

Cell cycle arrest & apoptosis of epithelial cell line by cytolethal distending toxin positive *Campylobacter jejuni*

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Background & objectives: *Campylobacter jejuni* is the leading cause of gastroenteritis worldwide; cytolethal distending toxin (CDT) being an important virulence determinant. As its role in pathogenesis remains unclear, this study aims to investigate cell cycle arrest and apoptosis by CDT (+ve) and CDT (-ve) *C. jejuni* isolates on HeLa cells.

Methods: Culture supernatants and lysates from 10 *C. jejuni* isolates [CDT (+ve) and CDT (-ve), five each] were incubated with HeLa cells. CDT activity on HeLa cells was confirmed by cell distension, cell cycle arrest by flowcytometry, and apoptosis by DNA fragmentation and flowcytometry.

Results: Culture supernatant and lysate of only CDT (+ve) *C. jejuni* isolates produced cell distension. For CDT (+ve) and CDT (-ve) isolates, the cells at G2/M phase after 24, 48 and 72 h were 25.8 ± 3.79 per cent and 11.2 ± 0.58 per cent, 72.9 ± 2.44 and 14.3 ± 1.88 per cent, 93.5 ± 0.54 per cent and 18.0 ± 1.80 per cent respectively ($P < 0.001$). All CDT (+ve) isolates induced DNA fragmentation. Apoptosis induced by CDT (+ve) *C. jejuni* was significantly greater than CDT (-ve) (26.3 ± 3.49 % vs. 10.4 ± 1.01 % at 24 h, 43.9 ± 2.40 % vs. 17.6 ± 0.88 % at 48 h, 68.4 ± 1.61 % vs. 28.4 ± 1.62 % at 72 h); ($P < 0.001$).

Interpretation & conclusion: The present study shows that CDT (+ve) *C. jejuni* contributes to the pathogenesis through epithelial cell G2/M phase arrest and apoptosis.

Key words Apoptosis - *Campylobacter jejuni* - cell cycle arrest - cytolethal distending toxin - epithelial cell line

Campylobacter jejuni, a leading bacterial cause of gastroenteritis worldwide has also been implicated in a variety of other human systemic diseases such as Gullian-Barré syndrome and reactive arthritis¹. Although the association of *Campylobacter* spp. with human enteric disease is known, the molecular mechanisms involved in the pathogenesis are poorly understood. Various virulence factors such as flagellin, adhesion, lipopolysaccharide (LPS) and

cytolethal distending toxin (CDT) have been reported in *Campylobacter* spp. Besides *Campylobacter* spp, CDT has been reported in a number of other bacterial pathogens, including *Escherichia coli*²⁻⁴, *Shigella* spp.⁵, *Haemophilus ducreyi*⁶ and *Actinobacillus actinomycetemcomitans*⁷. The best described action of CDT is its ability to inhibit cell cycle progression by blocking intoxicated cells in G2 phase⁸. CDT is encoded by three adjacent genes, *cdtA*, *cdtB* and *cdtC*.

CDT-B has a DNase like activity⁹⁻¹¹; it blocks cell cycle progression by activation of a DNA damage checkpoint^{12, 13} that eventually leads to apoptosis¹⁴⁻¹⁵.

Recent data suggest that induction of host cell apoptosis is an important mechanism of pathogenesis in bacterial infections. CDT produced by *A. actinomycetemcomitans* and *H. ducreyi* has been reported to induce cell cycle arrest in cells of non epithelial origin¹⁶. *C. jejuni* also induces apoptosis of macrophage cell line through pathways that are distinct from *Salmonella* Typhimurium and *Shigella flexneri*¹⁷. However, little is known on cell cycle arrest and apoptosis in mammalian epithelial cells by *C. jejuni* toxin. Therefore, we investigated cell cycle arrest and apoptosis induction in HeLa cell line by *C. jejuni* cell culture supernatant and cell lysate containing crude CDT.

