Immunochemistry of the Cell-Surface Carbohydrate Antigens of
Clostridium difficile

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Two carbohydrate cell-surface antigens were extracted from Clostridium difficile. One was extracted from pure cell walls by NaOH and contained glucose, mannose, galactosamine and phosphate in the approximate molar proportions of 2:0.65:1:0.63. The other antigen was extracted with phenol from the disrupted contents of whole cells and purified by chromatography on Sepharose 6B and an immunoabsorbent column; it contained glucose, glucosamine, phosphate and fatty acid in the approximate molar proportions of 2:1:1:6:0.04. Both antigens showed partial immunological identity and both cross-reacted with Clostridium sordellii antiserum. They are analogues of the wall and membrane teichoic acids of other Gram-positive bacteria.

INTRODUCTION

Clostridium difficile is a Gram-positive, motile, spore-forming, rod-shaped bacterium which, until recently, was regarded as an uncommon, non-pathogenic organism. In 1977 it was shown to be involved in the aetiology of antibiotic-associated colitis; it may also be involved in a significant number of antibiotic-associated diarrhoeas (Larson, 1979; Bartlett, 1979). A recent analysis of the EDTA-soluble antigens of this and related species by Poxton & Byrne (1981) showed that a carbohydrate antigen is shared by C. difficile and C. sordellii. The present study investigates the cell-surface carbohydrate antigens of C. difficile with a view to showing the location and nature of the common C. sordellii/C. difficile antigen, and also reports the first investigation of the secondary cell wall polymer (teichoic acid or analogue) and of the membrane lipocarbohydrate polymer (lipoteichoic acid or analogue) of a Clostridium species.

METHODS

Culture of organism and preparation of cell walls. Clostridium difficile NCTC 11223 was cultivated in 16-litre batches in an L.H. fermenter (L.H. Engineering, Stoke Poges, Berks.). Oxygen-free nitrogen was bubbled at the rate of 300 ml min⁻¹ through PPY medium (Holbrook et al., 1977) which contained 0.4% (w/v) sodium carbonate and 0.75% (w/v) cysteine hydrochloride, for 16 h at 37 °C. The medium was inoculated with 100 ml of a 16 h culture of C. difficile in PPY medium and incubated for 18 h at 37 °C with nitrogen bubbling. Sporulation was negligible under these conditions. Bacteria were harvested by centrifugation at 2000 g for 40 min and washed once in phosphate-buffered saline (PBS: 50 mm-phosphate buffer pH 7.5 containing 0.15 M-NaCl). The wet pellet was suspended to a concentration of approximately 30% (w/v) in PBS and the bacteria were disrupted by passage through a French Pressure Cell (Aminco, American Instrument Co. Inc., Silver Springs, Md., U.S.A.) at 6000–7000 lbf in⁻¹ (41–48 MPa). The suspension was centrifuged at 45000 g for 20 min at 4 °C. The supernate was removed, freeze-dried and retained for subsequent extraction of membrane antigen. The upper white layer of cell walls was carefully removed from the small pellet of unbroken cells, suspended in a small volume of water and heated to 80 °C for 5 min to inactivate the autolytic enzymes; it was then purified by treatment with 2.5% (w/v) sodium dodecyl sulphate (SDS) as described by Poxton et al. (1978).
Extraction of cell-wall antigen. Freeze-dried SDS-treated cell walls were extracted with 0·5 m-NaOH for 4 h at
20 °C by the method of Archibald et al. (1969). The NaOH was neutralized with dilute HCl and salts were
removed by extensive dialysis against distilled water. The extract was freeze-dried.

Extraction of membrane antigen. The freeze-dried supernate (8 g) obtained after a cell breakage was defatted
with 2 × 200 ml chloroform/methanol (2:1, v/v), over 24 h, extracted with cold 80% (w/w) phenol and partially
purified on a Sepharose 6B (Pharmacia) column (65 × 1·6 cm) by the method of Coley et al. (1975).

Preparation of antiserum. Antiserum against u.v.-killed, whole, washed cells of C. difficile NCTC 11223 and C.
sordellii NCTC 8780 were prepared as described previously (Poxton & Byrne, 1981).

