***Campylobacter upsaliensis* exerts a cytolethal distending toxin effect on HeLa cells and T lymphocytes**

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***Campylobacter upsaliensis* is an emerging human enteropathogen. However, little is known about the pathogenesis of *C. upsaliensis* infection. In this study the authors demonstrate that *C. upsaliensis* whole-cell preparations and extracts produce a cytolethal distending toxin (CDT)-like effect on HeLa cells characterized by progressive distension and nuclear fragmentation culminating in cell death over 5 d. To further delineate the nature of this toxic effect in relation to CDT from other pathogens, the effect of *C. upsaliensis* on cellular events in epithelial cells and immunocytes was investigated. *C. upsaliensis* lysate-treated HeLa cells subjected to FACScan analysis using carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) as a cell tracer demonstrated cell division arrest. Propidium iodide (PI) staining of HeLa cells revealed that cell cycle arrest occurred in G₂/M. Human T lymphocytes exposed to *C. upsaliensis* lysates also showed cell cycle arrest in G₂/M. Using a combination of Annexin V/PI staining and TUNEL assay, cytodistended HeLa cells were shown to undergo apoptotic cell death. These data provide the first insights into the virulence mechanisms of this novel enteropathogen.**

Keywords: enteropathogen, CDT, virulence factors, apoptosis

INTRODUCTION

Campylobacter upsaliensis is a recently described member of the genus *Campylobacter* that has been associated with enteric infection in humans (Steele *et al.*, 1985; Patton *et al.*, 1989; Lindblom *et al.*, 1995; Jimenez *et al.*, 1999). The precise prevalence of *C. upsaliensis*-associated diarrhoea is uncertain because this species is sensitive to antibiotics present in *Campylobacter*-selective media used in most clinical laboratory settings. However, when specifically sought using specially devised media or a filtration technique *C. upsaliensis* accounts for up to 20% of all *Campylobacter* isolates identified from stools (Bourke *et al.*, 1998). *C. upsaliensis* is particularly common in some groups, including those who are immunocomprised (Patton *et al.*, 1989; Jenkin & Tee, 1998), and among children (Lindblom *et al.*, 1995; Lastovica & LeRoux, 1993). *C. upsaliensis* has been associated with extra-enteric infections including septicæmia (Patton *et al.*, 1989; Chusid *et al.*, 1990),

haemolytic-uræmic syndrome (Carter & Cimolai, 1996) and more recently Guillain-Barre syndrome (Goddard *et al.*, 1997; Ho *et al.*, 1997).

Despite considerable evidence for a role of *C. upsaliensis* in human disease, little is known of the molecular biology or pathogenic mechanisms of this organism (Bourke *et al.*, 1995, 1996, 1998). In particular, few studies have investigated the mechanisms by which *C. upsaliensis* causes disease. It is known that the organism is motile and adheres to tissue culture cells *in vitro* (Bourke *et al.*, 1998). However, the mechanisms underlying adhesion and its contribution to virulence have not been explored.

There have been few reports of the investigation of toxin activity in *C. upsaliensis* (Bourke *et al.*, 1998). However, Pickett *et al.* (1996) noted both the presence of a cytolethal distending toxin B gene (*cdtB*) homologue and cytolethal distending toxin (CDT) activity in the *C. upsaliensis* type strain. Furthermore, CDT activity was demonstrated for two *C. upsaliensis* strains isolated from children with diarrhoea in Italy (Musmanno *et al.*, 1998). CDT, first described by Johnson & Lior (1988) is produced by a number of bacterial pathogens, including

Abbreviations: CDT, cytolethal distending toxin; CFDA-SE, carboxyfluorescein diacetate succinimidyl ester; CFSE, carboxyfluorescein succinimidyl ester; PHA, phytohaemagglutinin; PI, propidium iodide.

Escherichia coli (Scott & Kaper, 1994; Pickett *et al.*, 1996; Peres *et al.*, 1997), *Shigella* species (Okuda *et al.*, 1997), *Haemophilus ducreyi* (Cope *et al.*, 1997), *Actinobacillus actinomycetemcomitans* (Sugai *et al.*, 1998) and *Campylobacter jejuni* (Pickett *et al.*, 1996; Purdy *et al.*, 2000). This toxin alters host cell cytoskeletal structure (Aragon *et al.*, 1997) and causes cell cycle arrest in epithelial cells (Peres *et al.*, 1997; Whitehouse *et al.*, 1998) and T cells (Shenker *et al.*, 1999; Gelfanova *et al.*, 1999) through its effect on cyclin-dependent kinases (Comayras *et al.*, 1997; Whitehouse *et al.*, 1998). Because preliminary reports suggested that *C. upsaliensis* also harbours a CDT-like activity we investigated the effects of this species on tissue culture cells and human T cells.

