Immunochemical Studies on the Cell-wall Antigen of Group B Streptococcus, Type Ib

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A polysaccharide antigen of group B streptococcus type Ib was extracted from sodium dodecyl sulphate-treated cell walls by trichloroacetic acid. In crossed immunoelectrophoresis the polymer reacted with specific antisera to serotypes Ia, Ib and Ic of group B streptococci and with commercial grouping antiserum to give one precipitin line. However, the antigen did not react with antisera to types II and III. The evidence suggests that this polymer confers the group specificity to type Ib of group B streptococci. The polysaccharide, after purification on DEAE-cellulose, was shown to consist of rhamnose, glucosamine, galactose, glucose, sialic acid and a trace of phosphorus.

INTRODUCTION

The recognition that group B streptococci (GBS) play an important role in the pathogenesis of neonatal infections has generated much interest in these organisms. Their definitive identification is based on serological reaction of the group-specific carbohydrate antigen which is extracted from the cell wall by hot hydrochloric acid (Lancefield, 1933). Early reports indicated that the components of this antigen are polysaccharides of low molecular size and consist mainly of rhamnose (Slade & Slamp, 1962; Curtis & Krause, 1964a; Wittner & Hayashi, 1965). Immunochemical studies by sugar inhibition tests of the precipitin reaction between the GBS antigen and specific antisera showed that rhamnose is the principal immunodeterminant portion of the antigen; this suggests that it occupies a terminal position in the polysaccharide (Curtis & Krause, 1964b).

More recent studies have concentrated on the immunochemical characterization of the type-specific, or capsular antigens of GBS. since these surface components are theoretically most likely to be recognized immunologically by the infected host (Freimer, 1967; Wilkinson, 1975; Kasper et al., 1979). Capsular antigens extracted by the HCl technique (Lancefield, 1934) have been shown to be partially degraded and immunologically incomplete (Baker et al., 1976). Isolation of the complete native antigen by more subtle extraction procedures allows an acid-labile terminal sialic acid determinant to be identified. Tai et al. (1979) were able to isolate from whole cells the Ib type-specific antigen which contained galactose, glucose, N-acetylg glucosamine and sialic acid in the approximate molar ratio 2 : 1 : 1 : 1. The fact that this antigen cross-reacted with both type Ia and type Ic antisera is attributed to the common determinant Iabc (Lancefield et al., 1975). Absorption studies suggest that the Ib specific determinant and the Iabc determinant are on the same molecule but that sialic acid is not the cross-reactive determinant (Tai et al., 1979).

Various findings thus have shown that the character of GBS antigens is complex. The cell surfaces contain a range of poorly characterized carbohydrate and protein antigens which
METHODS

Strains of group B streptococci. The following strains, representative of the various serotypes, were obtained from the Streptococcal Reference Laboratory, Colindale, London: 0903 (type Ia), H36B (Ib), NCTC 11078 (Ic), NCTC 11079 (II), NCTC 11080 (III).

Antisera. Specific antisera for types Ia, Ib, II and III were raised in New Zealand White rabbits by intravenous injection of formalin-killed, trypsin-treated whole cell preparations. Heat-killed whole cells of strain NCTC 11078 were prepared to obtain antisera specific for type Ic bacteria. The immunization schedule has been described previously (Cumming et al., 1980). Group B antiserum was purchased from Wellcome Reagents. Antisera were not absorbed and their specificity was checked by capillary precipitin tests (Swift et al., 1943) and double diffusion in agarose gel against Lancefield extracts.

Preparation of cell walls and extraction of carbohydrate. Saline-washed bacteria from overnight cultures (6 l) in Todd-Hewitt broth were suspended in ice-cold water and disrupted by passing through a French press (Aminco Inc., Md., U.S.A.) at 48 MPa (7000 lb in -2). Cell walls and unbroken bacteria were collected by centrifugation at 52000 g for 15 min at 4 °C and then carefully separated. The suspension of cell walls was heated at 80 °C for 3 min to destroy autolytic enzymes, then washed in water at 4 °C. Protein and membrane components 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 (Poxton et al., 1978). To remove SDS, the cell walls were washed by centrifugation at 50000 g for 10 min in six successive changes of water at 20 °C. Carbohydrate was extracted from 75 mg freeze-dried walls with 10 ml 10% (w/v) trichloroacetic acid (TCA) for 48 h at 4 °C. Walls were collected by centrifugation at 50000 g for 10 min and TCA was removed from the supernatant fluid by six successive extractions with 10 ml volumes of diethyl ether. Water was removed by rotary evaporation and the extracted carbohydrate was dissolved in 1 ml water.

