BACTERIAL PATHOGENICITY

Identification and characterisation of a cytotoxic porin-lipopolysaccharide complex from *Campylobacter jejuni*

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A clinical isolate of *Campylobacter jejuni*, previously found to produce a toxin active in cell culture assays, was used for identification and characterisation of a cytotoxic porin-lipopolysaccharide (LPS) complex. This cytotoxic complex was isolated by high-performance liquid chromatography of crude concentrated culture supernate and DEAE-anion exchange chromatography. The complex had a toxic activity of 20.1 tissue culture dose₅₀ (TCD₅₀)/μg of protein for HEp-2 cells, 7.49 TCD₅₀/μg of protein for HeLa cells and 1.87 TCD₅₀/μg of protein for Chinese hamster ovary cells. Analysis by SDS-PAGE revealed a single protein band of 45 kDa and a high mol. wt carbohydrate moiety. The complex gave a positive result in the *Limulus* amoebocyte lysate test, indicating that the co-purifying carbohydrate was LPS, and had specificity for the lectins *Galanthus nivalis* agglutinin, *Maackia amurensis* agglutinin and *Datura stramonium* agglutinin. The cytotoxic activity associated with the complex was heat-labile at 70°C, resistant to inactivation with trypsin and retained activity after treatment with sodium metaperiodate and the glycosidases neuraminidase and N-glycosidase F. Sequencing of the N-terminus of the protein component of the complex revealed 97% homology with the major outer-membrane porin protein from *C. jejuni*. The cytotoxic activity of the complex was neutralised by a polyclonal, homologous antiserum, which reacted on Western blot with the 45-kDa protein, but not by polyclonal antisera raised against a number of other bacterial toxins.

Introduction

Campylobacter jejuni is a major cause of bacterial gastro-enteritis in developed and developing countries. Active surveys conducted in the USA have estimated the incidence of gastro-enteritis associated with *C. jejuni* to be 1 case per 100 of the population and this corresponds to c. 2.5 million cases per year [1]. This estimated rate is twice that reported for salmonellosis and four times that for shigellosis [2]. Although campylobacteriosis can persist for up to 7 days, most cases are self-limiting and only occasional instances require antibiotic and rehydration therapy for effective treatment.

Mechanisms of pathogenesis in *C. jejuni* infections remain controversial. The organism has been reported to be invasive [3] and various toxins have been implicated in disease [4, 5]. One such toxin is an enterotoxin with serological and ganglioside-recognition properties similar to those of cholera toxin [6, 7]. *C. jejuni* has also been found to produce another distinctive toxin, the cytolethal distending toxin (CDT), which induces slow and progressive cytological changes in Chinese hamster ovary (CHO) cells and other cells including Vero, HEp-2 and HeLa [8, 9]. Three genes, designated *cdtA*, *cdtB* and *cdtC* and encoding proteins of 30, 28 and 19 kDa, respectively, are necessary for the production of active CDT [9], but the role of this toxin in pathogenesis has yet to be established.

The production of other cytotoxins has also been described [6, 10–13] and some have been partially characterised [11, 14]. Little is known about the structure and function of these cytotoxins but, in

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general, they are reported to be heat-labile, sensitive to trypsin degradation and >14 kDa as determined by size exclusion chromatography [12]. Cytotoxic activity has also been found associated with the lipopolysaccharide (LPS)-rich fraction of the organism [14], but currently the role of LPS in toxicity is unknown. It is probable that a combination of virulence factors act in concert to realise the full pathogenic potential of *C. jejuni*.

Porins are an integral part of the cell membrane which permit cell-cell interaction as well as the exchange of material between the bacterial cell and the external environment [15, 16]. They usually exist as β -barrels with β -strands that traverse the membrane and form channels of *c.* 1 nm diameter [17] and an exclusion limit of 5000 Da [15]. Porins have been shown to activate splenocytes [18] and interleukin-1 (IL-1) and IL-6 gene expression in human monocytes [19]. *Salmonella typhi* porins stimulated a protective immune response in rabbits, by the induction of lymphokines leading to the activation of T cells [20].

The major outer-membrane protein (MOMP) of *C. jejuni* is a porin of 43 kDa and is present in *c.* 60% of pathogenic strains [21–23]. When the MOMP was purified and reconstituted in a lipid bilayer, it formed a channel with a conductance of 8.82 nS in 1 M KCl [23]. Conformational analysis of the assembled porin showed that it was a trimer formed by three folded monomers with a high degree of β -sheet secondary structure [22]. It was also found to be immunologically similar to the OmpC protein in *Escherichia coli* [21] and has been classified as part of the trimeric porin family [23].