Immunoelectrophoresis. Crossed immunoelectrophoresis (CIE) was performed by the procedure of Weeke
(1973) as described for C. difficile by Poxton & Byrne (1981). Fused rocket immuno-electrophoresis (FRIE) was
done by the method of Svensden (1973), with the same buffer system as used for CIE.

Preparation of immunoabsorbent column. Immunoglobulin G (IgG) was prepared from 3 ml antiserum to C.
difficile NCTC 11223 by batch treatment with DEAE-cellulose (Whatman DE 52) by the method of Hudson &
Hay (1976). IgG (50 mg) was coupled to 10·5 ml swollen CNBr-activated Sepharose 4B (Pharmacia) by the
method recommended by Pharmacia in Affinity Chromatography, Principles and Methods. The immuno-
absorbent was packed in a column (10 × 1 cm) and equilibrated for storage in 0·1 m-phosphate buffer pH 7·0
containing 0·5% (v/v) Tween 80 and 0·01% (w/v) Merthiolate (thimerosal).

Analytical techniques. Phosphorus was estimated by the method of Chen et al. (1956); sugars, as glucose
equivalents, were estimated by the method of Dubois et al. (1956); sialic acid was estimated by the method of

Paper chromatography. Acid hydrolysates of carbohydrates (2 m-HCl, 100 °C, 2 h) were dried several times in
vacuo over NaOH pellets and phosphorus pentoxide. They were chromatographed on Whatman no. 1 paper in
solvent A [butan-1-ol/pyridine/water (6:4:3, by vol.)] for reducing sugars and stained with the alkaline silver
nitrate reagents (Trevelyan et al., 1950) and in solvent B [propan-1-ol/aqueous ammonia (S.G. 0·88)/water
(6:3:1, by vol.)] for polyols and stained with the periodate–Schiff’s reagents (Baddiley et al., 1956).

Gas chromatography. (a) Sugars. Dry hydrolysates of carbohydrates were converted to alditol acetates by a
method modified from that of Lindberg et al. (1978). The hydrolysate (1–20 mg) was dissolved in 1 ml distilled
water, and 10 mg sodium borohydride was added. After 1 h at 20 °C, excess borohydride was destroyed by the
addition of a few drops of glacial acetic acid. After drying by rotary evaporation, borate, as methyl borate, was
removed by three distillations with methanol. The dry alditols were acetylated in 1 ml pyridine/acetric anhydride
(1:1, v/v) at 100 °C for 1 h. Excess reagents were removed by several co-distillations with toluene in a rotary
evaporator, and the alditol acetates were taken up in 1 ml chloroform, washed twice with water, dried by rotary
evaporation and finally dissolved in 0·5 ml chloroform. Samples (1 μl) were examined on columns of 3% OV225
on Gas-chrom Q (for amino sugar derivatives) and 3% SP2330 on Supelcoport (for pentose and hexose
derivatives) in a Pye-unicam model 104 gas chromatograph, with the temperature controlled between 190 °C and
240 °C by 2 °C min−1.

(b) Fatty acids. Membrane antigen which had been eluted from the immunoabsorbent column was dried and
methanolysed by heating with 0·5 m-HCl in methanol (1 ml) in a sealed vessel for 2 h at 65 °C. Water (2 ml) and
ether (4 ml) were added; after mixing, the phases were separated. The ether phase was brought to dryness in a
rotary evaporator, redissolved in ether (0·5 ml) and examined for methyl esters of fatty acids on a column of 3% SP2330 on Supelcoport at 150 °C.

RESULTS
Cell wall antigen

From 50 mg lyophilized cell walls, 20 mg was solubilized by 0·5 m-NaOH. When this wall antigen extract was examined by crossed-immunoelectrophoresis (CIE) against antiserum to whole cells of C. difficile NCTC 11223, one precipitin line was observed (Fig. 1a). This line did not correspond to any of the lines produced by the EDTA extract of whole cells (see Poxton & Byrne, 1981) but it did correspond to one produced by an autolysate of whole cells of C. difficile (Fig. 1b). When this wall antigen was run against antiserum to C. sordellii NCTC 8780 in CIE, an identical precipitin line was produced.

