.0) containing 10^{-3} M $MgCl_2$, under toluene for 48 h at 37°C. To ensure complete removal of contaminants, the aqueous phenol extraction procedure was repeated. Further purification of the extract was performed on a Sepharose 6B (Pharmacia Fine Chemicals, Sweden) column (60 cm × 1.6 cm). Fractions (3 ml) were eluted by an upward flow of 0.2 M ammonium acetate buffer, pH 6.9, containing sodium azide 0.01%. Fractions were analysed for carbohydrate and assessed for antigen content with GBS antisera by fused rocket immunoelectrophoresis.

Analytical techniques. Carbohydrate was estimated by the method of Dubois *et al.* (1956), phosphate analysis was by the method of Chen, Toribara and Warner (1956), and sialic acid was determined by the thiobarbituric acid method of Aminoff (1961).

Immunoelectrophoresis techniques. Electrophoresis was carried out with a Shandon Southern apparatus (Camberley, Surrey, UK). Fused rocket immunoelectrophoresis (Svendsen, 1973) against GBS type II antiserum in the gel was used to assess the antigen content of cell-wall and membrane fractions from the chromatography columns. Pooled antigen-containing fractions were subjected to crossed immunoelectrophoresis (CIE) techniques, described previously by Cumming *et al.* (1981). Simple immunodiffusion experiments in agar gel were performed by the method of Ouchterlony (1948).

Acid hydrolysis. Purified cell-wall and membrane samples were hydrolysed in 2 M HCl for 4 h at 100°C. Acid was removed by desiccation over NaOH and P_2O_5 in a vacuum and the samples were dissolved in 0.5 ml water.

Paper chromatography. Descending paper chromatography was performed on Whatman no. 1 paper with a butan-1-ol/pyridine/water (6:4:3, by volume) solvent. Aminosugars were detected by ninhydrin and reducing sugars by the alkaline silver nitrate reagents (Trevelyan, Procter and Harrison, 1950).

Preparation of methyl esters of fatty acids. Methyl esters of the fatty acids of the lipid portion of the cell-membrane extract were prepared by heating in 0.5 M HCl in methanol at 65°C for 2 h. The methyl esters were partitioned into ether and the constituents were analysed by gas-liquid chromatography (GLC) on columns of SP2330 3% on Chromosorb W.

Preparation of alditol acetates. Hydrolysed carbohydrates were converted to alditol acetates using methods described by Cumming *et al.* (1981).

RESULTS

Cell-wall and carbohydrate preparations

The yield of SDS-treated GBS type II cell walls from 10L of culture was 950 mg. Total carbohydrate extracted with trichloroacetic acid was approximately 10 mg.

Purification and analyses of cell-wall extract

Fractions eluted from the DEAE-cellulose column were collected and assayed for carbohydrate. One major peak (W1) was eluted between 0.2M and 0.26M pyridinium acetate buffer (fig. 1). Two additional peaks (W2 and W3) were detected; they were eluted between 0.38M–0.58M and 0.62M–0.82M pyridinium acetate buffer, respectively.

Chemical analyses were performed on material from peaks W1 and W2 only. Examination of acid hydrolysates of W1 by descending paper chromatography showed the presence of galactose, glucose, glucosamine and rhamnose. These constituents were confirmed by GLC of alditol acetate derivatives from which molar proportions were calculated. In addition, significant amounts of sialic acid and trace

FIG. 1.—Purification of a TCA extract of SDS-treated cell walls of GBS, type II by ion exchange chromatography on a DEAE-cellulose column. Fractions (2 ml) were eluted with a linear gradient (0 to 1 M) of pyridinium acetate, pH 5.3. Carbohydrate was assayed by the method of Dubois *et al.* (1956).

amounts of phosphorus were identified in the fraction. The complete chemical composition of the polymer W1, expressed as molar proportions, is shown in table 1.

Analyses of W2 showed the presence of ribose and trace amounts of those constituents found in extract W1, except sialic acid which was not detected.

