Demonstration of a cytotoxin from *Campylobacter jejuni*

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SUMMARY A 48-hour culture filtrate of *Campylobacter jejuni* was found to produce cytopathic effects on three human cell lines—that is, HeLa, MRC-5 and Hep-2. The cytopathic effects observed include cell rounding, loss of adherence and cell death after 24–48 h of incubation. Such morphological changes were observed with eight of the eleven strains of *Campylobacter jejuni* isolated from the blood/stools of patients who suffered from either acute gastroenteritis or septicaemia. The toxic factor did not retain its activity after treatment at 100°C for 30 min. Trypsinisation of the filtrate totally abolished its toxic activity thus indicating that it was probably protein in nature. It is most probably an extracellular toxin as bacterial sonicates did not produce any toxic effect. This study reports the finding of toxic factor(s) in the culture filtrate of *Campylobacter jejuni* which is cytotoxic to human tissue culture cells.

Campylobacter jejuni is a recently recognised cause of human acute/subacute diarrhoeal disease.^{1–3} The organism is responsible for 3–11% of cases of infective diarrhoea reported in Europe, Africa, Australia and North America.^{1,3,4,5} In Sweden, *Campylobacter* alone accounted for approximately 11% of diarrhoeal cases and was even more common than *Salmonella*.⁶ Despite considerable documentation of human infections with this organism, the exact mechanism(s) involved in disease production is not known. The presence of bacteraemia in some patients and the frequent finding of dysenteric stools similar to that seen in shigellosis suggest that *Campylobacter jejuni* may be an invasive organism. The possibility of direct tissue invasion is further supported by the demonstration, by electron microscopy, of the presence of vibrio-like organisms in chicken embryo cells as well as in the caecal wall of neonatal chickens following intragastric inoculation.^{7,8}

However, the significance of these findings has yet to be established, particularly in view of the failure of many investigators to demonstrate invasiveness by the classical Sereny test.^{9,10} The various clinical features of patients with *Campylobacter* enteritis raise the possibility of toxin-mediated effects¹¹—for example, tissue damage of the jejunum and colon

may be caused by a cytolytic exotoxin like that produced by *Clostridium difficile*, and the frequent occurrence of profuse watery stools in some diarrhoeal patients may probably be due to the presence of an enterotoxin.

It was thus the aim of this study to investigate the probable production of toxin by *Campylobacter jejuni* isolated from human cases. Various tissue culture cell lines have been employed to detect the presence of toxic factor(s) found in the culture filtrate of the organism.

ISOLATES OF *CAMPYLOBACTER JEJUNI*

Eleven isolates of *Campylobacter jejuni* were studied. All these isolates were obtained from the blood or stool cultures of infants and adults admitted to the University Hospital, Kuala Lumpur with acute gastroenteritis or septicaemia. Three strains (CJ01, CJ02, CJ03) were confirmed to be *Campylobacter jejuni* biotype 1 by Dr MB Skirrow (Public Health Laboratory, Worcester, United Kingdom). All other strains were confirmed as *Campylobacter jejuni* by standard biochemical tests but have not been biotyped. Isolates were stored in freeze-dried powder form, and none was passaged more than four times after isolation and before use in any of the tests described below.

PREPARATION OF CELL-FREE FILTRATE

Ten colonies of *Campylobacter jejuni* were grown in

10 ml of nutrient broth (Difco) at 42°C. After 48 h, cultures were centrifuged at 5000 g for 15 min and the supernatant were filtered (pore-size, 0.22 µm; Millipore Corp, USA). The resulting supernatant represented the cell-free filtrate which was then tested for sterility by streaking on *Campylobacter* selective medium or Skirrow's medium (5% lysed ox blood agar supplemented with vancomycin 10 µg/ml, polymyxin B 2.5 IU/ml, trimethoprim lactate 5 µg/ml, Oxoid, England) and incubated at 42°C in 10% CO₂.

For the heat stability test, the filtrate was heated at 56°C for 30 min or at 100°C for 30 min.

MAINTENANCE OF CELL LINES

MRC-5 (human embryonic fibroblasts), Hep-2 (human tumour epithelial cells), L929 (mouse fibroblasts) and Vero (African Green monkey kidney) cells were grown in Dulbecco's modification of Eagle's Medium (Flow Lab, USA) supplemented with 10% fetal bovine serum, 100 IU/ml of penicillin and 100 µg/ml of streptomycin (Gibco Lab, USA). The cells were seeded in tissue culture flasks (50 ml, Costar, USA) and incubated at 37°C under normal atmospheric conditions, except L929, which required 10% CO₂.