Chemical analysis of the wall antigen showed it to contain sugar (phenol/sulphuric acid assay method) and phosphorus in the molar ratio of 4·2:1. Analysis of the constituent sugars by paper chromatography of acid hydrolysates showed the presence of glucose, mannose and galactosamine. No ribitol or glycerol was detected. Gas chromatography of alditol acetate derivatives showed the presence of the same three sugars in the molar ratio
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Fig. 1. Crossed immunoelectrophoresis of (a) NaOH-soluble cell-wall antigen (6 µl of 5 mg ml⁻¹ solution) and (b) soluble fraction of autolysed whole cells of C. difficile (6 µl of supernate from cells allowed to autolyse in PBS for 24 h at 37 °C) run against 65 µl C. difficile NCTC 11223 antiserum. The precipitin line X is common to both preparations.

glucose : mannose : galactosamine of 2 : 0.65 : 1. As galactosamine does not react in the phenol/sulphuric acid assay this suggests a ratio of total sugar : phosphorus of 6 : 1 or glucose : mannose : galactosamine : phosphorus of 2 : 0.65 : 1 : 0.63. Sialic acid was absent. The polymer (1 mg) was treated with 4 units of phosphomonoesterase (Boehringer, Grade I) for 3 h at 37 °C in 0.5 ml 0.05 M-ammonium carbonate pH 9.5 under toluene. All the phosphate was shown to have been present in the monoester form. The dephosphorylated product and the untreated control gave an identical precipitin line when run in CIE against C. difficile antiserum.

Membrane antigen

The crude phenol extract of the lyophilized supernate from the broken cells was run in CIE against C. difficile whole-cell antiserum. Two precipitin lines were present and they appeared to be joined (Fig. 2). After fractionation on a Sepharose 6B column, fractions were analysed for carbohydrate, phosphate and nucleic acid and by fused-rocket immunoelectrophoresis (Fig. 3). The fractions corresponding to the two antigens were pooled and each was examined against C. difficile whole-cell antiserum by tandem CIE with an EDTA extract of whole cells of C. difficile NCTC 11223 (Poxton & Byrne, 1981) and with the wall antigen according to the method of Kröll (1973). The antigen that eluted from the Sepharose 6B column in the void volume (Peak I) gave a precipitin line that was identical with line 2 in the EDTA extract, i.e. the carbohydrate antigen that is common to C. difficile and C. sordellii. The antigen that was included by the Sepharose column (Peak II) gave a precipitin line that was identical with the purified wall antigen.

The material in Peak I contained one antigen, but as there was extensive contamination with nucleic acid, further purification was necessary before a chemical analysis could be made. The immunoabsorbent column, prepared as described in Methods, was equilibrated in borate-buffered saline (BBS: 0.1 M-NaCl, 0.2 M di-sodium tetra-borate, pH 7.3). This was to remove any phosphate buffer that might subsequently contaminate the eluted antigen. The material from Peak I (2.5 mg) was dissolved in BBS, applied to the column and washed with 20 ml BBS. The flow was reversed and the antigen was eluted with sodium carbonate buffer pH 9.6/ethylene glycol (1 : 1, v/v). Ten 4 ml fractions were collected and screened for antigen by fused-rocket immunoelectrophoresis. The fractions containing antigen were pooled and concentrated to 2 ml in an Amicon (Lexington, Mass., U.S.A.) ultrafiltration cell (PM10
Fig. 2. Crossed immunoelectrophoresis of phenol extract of *C. difficile* cell contents (6 μl of 5 mg carbohydrate ml⁻¹ solution) run against 65 μl *C. difficile* antiserum. Lines A and B correspond to the antigens separated in Fig. 3.

Fig. 3. (a) Purification of phenol extract of *C. difficile* cell contents run on a Sepharose 6B column (60 x 1.6 cm). Fractions (3 ml) were collected and screened for nucleic acid at 260 nm (○); 0.1 ml of each fraction was screened for phosphorus (●) and carbohydrate (□).