This study describes the identification and characterisation of a cytotoxic porin-LPS complex produced by *C. jejuni*. Although there have been several previous reports (reviewed by Sears and Kaper [4] and Wassenaar [5]) describing the production of cytotoxins by *C. jejuni*, the present report is the first to identify a complex composed of a porin protein and LPS. These findings may lead to a more comprehensive understanding of toxin-mediated enteritis in campylobacter infections.

Materials and methods

Bacterial strains and culture media

C. jejuni strain 2483, previously shown to produce a cytotoxin, was isolated from a patient with gastroenteritis [10, 11] and was characterised as Lior serotype 82, biotype 1 and Penner serotype O11. The strain was passed twice on tryptic soy agar (TSA) containing sheep blood 5% and was stored at -70°C in tryptic soy broth containing sheep blood 5%. Thawed samples were cultured on TSA with sheep blood 5% before inoculation into Brucella broth (BBL), pre-equilibrated

in an atmosphere of O₂ 5%, CO₂ 10% and N₂ 85%. For batch preparation, a suspension of the organism was made equivalent in density to a McFarland number 8 standard and inoculated into 4 L of Brucella broth at a concentration of 2 ml/L. Inoculated broths were incubated in stationary conditions in the gas mixture for 48 h at 37°C, at which time they were in the stationary phase of growth.

Isolation of cytotoxic complex

Bacteria were harvested by centrifugation at 12 000 *g* for 20 min at 4°C and the supernate was concentrated *c.* 40-fold by ultrafiltration at 4°C in a stirred cell apparatus with a YM30 membrane (30 000 mol. wt cut-off) (Amicon, Beverly, MA, USA). Protein was precipitated by the addition of ammonium sulphate to 80% saturation at 4°C, collected by centrifugation at 12 000 *g* for 30 min and resuspended in 50 mM Tris-HCl buffer, pH 7.0. The cytotoxic protein was purified with a Hewlett Packard 1050 series high-performance liquid chromatograph (HPLC) equipped with a diode-array detector. The protein concentrate was added at 1% of the total bed volume to a HiLoadTM 16/60 SuperdexTM 75 sizing column (Pharmacia Biotech, Uppsala, Sweden) and eluted with phosphate-buffered saline (PBS), pH 7.0, at a flow rate of 1 ml/min. Fractions were collected on a Gilson fraction collector and 50 μl of each was evaluated for cytotoxic activity. The molecular mass of the native cytotoxic complex was determined by calibrating the column with low mol. wt standards (Pharmacia Biotech) dissolved in PBS. Cytotoxin-containing fractions were pooled, concentrated with Centraprep-30 units (Amicon) and applied to a 7.5 $\times$ 75 mm TSK DEAE-5PW column (Pharmacia Biotech). Proteins were eluted with a linear gradient of 0.2–0.25 M NaCl in 50 mM Tris-HCl, pH 7.0, at a flow rate of 1 ml/min. Fractions were collected, desalted by spin dialysis in Centricon-30 units (Amicon) and 50 μl of each sample was assessed for cytotoxic activity.

The bacterial pellet recovered by centrifugation was suspended in 50 mM Tris-HCl, placed on ice and sonicated with a Branson Sonifier 450 sonicator (Branson Ultrasonic Corporation, Danbury, CT, USA), centrifuged at 12 000 *g* for 10 min and the cell-sonicate supernate was processed in the same manner as the culture supernate.

Polymerase chain reaction

Genomic DNA was isolated from *C. jejuni* strain 2483 by a standard procedure [24]. Polymerase chain reaction (PCR) was conducted as outlined previously [25] with 50 ng of genomic DNA and primers for *E. coli* verocytotoxins VT1a and VT1b, and VT2a and VT2b at annealing temperatures of 42°C, 45°C and 50°C. PCR was also performed with primers DZ3 and R009 specific for *Helicobacter pylori* *cagA* and

primers F6 and R20 specific for *H. pylori vacA* gene as outlined previously [26]. The *H. pylori* Penner reference serotypes O5 and O6 were used in PCR reactions as positive controls for *cagA* and *vacA*, respectively. The PCR methodology was as described for *cagA* [26] except that 100 ng of chromosomal DNA/reaction was used for amplification of the *vacA* gene with 35 cycles of 95°C for 1 min, 58°C for 1 min and 72°C for 2 min with a final extension of 10 min. PCR products were electrophoresed on an agarose 1% gel in Tris-acetate, EDTA-containing buffer (pH 8.3), stained with ethidium bromide and visualised on a transilluminator (Ultra-violet Products, San Gabriel, CA, USA).