Material & Methods

Bacterial isolates: The study was conducted in the Department of Microbiology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow. A total of 10 randomly selected *C. jejuni* isolates [5 CDT (+ve) and 5 CDT (-ve)] from a pool of 41 were included in the study. These isolates were obtained from stool samples of diarrhoeic individuals belonging to a rural community of Mohanlalganj block, Lucknow (UP, India) from July to December 2002. The details of these isolates and their isolation and identification protocols have already been published¹⁸. Presence / absence of *cdtB* in these isolates were confirmed by two different sets of primers, one set (forward- 5' GTTAAAATCCCCTGCTATCAACCA 3' and reverse- 5' GTTGGCACTTGGAATTTGCAAGGC 3') reported in an earlier study¹⁹ and the other set (forward- 5' GCGTTGATGTAGGAGCTAATCGTG 3' and reverse-5'GGTTGATCGCGTTGAGTTCGTT 3') designed in our laboratory (gene bank accession no DQ882648) using Biosoftware (Primer Premier, PREMIER Biosoft International, Palo Alto, CA, USA). The primers were commercially synthesized by Operon, Germany. The details of PCR protocol for the detection of *cdtB* gene, preparation of culture supernatant and lysate had been described in our earlier study²⁰. Culture supernatants and the lysates were dialyzed ten-fold using polyethylene glycol 600 (SRL, India) and stored at -20°C till use.

Demonstration of CDT activity in culture supernatant and bacterial lysate: CDT activity was demonstrated on HeLa (Human tumour epithelial) cell line following

standard protocol as described earlier²⁰. The cell line was obtained from National Centre for Cell Sciences, Pune (India). In brief, HeLa cell lines grown to confluent in Eagle's minimum essential medium (MEM) with 10 per cent foetal calf serum (FCS) (Gibco BRL, Germany), 2 mM L- glutamine (Sigma, MO, USA), 1.5 g/l Na-bicarbonate (Sigma, MO, USA), 10 mM N-(2-hydroxy-ethyl) piperazine-N'-ethanesulphonic acid (Sigma, MO, USA) were trypsinized and seeded into 24- well tissue culture plates (Nunc, Denmark) at a density of 2×10^4 cells per well in 0.5 ml medium. The plates were incubated overnight to allow the cells to attach to the bottom of the plates. Doubling dilutions of culture filtrates and bacterial lysates were prepared in MEM and 0.5 ml of each dilution was added to HeLa cell line and incubated for 72 h at 37 °C in an atmosphere of 5 per cent CO₂. The cells were examined under inverted microscope (Olympus- model CKX31; Japan) at every 24 h for demonstration of morphologic changes. CDT activity titre was defined as the reciprocal of the highest dilution that produced distension and rounding in > 50 per cent of the cells.

Analysis of cell cycle arrest: To measure cell cycle arrest induced by culture supernatant and lysate, 60 mm tissue culture plates were seeded with HeLa cells (2×10^5 cells/ml) in MEM containing 10 per cent FCS and allowed to adhere for 4 h. Culture supernatant and cell lysate (undiluted) were added and incubated for 24, 48 and 72 h. HeLa cells were washed with PBS and stained in the dark at 4°C for 1 h with propidium iodide (PI, 10 µg/ml) containing RNase (1 mg/ml). Flow cytometry analysis of DNA content of the cells was performed with a FACSCalibur flow cytometer (Becton Dickinson, USA) and 10,000 events were collected. Cell cycle analysis was performed by cellquest software (Becton Dickinson, USA).

Assessment of apoptosis: HeLa cells co-cultured with bacterial culture supernatant and lysate as described above were examined by microscopy for morphologic changes suggestive of apoptosis at different time points 24, 48 and 72 h; harvested cells were also processed for DNA fragmentation as described^{21,22}. Briefly, extracted DNA from lysed cells was dissolved in 10 µl distilled water and treated with RNase A (10 µg/ml at 37°C for 45 min), and electrophoresed on 1.5 per cent agarose gel containing 0.5 µg of ethidium bromide per ml. DNA fragments were visualized under UV light transilluminator (UVS System, Hammond, USA). Cells exposed to UV light at 25 joule for 3 min were used as positive control.