CIE of cell-wall fractions

The antigenic nature of the cell-wall fractions was assessed with 'type' and 'group-specific' GBS antisera by CIE. Material from peak W1 produced a single precipitin line in gel with type II and commercial GBS grouping antisera. No reaction occurred with type Ia, Ib, Ic or III 'type-specific' antisera. Similar results were obtained with peak W2 and the GBS antisera. There was however, a slight difference

TABLE I

Chemical composition of material from peak W1 from DEAE chromatography of a TCA extract of SDS-treated cell walls of GBS, type II

Component	Molar proportions
Galactose	1.7
Glucose	1.0
Rhamnose	3.4
Glucosamine	0.9
Sialic acid	0.21
Phosphorus	trace

FIG. 2.—Purification of a phenol extract of the cell membranes of GBS, type II on a Sepharose 6B column. Fractions (3 ml) were collected and assayed for nucleic acid at 260 nm (●) and carbohydrate (○).

in electrophoretic mobility in gel between the two fractions when they were run in tandem gels against commercial grouping antiserum.

No reaction was detected between material from peak W3 and the GBS antisera.

Cell-membrane preparation

The yield of freeze-dried cell membranes from an overnight culture of GBS type II in 10L of Todd-Hewitt broth was 1.26 g. Following phenol-extraction procedures, 47.5 mg of extract was collected.

Analyses of cell-membrane extract

Material extracted by phenol from type II cell membranes was applied to a Sepharose 6B column and the fractions assayed for carbohydrate, phosphorus and nucleic acid. The elution profile is shown in fig. 2. Three distinct carbohydrate peaks

TABLE II

Carbohydrate composition of material from peak M1 from Sepharose 6B chromatography of a cold aqueous phenol extract of cell membranes of GBS type II

Component	Molar proportion
Galactose	1.6
Glucose	1.0
Glucosamine	0.35
Phosphorus	2.6

TABLE III

Lipid composition of material from peak M1 from Sepharose 6B chromatography of a cold aqueous phenol extract of cell membranes of GBS type II

Component	Percentage of total lipid
Palmitic acid (C:16)	74
Stearic acid (C:18)	17.5
Oleic acid (C:18.1)	8.5

(M1, M2 and M3) were detected. Analysis of peak M1 for carbohydrate and lipid was performed (tables 2 and 3). Sialic acid was not detected. Chemical analyses of material from peaks M2 and M3 were not performed.

CIE of cell-membrane fractions

Material from peak M1 reacted only with antiserum against type II GBS to produce one characteristic precipitin line in agar gel, but material from M2 reacted with type II antiserum and with commercial grouping antiserum forming single precipitin lines. Simple immunodiffusion experiments indicated that with both type II and grouping antisera, material from peak M2 formed precipitin lines of complete identity with material from cell-wall peak W1. Lines of identity were not detected with either of these extracts or material from peak M1.

DISCUSSION

The results described in this study indicate that two distinct antigenic carbohydrate polymers can be isolated from cell walls and membranes of GBS, type II. The chemical composition of the main antigenic peak (W1) from the TCA extract of SDS treated cell walls is qualitatively similar to the group-specific antigen isolated from GBS type Ib (Cumming *et al.*, 1981). The second antigenic cell-wall polymer (W2) differed chemically from the material in peak W1 only in its lack of sialic acid. The absence of sialic acid in this polymer almost certainly accounted for the difference in electrophoretic mobility between the two antigens. In simple immunodiffusion experiments, however, the two antigens produced lines of partial identity when reacted with commercial group-specific antiserum. This pattern of serological activity is similar to that of the group-specific extracts from GBS, type Ia (Cumming, Ross and Poxton, 1982).

The fact that type-specific antigens were removed from cell walls by SDS treatment indicates that these antigens are not covalently bound to peptidoglycan. This opposes the view of Tai *et al.* (1979) who suggested that the capsular type-specific antigens are covalently bound to the cell wall.

Two distinct antigenic polymers were detected in material extracted from type II membranes by cold phenol. Serological reaction of material from peak M1 showed its specificity for GBS type II only, whereas peak M2 produced the same serological profile as the group-specific cell-wall polymer (W1). Immunodiffusion experiments

with material from the two peaks (W1 and M2) confirmed their serological similarity. This agrees with the observation of Coley *et al.* (1975) that wall teichoic acids of certain gram-positive bacteria are often found in the second peak eluted from a Sepharose 6B column.

Chemical analysis of the type-specific antigen (M1) showed it to be a phosphorus containing lipocarbohydrate. This polymer should not be classified as a teichoic acid, however, since it does not contain ribitol or glycerol phosphate. The composition of the carbohydrate portion of the molecule is similar to the type-specific antigen of GBS type II described by Freimer (1967), except for the absence of sialic acid in the membrane polymer. This result implies that the GBS type-specific antigens, commonly referred to as capsular, surface, or cell wall associated antigens, are indeed membrane bound structures.

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