Cytotoxic activity and protein determination

The amount of protein at each stage of the isolation procedure was quantified with the BCA protein assay (Pierce, Rockford, IL, USA). HEP-2, HeLa and CHO cells were grown in T-75 cell culture flasks (Costar, Cambridge, MA, USA) in Eagle's Minimal Essential Media (MEM) supplemented with fetal bovine serum (FBS; Sigma) 10%. Cells were subcultured into 96-well plates 24 h before determination of cytotoxic activity. Cytotoxic activity for the three cell lines was quantified as described previously [8] and activities were expressed after incubation for 24 h as tissue culture dose 50 (TCD50). A TCD50 was defined as the amount of toxin, in μg of protein, required to induce cell rounding, cytoplasmic blebbing or vacuolation of the cytoplasm in 50% of the cells. Cell cultures were fixed for 10 min in absolute methanol and stained for 30 min with Giemsa (Gibco BRL, Grand Island, NY, USA). The specific activities were determined on culture supernates of *C. jejuni* and on preparations after each step of the purification procedure and expressed as number of TCD50/ μg of protein. Culture supernates of *E. coli* O157:H7 strains LCDC 3787 (H19), positive for verocytotoxin VT1 and LCDC 90-2380, positive for VT2, were used as controls for TCD50 determination in HEP-2 and HeLa cells. *Vibrio cholerae* O1, strain 755, a cholera toxin-producing isolate, was used as a control in the CHO cell assay.

Physical characterisation of the cytotoxic complex

One μg of protein of the isolated cytotoxic material was mixed with an equal volume of $2\times$ sample buffer containing β -mercaptoethanol and sodium dodecyl sulphate. The sample was boiled for 5 min and separated by SDS-PAGE on a homogeneous 12% gel, along with kaleidoscope pre-stained low mol. wt standards, and silver stained with a commercial kit (BioRad, Hercules, CA, USA). One- μg samples of the isolated cytotoxic material were either heated at 70°C for 30 min or treated with trypsin in PBS at concentrations ranging from 0.03 to 1.25% w/v for 2 h at 37°C. Residual trypsin activity was inactivated by addition of FBS to give a final concentration of

20% v/v and incubated for 1 h at 37°C. Heated and trypsin-treated samples were serially diluted two-fold in PBS before cell culture assay to determine the degree of activity remaining after the treatments. Heat-inactivated trypsin and FBS alone were used as negative controls.

N-Terminal sequencing of cytotoxin

After electrophoresis of the cytotoxic material as above, the protein was transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (BioRad) for 18 h at 100 mA in 10 mM 3-(cyclohexylamino)-1-propane sulphonic acid (CAPS; Sigma) buffer, pH 11.0, containing methanol 10% v/v. The blot was stained with Coomassie Blue R-250 (BioRad) 0.1 w/v in methanol 50% v/v for 5 min and destained with methanol 50% v/v and acetic acid 10% v/v. The immobilised protein band was excised and the protein was sequenced by Edman degradation on an Applied Biosystems model 473A protein sequencer (CHUL Research Center, Saint-Foy, Quebec, Canada) and analysed with Lasergene software from DNASTar, (Madison, WI, USA).