Table I. Screening of *C. jejuni* isolates for the presence of cytolethal distending toxin in culture supernatant using HeLa cell line

Isolate No.	Dilution	Cytotoxicity on HeLa cell line at different dilutions and hours of incubation														
		0			8			16			32			64		
		24	48	72	24	48	72	24	48	72	24	48	72	24	48	72
CDT +ve																
837		++	+++	+++	++	+++	+++	+	++	+++	+	++	+++	+	+	++
556		++	+++	+++	++	+++	+++	+	++	+++	+	+	++	+	+	++
314		++	+++	+++	++	++	+++	-	++	+++	-	+	++	-	+	++
1315		++	+++	+++	++	++	+++	-	++	++	-	+	++	-	-	+
668		++	++	+++	++	++	+++	-	++	++	-	+	++	-	-	+
CDT -ve																
356		+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
130		+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
584		+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
978		+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
701		+	+	+	-	-	-	-	-	-	-	-	-	-	-	-

The degree of cell distension and rounding at different dilutions (8, 16, 32 and 64) after different time points (24, 48 and 72 h). +, <50%; ++, 50-75%; +++, >75% distension and rounding of cells; -, no visible morphological changes

Flowcytometric analysis of apoptosis: At 24, 48 and 72 h post inoculation with culture supernatant and cell lysate, HeLa cells on 60-mm tissue culture plate were treated with 1× trypsin-EDTA (Invitrogen, USA) for 10 min at 37°C; cells were then removed from plate by gentle scrapping, dispensed into cold PBS and washed twice. The cells were resuspended in 1× binding buffer at a concentration of approximately 1×10⁶ cells/ml; 5 µl of Annexin V- FITC and 10 µl of PI to each cell suspension were added and incubated at room temperature for 10 min in the dark. The cells were subjected to fluorescence estimation using a FACSCalibur flow cytometer. Uninoculated HeLa cells stained with Annexin V- FITC and PI were used to determine background levels of apoptosis.

Statistical analysis: Data were analyzed with SPSS statistical software, version 12.0 (SPSS Inc., Chicago, IL, USA). Results were expressed as means ± standard deviation (SD). Comparison of cell cycle arrest and apoptosis among CDT (+ve) and CDT (-ve) culture supernatants were performed using Student-Newman-Keuls (SNK) *post hoc* test and *P*<0.05 was considered significant.

Results

Bacterial isolates: Of the 41 *C. jejuni* isolates, 36 were positive and 5 were negative for *cdtB* gene by both sets of primers. All 5 *cdtB* gene negative and five randomly selected *cdtB* gene positive *C. jejuni* isolates were analysed for CDT activity and HeLa cell apoptosis.

Table II. Cell cycle analysis of *C. jejuni* culture filtrate treated HeLa cells after different time of incubation

Isolate no.	Cell cycle arrest at G2/M phase (%)		
	24 h	48 h	72 h
CDT (+ve)			
837	26.72	74.0	94.0
556	32.05	72.71	92.97
314	22.38	76.27	94.31
1315	23.77	69.58	93.43
668	24.42	72.28	93.26
Mean ± SD	25.8± 3.79	72.9± 2.44	93.5± 0.54
CDT (-ve)			
356	11.38	14.05	14.71
130	12.25	16.87	18.92
584	10.76	13.86	19.54
978	11.24	12.45	19.38
701	10.58	12.62	18.10
Mean ± SD	11.2± 0.58	14.3± 1.88	18.0± 1.80
Negative control	11.40	16.48	17.36

CDT (+ve) vs. CDT (-ve), *P*<0.001

CDT activity of culture supernatant and lysate: Both the culture supernatant and lysate of all 5 CDT (+ve) *C. jejuni* isolates produced distension and rounding in HeLa cells; 3 isolates had CDT activity titres 1 in 64 and two had 1 in 32. None of the CDT (-ve) isolates showed CDT activity (Table I).

Effect of *C. jejuni* supernatant on the cell cycle: Cell cycle arrest of HeLa cells treated with culture supernatant of CDT (+ve) isolates at G2/M phase by