Purification of carbohydrates was performed by ion-exchange chromatography. Samples of carbohydrate in 5 ml water were applied to a column of DEAE-cellulose previously treated with 1 M-pyridinium acetate (pH 5-3) and eluted by a linear gradient of 100 ml pyridinium acetate (pH 5-3) from 0 to 1 M concentration. Fifty fractions were collected and samples were analysed for carbohydrate.

Lancefield extracts from whole cells. The group antigen extraction was performed by a modified method of Lancefield and Freimer, described previously by Cumming et al. (1980).

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

Crossed immunoelectrophoresis (CIE) techniques. Electrophoresis was carried out with a Shandon Southern apparatus (Camberley, Surrey, U.K.). The technique used was similar to that described by Weeke (1973b). Antigen samples (10 μl, containing 1 mg carbohydrate ml -1) were applied to gels of 1% (w/v) agarose (BDH electrophoresis grade). A barbital/glycine/Tris buffer, pH 8.8, described by Weeke (1973a) was used throughout. Electrophoresis was at 12-5 V cm -1 for 2 h in the first dimension and at 12 V cm -1 for 16 h in the second dimension, both at 4 °C. After washing, pressing and drying of the gels, precipitin lines were detected by Coomassie blue stain (Weeke, 1973a).

Acid hydrolysis. Samples eluted from the DEAE-cellulose column were hydrolysed in either 2 M-HCl for 4 h at 100 °C or 4 M-HCl for 18 h at 100 °C. Acid was removed by desiccation over NaOH and P 2 O 5 in vacuo and the samples were dissolved in 0.5 ml water.

Paper chromatography. Descending paper chromatography was performed on Whatman no. 1 paper with a pyridine/acetate acid/water (6:4:3, by vol) solvent. Aminosugars were detected by ninhydrin and reducing sugars by the alkaline silver nitrate reagents (Trevelyan et al., 1950).

Preparation of alditol acetates and gas-liquid chromatography (g.l.c.). Hydrolysed samples were converted to alditol acetates as follows. Hydrolysates (1 ml) were reduced by 10 mg potassium borohydride for 1 h at 20 °C. Excess borohydride was destroyed by addition of glacial acetic acid. Methyl borate was removed from the samples by three successive distillations with methanol. The residue of each sample was then dissolved in 0.5 ml pyridine plus 0.5 ml acetic anhydride and heated for 1 h at 100 °C. Pyridine and excess acetic anhydride were removed by rotary evaporation with successive volumes of toluene until the acetate residues were dry. To remove impurities in the residues, 2 ml chloroform was added followed by vigorous mixing with water using a vortex mixer. The chloroform phase was subjected to rotary evaporation and the resulting samples were redissolved in 0.5 ml chloroform and analysed by g.l.c.
Cell-wall antigen of group B streptococcus

Alditol acetates were identified in a Pye Unicam series 104 chromatograph. A column (2.1 m x 4 mm) containing 3% OV-225 on 100-120 mesh Gas Chrom Q was used for identification of amino sugars; hexoses were identified on a column (1.5 m x 2 mm) of 3% SP-2330 on 100-120 mesh Supelcoport. Gas flow rates for the OV-225 column were 35 ml N₂ min⁻¹, 35 ml H₂ min⁻¹ and 525 ml air min⁻¹. Flow rates for SP-2330 were 20 ml N₂ min⁻¹, 20 ml H₂ min⁻¹ and 525 ml air min⁻¹. The temperature of both columns was 180 °C rising by 2 °C min⁻¹ to 240 °C.

RESULTS

Cell wall and carbohydrate preparations

The yield of SDS-purified cell walls obtained from a 6 l culture volume of type Ib cells was 600 mg. Total carbohydrate extracted from the cell walls was approximately 6 mg. The amount of reducing sugar in the sample was estimated as 3-0 mg by the phenol/sulphuric acid assay.

CIE of TCA-prepared wall antigens

Preliminary studies indicated that the optimum antigen concentration for electrophoresis was 10 μl carbohydrate adjusted to 1 mg ml⁻¹ estimated by the phenol/sulphuric acid assay (Dubois et al., 1956). The optimum antiserum concentration to obtain clear precipitin lines in the second dimension of CIE was 0.5 ml antiserum in 3 ml agarose.

Electrophoresis of the Ib antigen extracted from cell walls was performed with sera raised against the five main serotypes of GBS and with commercial grouping antiserum. Single precipitin lines were observed with Ia, Ib (Fig. 1) and Ic antisera and also with the Wellcome grouping antiserum. No reaction was observed with type II or III typing antisera.

To check the specificity of the antisera, 'Lancefield' group antigens were prepared from whole cells of the five serotypes of GBS and examined by the Ouchterlony technique. These group antigen extracts resulted in one line of identity being produced between antigens from all serotypes and the Wellcome grouping antiserum. However, when they were reacted against the typing sera, Ia, Ib and Ic extracts reacted with each other's typing antiserum but not with antisera against types II and III, while group extracts from II and III reacted only with their homologous typing antiserum. This showed that the typing antisera were devoid of group antibodies.