Neutralisation and Western blot analysis

Neutralisation studies were performed on the cytotoxic complex from *C. jejuni* with homologous polyclonal antiserum and with antisera against *E. coli* VT1, *E. coli* VT2, CDT from *C. jejuni* [8] and CDT from *E. coli* [8], cholera toxin from *V. cholerae* (Sigma) and cytotoxin B from *Clostridium difficile* (Techlab, Blacksburg, VA, USA). Normal rabbit serum was used as a negative control. Homologous antiserum was raised by intramuscular inoculation of New Zealand White rabbits with 0.5 ml of a preparation of isolated cytotoxic material at 5 $\mu\text{g}/\text{ml}$ emulsified in 0.5 ml of Freund's incomplete adjuvant (FIA). This was followed, at weekly intervals for 4 weeks, by subcutaneous injection of the same antigen preparation in FIA. A 1 in 10 dilution of each antiserum was added to serial two-fold dilutions of the isolated cytotoxic material, starting at 1 $\mu\text{g}/\text{ml}$ of protein. After incubation for 1 h at 37°C, aliquots of each were added to HEP-2 cells and incubated for 24 h at 37°C. Each antiserum was also assayed by Western blot analysis. *C. jejuni* cytotoxic complex, *E. coli* VT1 and VT2, *C. jejuni* CDT, *E. coli* CDT, *C. difficile* cytotoxin B and cholera toxin were each separated by SDS-PAGE and transferred to PVDF. These were washed with skimmed milk 5% for 1 h to prevent non-specific binding of antibody. A 1 in 500 dilution of each of the antisera in skimmed milk 1% containing Tween-20 0.05% v/v was added to membranes for 2 h at room temperature. Membranes were probed with alkaline phosphatase-conjugated goat anti-rabbit antibody (Boehringer Mannheim) 200 mU/ml for 1 h at room temperature and developed with 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium. Western blot analysis

and cytotoxicity assays were also performed on crude concentrated culture supernates from *C. jejuni* and other bacterial strains.

Carbohydrate characterisation

A total of 2 µg (protein content) of the isolated cytotoxic material was assayed for the presence of LPS by the *Limulus* amoebocyte lysate (LAL) test according to the manufacturer's instructions (Pyrotell, Associates of Cape Cod, MA, USA). The toxic material and *E. coli* were each diluted 10-fold with pyrogen-free water in duplicate and 100 µl of each dilution were incubated with 100 µl of Pyrotell in a 37°C water bath for 1 h. Tubes were inverted and those containing a solid clot were considered positive. To determine whether LPS contributed to the cytotoxic activity of the material, 1-µg (protein content) amounts of the sample were incubated for 1 h at 37°C with 5 U of neuraminidase (Sigma) at pH 5.0, 3 U of N-glycosidase F (Boehringer Mannheim) at pH 7.2, or 10 mM sodium metaperiodate (Sigma) for 90 min at room temperature. The residual cytotoxic activity was then assayed with HEp-2 and HeLa cells.

The carbohydrate moiety was identified with a glycan differentiation kit (Boehringer Mannheim) containing five unique digoxigenin-labelled lectins (Table 1).

Approximately 1 µg of the isolated cytotoxic protein and 5 µg of each carbohydrate standard were spotted on PVDF membranes and allowed to dry overnight at 37°C. Membranes were probed for the co-purifying LPS according to the manufacturer's instructions. Those lectins that gave positive results were further examined by Western blot analysis with 15 µg of each carbohydrate standard and 8 µg of the test carbohydrate from the purified preparation. The isolated cytotoxic material from three separate batches was assayed for carbohydrate with a phenol-sulphuric acid assay [27]. SDS-PAGE and native PAGE were done with 10 µg of the carbohydrate and gels were double stained, first with periodic acid-Schiff (PAS) [28] then with Coomassie Blue.

Results

Identification and molecular characterisation of cytotoxic complex

Cytological signs of intoxication caused by the cytotoxic complex included the formation of vacuoles in the cytoplasm of HEp-2 cells as compared with normal unaffected cells (Fig. 1a and b). Similar results were found with HeLa cells. The number of vacuoles in each cell ranged from one to five with ≥50% of the cytoplasm of some cells being affected. After 24 h, the

Table 1. Specificities and the reactions of the lectins used in the carbohydrate determination

Lectins	Specificity (linkage)	Reactivity
<i>Galanthus nivalis</i> agglutinin (GNA)	Manα(1-3), α(1-6) or α(1-2)-Man (terminally linked mannose)	+++
<i>Maackia amurensis</i> agglutinin (MAA)	Neu5Aca(2-3)-Gal (sialic acid terminally linked α(2-3) to galactose)	+
<i>Datura stramonium</i> agglutinin (DSA)	Galβ(1-4)GlcNac (galactose-β(1-4)-N-acetylglucosamine)	+
<i>Arachis hypogaea</i> (peanut) agglutinin (PNA)	Galβ(1-3)GalNac (galactose-β(1-3)-N-acetylgalactosamine)	-
<i>Sambucus nigra</i> agglutinin (SNA)	Neu5Aca(2-6)-Gal or GalNac (sialic acid terminally linked α(2-6) to galactose or N-acetylgalactosamine)	-