HeLa cells (human tumour epithelial cells) were grown in Eagle's Minimal Essential Medium (Flow Lab, USA) supplemented with 10% fetal bovine serum, 100 IU/ml of penicillin and 100 µg/ml of streptomycin. Both PMK (primary monkey kidney) and MK-2 (Rhesus monkey kidney) cells required medium 199, 5% glutamine and in the case of PMK, Hepes buffer was added. These three cell lines were grown in sterile screw capped glass tubes at 37°C.

When the cells become confluent, the growth medium was removed and replaced with minimal medium containing only 2% serum.

TESTING OF CYTOTOXIC ACTIVITY

The seven cell lines were used for the detection of cytotoxicity. Confluent monolayers of these cell lines were incubated with the cell-free filtrate at various dilutions at 37°C. Twofold dilutions of the filtrate were tested (1/2 to 1/32). Control groups were included in which sterile nutrient broth incubated at 42°C for 48 h was inoculated onto the cell cultures. Cytotoxicity was assessed morphologically at six-hourly intervals by the presence of destructive changes and by the failure to exclude trypan blue.

TRYPSINISATION OF CELL-FREE FILTRATE

Protein content of the cell-free filtrate was determined by the Biuret method. 10 mg/ml of the filtrate was mixed with equal amount of trypsin (Sigma, USA) and incubated at 37°C for two hours

(pH 7.2). Soybean trypsin inhibitor (Sigma, USA) was added to the mixture before determination of cytotoxicity.

SONICATED BACTERIAL CELL PREPARATION

Ten colonies of each strain of *Campylobacter jejuni* picked from blood agar plates were suspended into 1.0 ml distilled water in a small glass tube. The bacterial suspension was then sonicated (20 kc/s, Branson cell disruptor, USA) for 15–20 s on ice. The suspension was then centrifuged at 4°C for 20 min at 5000 g and filtered. The filtrate was then tested for cytotoxicity using HeLa and Hep-2 cell monolayers.

Results

On human cell lines (HeLa, MRC-5 and Hep-2), cell destruction occurred after incubation with untreated filtrate of CJ01, CJ02 and CJ14 strains for 24–48 h, but not with the uninoculated control broth (Table 1). Cell rounding associated with nuclear pyknosis (Fig 1b) was a distinctive feature of the cytopathic effects observed. There was loss of cell adherence of the monolayers and many of the floating cells were found to be dead by trypan blue exclusion. However, none of the animal cell lines was affected by these three culture filtrates (Table 1). Culture filtrate of CJ03 strain showed no cytopathic effects on both the human as well as animal cell lines. Seven other strains were tested only on HeLa and Hep-2 cells. Culture filtrates from five out of these seven strains produced cytopathic effects identical to that already described. When cell-free filtrates of CJ01, CJ02 and CJ14 strains were heated at 56°C for 30 min, cytopathic effects on HeLa and Hep-2 cells could still be observed (Table 2). However, treatment at 100°C for 30 min, abolished the toxic activity (Table 2). This indicated that the toxic factor was a heat labile substance. In addition, pretreatment of the filtrate with trypsin

Table 1 Result of a 1/8 dilution of cell-free filtrates of four *C. jejuni* on tissue culture cells

Cell lines	Origin	Strain of <i>C. jejuni</i> tested			
		CJ01	CJ02	CJ03	CJ14
HeLa	Man	+	+	–	+
Hep-2	Man	+	+	–	+
MRC-5	Man	+	+	–	+
Vero	Monkey	–	–	–	–
MK2	Monkey	–	–	–	–
PMK	Monkey	–	–	–	–
L929	Mouse	–	–	–	–

+ = presence of cytopathic effect.

– = absence of cytopathic effect.

Cytopathic effect was also detected at filtrate dilutions of 1/2 and 1/4 but not at 1/16 or 1/32.

Fig. 1 (a) HeLa cell monolayer incubated with control (uninoculated) culture broth. (b) HeLa cell monolayer incubated with cell-free filtrate of *CJ01* for 24–48 h. Original magnification $\times 500$.

Table 2 Characterisation of the cell-free filtrate of three strains of *C. jejuni* showing toxic activity

Treatment of cell-free filtrate	Cytopathic effect observed	
	HeLa	Hep-2
Untreated	+	+
Heated for 30 min at 56°C	+	+
100°C	—	—
Trypsinisation	—	—
Bacterial sonicates	—	—

totally prevented the cytopathic effects on HeLa and Hep-2 cells, thus indicating that the toxic factor was probably protein in nature (Table 2). Finally, bacterial sonicates did not contain any toxic factor as shown by the absence of cytopathic effects on HeLa and Hep-2 cells thus suggesting that the toxic factor was most probably an extracellular product (exocytotoxin) rather than an endotoxin.