Sialic acid determinations

Purified Ib cell walls were treated with 0-02 M-HCl at 80 °C for periods up to 24 h to extract sialic acid. From a sample (10 mg) of freeze-dried walls 120 μg sialic acid was liberated after 4 h, whereas after 24 h only 81 μg sialic acid was detected. After TCA treatment of walls, the sialic acid content of the extract was 63 μg mg⁻¹; of this, 30-5 μg was in the bound form. Control samples of sialic acid subjected to the same conditions as the test samples showed that, after 4 h heating in HCl, there was a 30% loss of sialic acid, and after 24 h, a 70% loss. The results obtained from cell walls and TCA extract were corrected accordingly for destruction of sialic acid.

Purification and analysis of TCA extract

Extracted carbohydrate preparations from cell walls were subjected to DEAE-cellulose chromatography and eluted with increasing molarities of pyridinium acetate (pH 5-3). Analysis of each sample from the column detected one carbohydrate fraction only, eluting between 0-05 M- and 0-2 M-pyridinium acetate. Crossed immunoelectrophoresis of this fraction showed it to be immunologically similar to the unpurified extract.

Paper chromatography of the purified sample hydrolysed in 2 M-HCl for 4 h at 100 °C resulted in the detection of glucosamine and rhamnose; after hydrolysis in 4 M-HCl for 18 h at 100 °C only glucosamine was detected. Confirmation of these results was obtained by
Fig. 1. Crossed immunoelectrophoresis of TCA-extracted GBS type Ib cell-wall antigen (10 μl) against antiserum to GBS type Ib (0.3 ml). This shows a single antigen which is heterogeneous for molecular weight.

Table 1. Chemical composition of cell wall extract of type Ib GBS, strain H36B

<table>
<thead>
<tr>
<th>Component</th>
<th>Molar proportion</th>
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<tbody>
<tr>
<td>Galactose</td>
<td>0.6</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>1.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.2</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>1.9</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>0.03</td>
</tr>
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</table>

analysis of the derivatized hydrolysate by g.l.c. In addition to glucosamine and rhamnose, small amounts of galactose and glucose were identified.

Analysis for phosphate in the sample detected trace amounts only. The complete chemical profile of this antigen, expressed as molar proportions, is summarized in Table 1.

DISCUSSION

In this study an attempt has been made to examine the nature of an antigenic carbohydrate polymer which is covalently bound to the peptidoglycan component of the cell wall of group B streptococci (GBS) of type Ib. Unlike previous reports in which antigens were extracted from untreated whole cells, in this study cell walls were collected and purified by SDS treatment, thereby removing all non-covalently bound polymers. Only one carbohydrate fraction was extracted from the walls after prolonged treatment with TCA; this polymer was shown to have a net negative charge by its binding capacity to DEAE-cellulose. Chemical analysis of the extract resulted in the identification of rhamnose, glucosamine, galactose, glucose, sialic acid and a trace of phosphorus. The relative proportions of these compounds are essentially similar to the composition of the group-specific antigen isolated from whole cells of GBS type Ia by Kane & Karakawa (1978). The presence of trace amounts of phosphorus and the TCA lability of the bond between the isolated carbohydrate and peptidoglycan suggest that a linkage unit containing phosphodiester bonds might be present. This would be analogous to the peptidoglycan-teichoic acid linkage in other Gram-positive bacteria (Coley et al., 1978).
Specific antigen–antibody reactions were demonstrated by CIE. Single precipitin lines were detected when the Ib antigen was reacted with antisera raised against serotypes Ia, Ib, Ic and with the commercial grouping antisera. No reaction between the extracted antigen and typing antisera for types II and III could be detected.

These findings indicate that type I strains of GBS possess a carbohydrate antigen which is covalently linked to the cell wall peptidoglycan in type Ib streptococci. This antigen does not confer sub-type specificity (Ia, Ib, Ic), and antibodies to this antigen do not appear to be provoked by whole cell antigen preparations of types II or III. It is surprising that the group-specific antigen of type I strains (i.e. the carbohydrate cell wall antigen) does not react with typing antisera to types II or III, although grouping antigens of types II and III react with commercially available group B serum. The type-specific antigens present in the ‘Lancefield’ extract are not covalently bound to the cell wall, as they can be removed by the SDS procedure. These are probably capsular or membrane-bound in their location within the B complex.

During preparation of this manuscript, Wagner et al. (1980) demonstrated by immunoelectron microscopy that the group-specific polysaccharide antigen of GBS traverses the whole of the cell wall. This is in complete agreement with the findings in this study, which indicate that the group-specific polysaccharide is the secondary wall polymer covalently bound to peptidoglycan.

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REFERENCES