+++ , strong positive result; + , weak positive result; - , negative result

Fig. 1. Morphological changes induced in HEp-2 cells after treatment for 48 h with isolated *C. jejuni* cytotoxic porin-LPS complex: **A**, control cells; **B**, cells intoxicated with 1 µg (protein content) of cytotoxic complex, note the cytoplasmic vacuoles (arrowed). **C**, cells intoxicated with 10 µg (protein content) of cytotoxic complex, note the cytoplasmic blebbing (arrowed and inset). (magnification ×140).

vacuoles diminished in size and the cells developed a rounded, highly refractile appearance. By 48 h, cytoplasmic blebbing and nuclear condensation became more evident along with cell loss from the monolayer (Fig. 1c and inset). Toxicity was dose-dependent: at 1 μ g of protein/well, the vacuoles persisted up to 48 h while rounding occurred up to 72 h; at 10 μ g of protein/well, vacuoles formed and dissipated within the first 12 h, >50% of the cells were rounded and refractile by 24 h and, by 48 h, 80–100% of the cells had become rounded (Fig. 1c). Similar cytological changes were observed in all the cell lines when the whole bacterial cell sonicate was assayed for toxicity. *C. jejuni* strain 2483 produced low levels of CDT in the crude concentrate; however, this was neutralised with polyclonal antisera raised against either *C. jejuni* or *E. coli* CDT (data not shown).

Concentrated proteins from the culture supernate of *C. jejuni* strain 2483 possessing high levels of cytotoxic activity were found to elute from the Superdex 75 gel filtration column in the void volume, with a calculated native mol. wt of >100 kDa (Fig. 2a). This peak (peak A) was collected and applied to the TSK DEAE-5PW column. Cytotoxic activities of the TSK DEAE-5PW fractions showed that the toxin eluted at *c.* 0.21–0.22 M NaCl (Fig. 2b, peak B). The two-column purification procedure produced a single protein band of 45 kDa which was revealed by silver staining (Fig. 2c). The toxic activities at each stage of the purification procedure are shown in Table 2. The isolated cytotoxic complex demonstrated greatest toxic activity for HEp-2 cells and the lowest for CHO cells. The cytotoxin was inactivated by heat treatment at 70°C for 30 min, but was resistant to trypsin at the concentrations tested.

Polymerase chain reaction

Oligonucleotide primers specific for *E. coli* VT1 and VT2 failed to produce amplicons corresponding to A- and B-subunits of mature verotoxin types 1 and 2. Also, primers specific for the *cagA* and *vacA* genes of *H. pylori* failed to generate amplicons with *C. jejuni* DNA. All primers gave positive reactions with their corresponding control DNA.

Protein alignment

The cytotoxic protein consisted of a single protein with a calculated mol. wt of 45 kDa. The excised band was subjected to N-terminal sequencing and a total of 31 amino acid residues was elucidated: TPLEEAIKDV-DVSGVLRVRYDTGNFDKNFVN. This sequence contained several hydrophobic and charged residues and had a predicted iso-electric point of 4.35. A search through the Beckman Center for Molecular and Genetic Medicine (Stanford University) revealed that the protein had 97% homology with the MOMP (TPLEEAIKDV-DVSGVLRVRYDTGNFDKNF*N) from

Fig. 2. Isolation of the cytotoxic porin-LPS complex from proteins concentrated from the culture supernate: **a**, fractionation on a Superdex 75 gel filtration column; **b**, fractionation of peak A on a TSK DEAE-5PW column; ++, >70% of HEp-2 cells rounded by 48 h; +, 50–70% cells rounded by 48 h; +, <50% cells rounded by 48 h; **c**, silver-stained SDS-PAGE. Lanes 1 and 3, mol. wt standards (kDa); 2, 1 μ g (protein content) of *C. jejuni* isolated cytotoxic complex (peak B from the DEAE column).

C. jejuni [22]. This MOMP has been characterised as a porin. The cytotoxic porin detected in the present studies also shared 56% and 63% sequence homology with the 45-kDa and 51-kDa OMPs, respectively, from *Wolinella recta* [29].