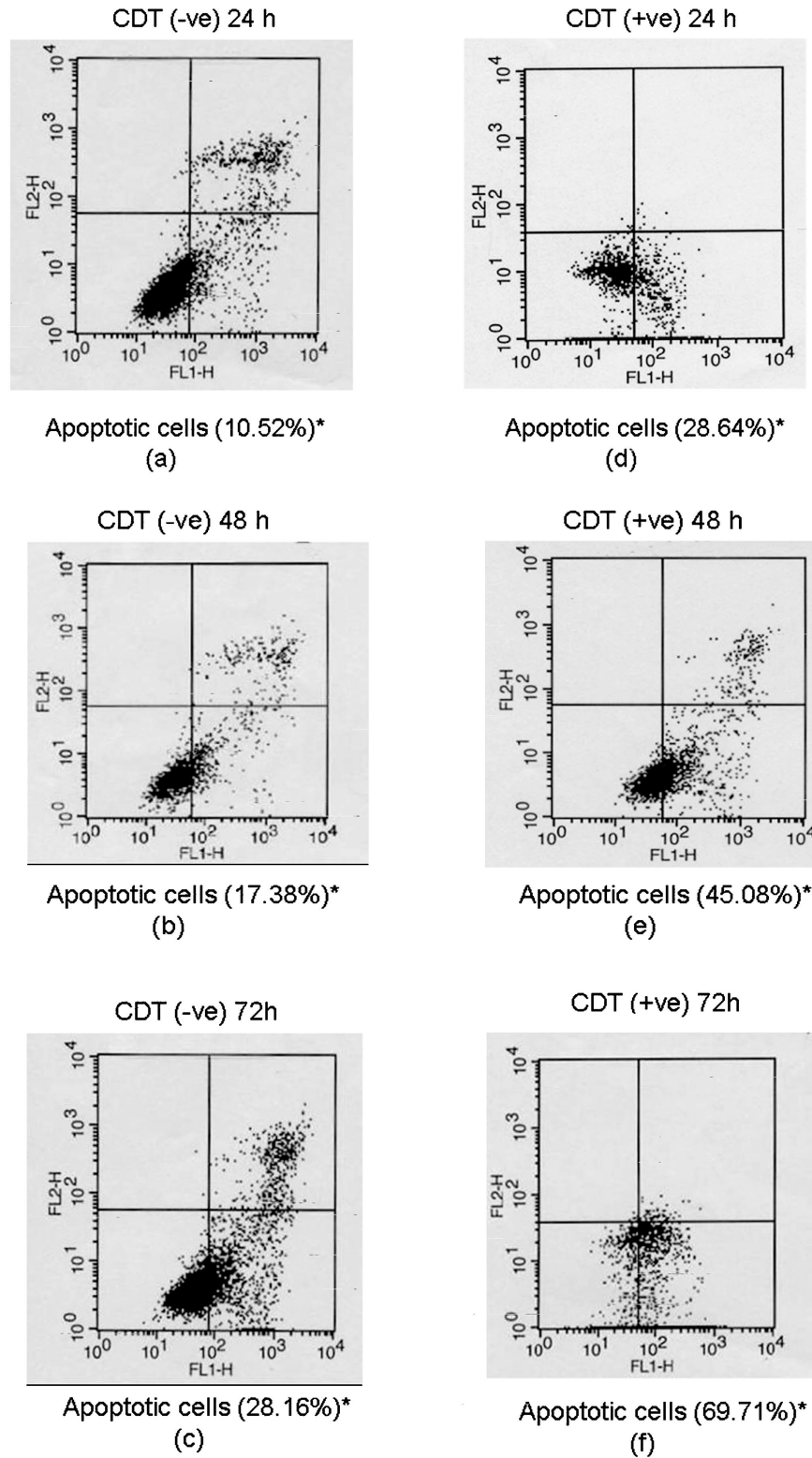


Fig. Effect of culture supernatant of a CDT (+ve) and a CDT (-ve) *C. jejuni* isolate on HeLa cells showing per cent of apoptotic cells. Annexin V/PI staining of HeLa cells after co-culture with culture supernatant of CDT (-ve) (a to c), and CDT (+ve) isolate (d to f) at different time period.

flowcytometry after 24, 48 and 72 h were as follows: 25.8 ± 3.79 , 72.9 ± 2.44 and 93.5 ± 0.54 per cent respectively, and with CDT (-ve) isolates the cell cycle arrest values at G2/M phase were 11.2 ± 0.58 at 24 h, 14.3 ± 1.88 at 48 h and 18.0 ± 1.80 per cent at 72 h (Table II). Similar consistent results were also observed with the lysate (data not shown).

Demonstration of cell apoptosis: Culture supernatants and lysates of only CDT (+ve) isolates induced apoptotic features such as cell shrinkage and apoptotic bodies, and DNA fragmentations in HeLa cells after 24 h co-culture. Apoptotic cells were quantified in CDT (-ve) and CDT (+ve) culture supernatants and lysates treated cells by flowcytometry. Apoptotic cells following co-culture with CDT (-ve) and CDT (+ve) culture supernatant at 24, 48 and 72 h was 10.4 ± 1.01 and 26.3 ± 3.49 per cent, 17.6 ± 0.88 and 43.9 ± 2.40 per cent, 28.4 ± 1.62 and 68.4 ± 1.61 per cent, respectively. Results of apoptosis induced by a CDT (-ve) and a CDT (+ve) isolate are shown in Fig. Both cell cycle arrest and the apoptosis was significantly higher in CDT (+ve) isolates as compared to CDT (-ve) at 24, 48 and 72 h ($P < 0.001$).