Discussion

The large global increase in the number of reports of enteric diseases caused by *Campylobacter jejuni* has stimulated many investigators to search for possible pathogenic mechanism(s) for this organism. Various studies suggest that the organism is enteropathogenic in man by means of its invasive properties. Evidence to support this proposal comes mainly from clinical studies such as the rapid rise of antibody titres post infection,¹² the recovery of the organism from patients' blood in the acute phase of the disease^{12 13} and the presence of intestinal congestion and haemorrhage at necropsy of a patient who died from *Campylobacter* infection.¹⁴ While the hypothesis of invasiveness is not firmly established, attempts to detect the production of a heat stable or heat labile enterotoxin analogous to those produced

by *Vibrio cholerae* and certain strains of *Escherichia coli* have not been fully successful.^{7 9 10}

Skirrow has reported in his preliminary studies that 16 out of a 100 clinical *Campylobacter* isolates showed evidence of heat stable enterotoxin production by the suckling mouse assay.⁷ However, none of the culture filtrates of these isolates demonstrated enterotoxin activity on Y₁ adrenal cells indicating the absence of a thermolabile toxin.⁷ Manninen *et al*¹⁰ in their recent studies, were unable to demonstrate any enterotoxin activity from the culture filtrate of both human and animal *Campylobacter* species. However, it is hazardous to deduce that an enterotoxin is absent merely on the failure to demonstrate activity in an in vitro system. In some cases, as with *Salmonella* and *Escherichia coli* culture filtrates, partial purification is necessary or at least helpful in demonstrating an enterotoxin-like effect.¹⁵

Despite the failure in vitro to detect any classical enterotoxin production, the ability of this organism to produce cytotoxin(s) should not be overlooked. Some investigators have suggested that a cytolytic exotoxin similar to that produced by *Clostridium difficile* would probably account for the production of intestinal epithelial damage seen in gnotobiotic dogs infected with *Campylobacter jejuni*.¹⁶

Tissue culture techniques are being increasingly employed in the study of microbial toxins and provide a useful, sensitive and reproducible experimental system for the study of pathogenic mechanisms. In this present study, we were able to demonstrate that eight of eleven pathogenic strains of *Campylobacter jejuni* produced a substance which is cytotoxic to human cells in tissue culture. We believe that the results obtained in the present study probably represents the first report of an in vitro demonstration of cytotoxin in the cell-free filtrate of *Campylobacter jejuni*. The reproducible and constant occurrence of the morphological changes (cell rounding, loss of

adherence and cell death) can be used as the basis of a biological assay for this substance. It is interesting to note that only the human cell lines were affected by the toxic factor. This may involve direct binding of the toxin to membrane receptors found in these cells but which are lacking in animal cells. However, the exact mechanism responsible for these cytopathic effects remains to be determined.

The sensitivity to trypsin and heat (100°C) indicated that the toxic factor was protein in nature. By virtue of the fact that cytopathic effects were not observed with sonicated bacterial cell suggests that the toxin is an extracellular cytotoxin and is not cell associated.

It is well documented that an exotoxin of *Shigella dysenteriae* type 1 not only possesses cytotoxic activity to HeLa cells, but is found to also have enterotoxigenicity and neurotoxicity.¹⁷ Thus the possibility of this cytotoxin being an enterotoxin cannot be ruled out. Other biological activities will be looked into such as detection of thermolabile or thermostable enterotoxin by the classical assay (Y1 adrenal cell, Chinese hamster ovary cell and suckling mouse test).

Although only a small number of strains were tested in the present study it does appear that cytotoxin-producing capacity was detected in most of the strains tested (eight out of eleven). This is in agreement with a study of cytotoxin produced by *Aeromonas hydrophila* where 19 of 23 strains tested showed cytopathic effect on tissue culture cells.¹⁸ The exact reasons for cytotoxin non-production in the small number of negative cytopathic effect strains is not clear and it remains to be seen whether cytotoxin production is associated with pathogenesis and a more severe type of disease. It would also be of interest to determine whether strains of *Campylobacter* isolated from animals and from asymptomatic carriers are toxin producers. Detection of cytotoxic factor(s) from the stool samples of diarrhoeal cases due to *Campylobacter jejuni* should also be attempted.

The nature and role of this toxin await further purification and characterisation. Resolution of the crude culture filtrate by Sephadex gel chromatography is currently being studied so as to obtain a purer toxin preparation. Purification is important because the toxin may represent a minor component of the mixture of protein in the crude preparation as seen with the exotoxin of *Shigella dysenteriae* type 1 in contrast with the relative prominence of exotoxin in cultures of *Vibrio cholerae*, *Corynebacterium diph-*

theriae and *Clostridium tetani*. The role of this toxin as a virulence factor needs to be investigated further to determine if the cytotoxin is directly involved in pathogenesis or whether it indirectly facilitates invasion and spread of the organism following entry via the alimentary tract.

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