Table 2. Cytotoxic activity of *C. jejuni* preparations at each step of purification

Organism and preparation	Cell culture specific activity (No. of TCD50/ μ g protein)		
	HEp-2	HeLa	CHO
<i>C. jejuni</i> strain 2483			
Culture supernate	1.56	0.51	0.51
Superdex 75 16/60	1.61	3.88	0.97
TSK DEAE-5PW	20.1	7.49	1.87
Toxin controls*			
<i>E. coli</i> strain LCDC 3787 (H19) (VT1)	0.35	0.17	ND
<i>E. coli</i> strain LCDC 90-2380 (VT2)	1.48	2.89	ND
<i>V. cholerae</i> O1, strain 755 (cholera toxin)	ND	ND	0.48

ND, not determined.

* Assayed as concentrated culture supernates.

Neutralisation and Western blot analysis

Polyclonal antisera raised against *E. coli* VT1 and VT2, *C. jejuni* and *E. coli* CDT, cholera toxin and *C. difficile* cytotoxin B failed to neutralise the cytotoxic effects elicited by the *C. jejuni* toxic complex in cell culture. However, when this cytotoxic complex was serially diluted, incubated with rabbit polyclonal antiserum raised against the cytotoxic protein and added to HEp-2 cells, the TCD50 occurred at a dilution of 1 in 2 of the toxin preparation, whereas the TCD50 in the presence of the normal rabbit serum was at a dilution of 1 in 32. Antiserum raised against the

cytotoxic protein showed immunological reactivity in Western blots with the cytotoxic 45-kDa protein in the purified preparation after TSK-DEAE fractionation. Antisera raised to the other toxins showed no cross-reactivity with either the cytotoxin or carbohydrate by immunoblot analysis. Western blots of crude concentrated filtrates from various cytotoxic strains of *Campylobacter* spp. showed the presence of a protein with a mol. wt similar to that of the porin (Fig. 3), whereas no bands were detected in the blots from the uninoculated broth or in the filtrates from *Aeromonas veronii* and *E. coli* VT1 strains used as controls.

Fig. 3. Western blot analysis of the isolated cytotoxic porin-LPS complex from *Campylobacter* spp. with 40 μ g (protein content) of crude, concentrated filtrate and homologous rabbit antiserum. Lanes 1 and 9, pre-stained standards (kDa); 2, uninoculated broth; 3, *Aeromonas veronii* LCDC A2297; 4, *C. coli* strain 8682; 5, *C. jejuni* LCDC 16336; 6, *C. jejuni* LCDC 3969; 7, *C. jejuni* strain 2483; 8, *E. coli* LCDC 3787 (VT1 producer).

Lipopolysaccharide identification and carbohydrate analysis

The isolated cytotoxic material and *E. coli* LPS were assayed for the presence of endotoxin by a *Limulus* amoebocyte lysate test. The cytotoxic material produced a strong positive result at a dilution of 1 in 128 000, signifying the presence of LPS. The *E. coli* LPS also gave a positive result. To determine whether or not the cytotoxic activity associated with the complex resided in the protein component, samples were incubated with 10 mM sodium metaperiodate, to oxidise the free hydroxyl groups present on visceral hexoses, with 5 U of neuraminidase, to cleave sialic acid residues, and with 3 U of N-glycosidase F, to cleave asparagine bound N-glycans. Dilution of the samples were then assayed for cytotoxic activity in HEp-2 and HeLa cells and expressed as TCD50 endpoints. The titres for purified toxin incubated with and without metaperiodate or the two enzymes were 32 in Hep-2 and 28 in HeLa cells. This indicated that the degradation of the carbohydrate component of the LPS had no effect on the toxicity elicited by the porin.

The carbohydrate component of the LPS was characterised with digoxigenin-labelled lectins (Table 1) and the data revealed a complex of different subunits. Lectin GNA reacted strongly with the purified material and suggested a high proportion of terminally linked mannose. The lectins MAA and DSA also gave positive but weaker results, indicating the presence of sialic acid terminally linked $\alpha(2-3)$ to galactose and galactose- $\beta(1-4)$ -N-acetylglucosamine as well as hybrid N-glycan structures in the complex, respectively. The remaining lectins showed no reactivity for

the carbohydrate complex. The proportion of carbohydrate to protein in the purified material was calculated to be 4:1. PAS staining revealed a high mol. wt carbohydrate which did not appear as a discrete band as did the protein component of the complex but, instead, occupied a broad range of sizes (Fig. 4). Double staining of purified cytotoxin in native PAGE gels showed no protein component, in contrast to samples boiled in denaturing buffer before SDS-PAGE (Fig. 4). Western blots with the lectins (Fig. 5) showed that the high mol. wt smear seen following PAS staining (Fig. 4) was carbohydrate in nature with high reactivity for GNA (Fig. 5).