Discussion

The present study demonstrated the differences in cytotoxicity and apoptotic properties of CDT (+ve) and CDT (-ve) isolates of *C. jejuni*. CDT activity in culture supernatant and cell lysates of *C. jejuni* isolates was confirmed on HeLa cells by distension in > 50 per cent of the cells. All the five CDT (+ve) isolates showed CDT activity at higher dilutions (> 1 in 32) whereas none of the CDT (-ve) isolates showed CDT activity. The cytotoxicity of CDT (+ve) *Campylobacter* isolates in the present study was similar to the previous studies^{19,23}. However, one study had reported the cytotoxicity in 17.0 per cent²⁴ and another in 28.6 per cent of CDT (+ve) *C. jejuni* isolates²⁵. More prominent cytotoxic effects have been reported in freshly seeded HeLa cells than in semi confluent monolayers²⁶. Freshly seeded cells may undergo active growth and protein synthesis and may, therefore, be more susceptible to the toxin. In the present study, all the CDT (+ve) isolates caused distension and rounding in freshly seeded HeLa cells at dilutions (≥ 1 in 32) suggesting that HeLa cells are good markers for detection of cytotoxic activity of *C. jejuni*.

Cell cycle arrest at G2/M phase in different cell lines by CDT of important bacterial pathogens including *C. jejuni* had been reported in literature^{16,27}. In the present study, culture supernatant and sonicated

cell lysates of CDT (+ve) isolates induced cell cycle arrest at G2/M phase in almost similar proportions, which progressively increased over time; however, CDT (-ve) isolates did not induce cell cycle arrest even after 72 h. Our result strongly suggested that CDT was the cytotoxic factor present both in the culture supernatant and the sonicated cell lysates of CDT (+ve) isolates. This observation further substantiates that an important function of CDT is to block cell cycle at G2/M phase. Comayras *et al*⁸ reported that CDT treatment caused HeLa cells to accumulate the inactive, tyrosine-phosphorylated form of cyclin-dependent kinase (CDC2). Whitehouse *et al*²⁷ further confirmed that the treatment of cells with CDT produced by *C. jejuni* failed to activate CDC2, which resulted to cell cycle arrest in G2 phase. These studies also suggested that CDT triggered cell cycle arrest by way of a DNA damage checkpoint.

Besides cell cycle arrest, we also studied apoptosis of HeLa cells treated with CDT (+ve) and CDT (-ve) *C. jejuni* supernatant and lysate. Presence of apoptotic DNA fragments in CDT (+ve) treated cells was demonstrated. CDT (+ve) isolates induced apoptosis in significantly higher proportions as compared to CDT (-ve) isolates. The proportions of apoptosis in CDT (+ve) and CDT (-ve) isolates suggested that culture supernatant and cell lysate of *C. jejuni* with CDT activity induced apoptosis in cells of epithelial origin. Apoptosis in HeLa cells by CDT (+ve) *C. jejuni* highlights the role of CDT in the pathogenesis. Mooney *et al*²⁸ reported that *C. upsaliensis* cell lysates produced distension, cell cycle arrest and apoptosis in HeLa and human T cells. Previous studies showed that abnormal chromatin condensation and nuclear fragmentation occurred during cell death following exposure to CDTs of *C. jejuni* and other bacteria^{7,27}. Siegesmund *et al*¹⁶ have shown that a *C. jejuni* wild isolate induced apoptosis in 63 per cent of human monocytic cell lines.

In conclusion, both the culture supernatant and the cell lysate of only CDT (+ve) *C. jejuni* isolates induced distension, G2/M phase arrest and apoptosis in HeLa cell lines. These observations emphasize the role of *C. jejuni* CDT in pathogenesis of enteritis in human and call for further study to elucidate the exact mechanism of cell cycle arrest and apoptosis in epithelial cells with purified CDT. *In vivo* demonstration of apoptosis on gut epithelium, the interaction between host cell and *C. jejuni* CDT, and their clinical relevance are the other important areas for investigations.

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