(b) Drawing of fused rocket immunoelectrophoresis of fractions run against antiserum to *C. difficile* NCTC 11223. Lines A and B correspond to the same lines in Fig. 2.

membrane). Paper chromatography of acid hydrolysates showed the presence of glucose and glucosamine. No glycerol or ribitol was detected. Gas chromatography showed the presence of the same sugars in the molar ratio glucose:glucosamine of 2:1. After methanolysis (see Methods) the ether phase was analysed by gas chromatography and the fatty acids were shown to consist of C16:0, 25.1%; C16:1, 7.1%; C18:0, 8.6%; C18:1, 43.8% and
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C18:2, 15.3\%. Percentages were calculated by measuring the area of the peaks of the gas-chromatography trace. Total fatty acid was calculated by comparison with methyl stearate. Phosphate and carbohydrate (phenol/sulphuric acid) were estimated on the aqueous phase of the methanolysate. Results showed the overall composition of the antigen to be glucose, glucosamine, phosphate and fatty acid in the molar proportions 2:1:1.6:0.04.

Treatment of the membrane antigen with mild alkali (5 mM-lysine, 140 mM-KCl, pH 11.5) for 2 h at 20 °C released the lipid moiety for the carbohydrate portion, which then became indistinguishable from the wall antigen in crossed immunoelectrophoresis.

\textbf{DISCUSSION}

\textit{Clostridium difficile} possesses two carbohydrate cell-surface antigens. The secondary cell wall polymer or teichoic acid analogue is a major component of the cell wall (40\% by weight of SDS-purified walls). It was not released by EDTA but was solubilized during autolysis. This is similar to the behaviour of teichoic acids, which appear to be released but not degraded by autolytic enzymes (Duckworth, 1977). Its composition is unusual but it resembles the wall polymer described by Partridge \textit{et al.} (1971), which was found in the walls of a \textit{Micrococcus} species. After extraction with alkali all the phosphorus was present as monoester and its removal by phosphomonoesterase did not alter its antigenic properties in crossed immunoelectrophoresis. This means that the phosphorus is not part of the antigenic determinant; as structural determinations have not been possible, the location of the phosphorus is as yet unknown.

The other carbohydrate polymer was extracted by methods developed for the isolation of membrane or lipoteichoic acids. The material excluded by Sepharose 6B and further purified by immunosorbent chromatography was the polymer analogous to the membrane teichoic acid. Its composition is unlike any polymer previously described. It is the common \textit{C. difficile}, \textit{C. sordellii}/\textit{C. bifermentans} EDTA-soluble carbohydrate antigen (Poxton & Byrne, 1981) which was released due to the loss of membrane integrity following chelation of divalent cations by EDTA. The antigen in Peak II from the Sepharose column was immunologically identical to the wall carbohydrate antigen. This agrees with the observation of Coley \textit{et al.} (1975) that the wall teichoic acids of several Gram-positive bacteria are often found in Peak II. When the lipocarbohydrate was deacylated with mild alkali, the carbohydrate fraction became immunologically identical to the wall polymer. It is therefore reasonable to ask whether the antigenic material in Peak II from the Sepharose 6B column is wall antigen or deacylated membrane antigen.

As both wall and membrane carbohydrate share immunological determinants, and both react with \textit{C. sordellii} antiserum, they cannot be exploited for the detection of \textit{C. difficile} by immunological means. A specific serological system for detection of \textit{C. difficile} and distinction from \textit{C. sordellii} would require an antiserum devoid of antibodies directed against either wall or membrane carbohydrate antigens.

\textit{C. difficile} thus appears similar to aerobic Gram-positive bacteria in possessing a secondary cell wall carbohydrate and a membrane-bound lipocarbohydrate. These polymers are not strict teichoic acids as they do not contain ribitol or glycerol phosphate. They are, however, phosphate-containing carbohydrates. Their composition is more complex than any related phosphorylated carbohydrates yet described.

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