Discussion

A cytotoxin from strains of *C. jejuni* that was heat-labile, trypsin-sensitive and induced characteristic rounding of HEp-2, HeLa and MRC-5 cells was first documented by Yeen *et al.* [30]. Guerrant *et al.* [12] also described a cytotoxic component which was heat labile at 60°C, was partially sensitive to trypsin 0.25% and had a mol. wt >14 kDa. The cytotoxic component identified by these workers could not be neutralised with antisera raised against *E. coli* verocytotoxins or *C. difficile* toxin B [12]. A subsequent report indicated the presence of a Shiga-like cytotoxin from *C. jejuni* which could be neutralised with monoclonal antibodies directed against the B subunit of the mature Shiga-toxin; however, these workers also detected a cytotoxin which could not be neutralised by the same monoclonal antibody [31]. In addition, Guerrant and colleagues [12], unlike Yeen *et al.* [30], found cytotoxic activity in

Fig. 4. Double staining of native-PAGE (lanes 1 and 2) and SDS-PAGE (lanes 3 and 4) gels with periodic acid-Schiff (PAS) and Coomassie Blue. Lane 1, native mol. wt standards (Pharmacia); 2, 10 μ g (carbohydrate content) of native cytotoxic complex; 3, 10 μ g (carbohydrate content) of cytotoxic complex denatured by heat and SDS; 4, kaleidoscope pre-stained mol. wt standards (kDa).

Fig. 5. Western blot analysis of the isolated cytotoxic complex with the digoxigenin-labelled lectin GNA. Lanes 1 and 4, kaleidoscope pre-stained standards (kDa); 2, 10 μ g (carbohydrate content) of the cytotoxic complex; 3, 15 μ g carboxypeptidase Y served as a positive control by binding the lectin GNA through its terminal mannose.

sonicated whole bacterial cell preparations. In the present study cytotoxic activity was detected both in culture supernates and in supernates of sonicated *C. jejuni* strain 2483 cells (data not shown).

A cytotoxic complex comprising a porin and LPS was isolated and characterised. Previous studies showed a cytotoxic factor present in LPS-rich fractions from *C. jejuni* [14]; however, it was not known what role the LPS played in toxicity. Misawa *et al.* [32] found that the expression of their cytotoxin was elevated when the *C. jejuni* was grown in Brucella broth. However, contrary to their additional findings regarding the activity of their cytotoxin in various culture media [32], the present studies indicated that the cytotoxic effects elicited by the porin had a more profound cytopathic effect on HEp-2-cells than on CHO cells when the cells were maintained in media containing FBS. The increase in activities observed in the different cell lines may be caused by the relative amounts of the receptor required for binding of the porin-LPS complex. Previous reports have implicated LPS in the adhesion of *C. jejuni* to epithelial cells as well as to intestinal mucus and also showed that this process could be inhibited by periodate oxidation [3]. As the cytotoxic activity of the porin-LPS was maintained following treatment with periodate in both HEp-2 and HeLa cells, it would appear that adhesion of the toxic complex is facilitated by components other than LPS. It is possible that expression of the porin protein may be involved in binding the organism to host cells; however, Fauchere *et al.* [33] indicated that the MOMP was not involved in adherence to HeLa cells. From these studies it would appear that,

although the LPS may mediate attachment of organisms to host cells [3], the porin component binds the cytotoxic complex.

Although the mode of action of the cytotoxic porin remains unclear, the morphological changes induced by it are similar in nature to those induced by other, well-characterised bacterial cytotoxins. During early stages of intoxication the cytotoxic porin induced vacuole formation in HEp-2 and HeLa cells similar to that produced by *H. pylori* vacuolating toxin [34]. However, no PCR products were generated with primers specific for *cagA* and *vacA* genes of *H. pylori* [26] suggesting that the genes encoding vacuole induction by the *C. jejuni* porin were distinct from those in *H. pylori*.

When intoxication of host cells with the *C. jejuni* cytotoxic porin was extended beyond 24 h, vacuoles dissipated while the cytoplasmic blebbing and nuclear condensation typical of verocytotoxin and diphtheria toxin became more evident. Verocytotoxin and diphtheria toxin are both known to interfere with protein synthesis, leading to programmed cell death or apoptosis [35, 36]. PCR-based screening of *C. jejuni* with verocytotoxin-specific primers was negative and confirmed the low stringency hybridisation experiments of Moore *et al.* [31] and this suggested that the *C. jejuni* cytotoxic complex was distinct from verocytotoxin. It is possible that the porin from *C. jejuni* induces holes in the cell membrane in a manner similar to that resulting from *Staphylococcus aureus* α -toxin [36]. Recently, the cytotoxic effects elicited by *Salmonella* Typhimurium porin suggested that porins directly affect the cytoskeleton and the membrane ultrastructure of HEp-2 cells [37]. In addition, porins from *Neisseria* spp. have also been shown to inhibit polymerisation of actin in human neutrophils [38] while porins from *S. Typhimurium* have been found to induce both an inflammatory response [39] and the release of cytokines from human monocytes and lymphocytes [40].

Isolation of the cytotoxic porin protein by chromatofocusing and determination of its iso-electric point by Coomassie Blue staining and Western blotting of iso-electric focus gels (data not shown) were unsuccessful, probably because of interference from LPS. The isolation protocol was also applied to a culture supernate from *C. jejuni* strain 3969 which had been reported previously to produce a cytotoxin [13, 32, 41]. Although the strain produced a lower cytotoxic activity, similar morphological changes were observed in HEp-2 cells and a protein of similar size was observed following SDS-PAGE. A protein of comparable mol. wt was also present in crude concentrated culture supernates from other cytotoxic strains of *Campylobacter* spp., indicating that the release of the porin-LPS complex was not unique to *C. jejuni* strain 2483. Carbohydrates were also present

in the cytotoxic product isolated from *C. jejuni* strain 3969. The difference in cytotoxic activity between strain 3969 (low toxin activity) and 2483 (high toxin activity) could be a growth-rate dependent phenomenon, as the release of the porin-LPS complex may occur most avidly during cell death or may be lost during active replication of the organism. Therefore, strains with a higher growth rate could produce quantitatively more cytotoxic complex [42]. A vacuolating cytotoxin similar to that produced by *H. pylori* was detected in the stools of children with diarrhoea in the absence of a disease agent [43]. Indeed, it is possible that many bacteria possess the ability to induce vacuole formation in host cells by the release of porin material from dead or dying organisms [42].

Lectin studies showed that the carbohydrate portion of the LPS which co-purified with the porin possessed terminally linked mannose as well as sialic acid terminally linked $\alpha(2-3)$ to galactose and to galactose- $\beta(1-4)$ -N-acetylglucosamine complexed with hybrid N-glycan structures. Based on the thermostable somatic (O) antigen, the strain of *C. jejuni* used in this study was type O11. The positive result with the lectin MAA suggests that the strain may be related to serotype O19 [44]. The LPS from the O19 serostrain of *C. jejuni* has core structures that mimic those present on GM1 and GD1a gangliosides and other strains of O19 have been linked to post-infectious neuropathies [45]. The presence of terminally linked mannose in the LPS of the O11 serotype in this study may be significant. Treatment of the isolated complex with sodium metaperiodate, neuraminidase and N-glycosidase F had no effect on the toxicity elicited by the complex, suggesting that the LPS is not an integral component of the cytotoxic activity but that it may play a protective role. Indeed, it is possible that it may have interfered with the enzymic degradation by trypsin and may offer an explanation for the disparity in trypsin inactivation data of previous reports. Under native conditions, the LPS probably forms complexes with the porin and protects it from discrete staining with Coomassie Blue. The cytotoxic protein is revealed by Coomassie Blue or silver staining only after boiling in sample buffer containing SDS and β -mercaptoethanol before SDS-PAGE. As the porin from *C. jejuni* has been classified as part of the trimeric porin family, it was not unexpected that it must be heat denatured in order to resolve the protein component of the cytotoxic complex [17].

When characterisation of the cytotoxic porin is complete and the encoding gene has been cloned and sequenced, a fuller understanding of the role of the porin in clinical campylobacteriosis will be forthcoming. Such evaluations may suggest potential roles for the porin-LPS complex as a diagnostic tool for the detection of either the organism or its cytotoxin or, additionally, as a recombinant vaccine for prevention and control of campylobacter disease.

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