METHODS

Bacterial strains media and culture. The bacterial isolates used in this study are indicated in Table 1. The *C. upsaliensis* isolates comprised four human clinical strains obtained from the Laboratory Centre For Disease Control, Winnipeg, Canada, and the ATCC type strain. For the toxin and hybridization studies, isolates of *Campylobacter jejuni* and *Helicobacter pylori* (Table 1) were used as positive and negative controls, respectively. *Campylobacter* and *Helicobacter* strains were grown on Columbia blood agar plates at 37 °C under microaerobic conditions.

Preparation of bacterial lysate and cell culture supernate. *C. upsaliensis* organisms were harvested from blood agar plates in RPMI 1640 cell culture medium. The volume of medium used was adjusted so that the OD₆₀₀ of the bacterial suspension was 0.125 (2×10^8 c.f.u. ml⁻¹). Bacteria were centrifuged at 4800 r.p.m. for 10 min and the supernatant collected. The bacterial pellet was resuspended in RPMI 1640 tissue culture medium and the bacteria lysed by sonication (4 × 30 s bursts with 30 s intervals between each burst). Cell debris and unlysed bacteria were then removed by centrifugation, 10000 r.p.m. for 20 min. Bacterial supernate and cell lysate were sterilized by passage through a 0.2 µm filter, split into aliquots and stored at -20 °C.

Detection of cytotoxic activity in culture supernatants and

bacterial lysates. HeLa cells were grown to confluence at 37 °C in RPMI 1640 tissue culture medium containing 5% fetal calf serum. Cells were trypsinized and seeded into 24-well tissue culture plates at a density of 2.5×10^4 cells per well in 500 µl medium. Cells were allowed to attach to the base of the wells overnight prior to addition of *C. upsaliensis* organisms, culture supernate or bacterial lysate. Doubling dilutions of the bacterial cultures, supernate and lysate were made and 500 µl amounts added to the wells; the plates were then incubated at 37 °C in an atmosphere of 5% CO₂ for up to 5 d. For experiments using unlysed bacteria, 500 µl of organisms (equivalent to 1×10^8 c.f.u.) suspended in RPMI were incubated for 4 h with HeLa cells and then the suspension was removed and replaced with tissue culture medium. Each day a plate was removed from the incubator and morphological changes in the cells were examined by Giemsa staining and microscopy. Toxin titres were expressed as the reciprocal of the highest dilution that distended 50–70% of cells in the well. Toxin assays were performed in duplicate.

Demonstration of HeLa cell nuclear changes. HeLa cells (2.5×10^5) were grown on glass coverslips for 24 h prior to the addition of cell lysate. At daily intervals cells were stained with ethidium bromide and acridine orange; the coverslips were mounted in DPX and subsequently cells were viewed by fluorescence microscopy.

Demonstration of cell cycle arrest. HeLa cells were grown overnight in 25 cm² flasks (5×10^5 cells per flask). The following day, cells were labelled with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE; Molecular Probes). After diffusion into host cells CFDA-SE undergoes acetate modification. The probe is colourless and non-fluorescent until its acetate groups are cleaved by intracellular esterases to yield highly fluorescent, amine-reactive carboxyfluorescein succinimidyl ester (CFSE). The dye-protein adducts that form in labelled cells are retained by the cells throughout development. After each cell division the amount of label inherited by the daughter cells is halved. Cells were washed with prewarmed PBS (37 °C) containing the CFDA-SE probe (0.01 mM) and incubated at 37 °C for 15 min. The probe was replaced with fresh prewarmed cell culture medium and cells were further incubated for 30 min at 37 °C. Cell lysate was then added to the cells. At daily intervals a flask of cells was removed from the incubator, trypsinized, and fluorescence of the cells monitored using flow cytometry. A total of 10000 events was collected and analysed using the LYSIS II software from Becton Dickinson.

Cell cycle arrest of HeLa cells was also monitored by propidium iodide (PI) staining of lysate-treated cells. Cells were removed from the flasks by trypsinization, and stained with PI using the DNAcon3 (Consul TS, TO, Italy) kit according to the manufacturer's instructions. Flow cytometry analysis of the DNA content of the cells was performed with a FACScan flow cytometer (Becton Dickinson). Data from 50000 cells were collected and analysed using the LYSIS II software. The signal of orange fluorescence (FL2, corresponding to PI) was measured by linear amplification.

Human T cell isolation and demonstration of T cell cycle alteration. Venous peripheral blood was obtained from healthy adult volunteers. Mononuclear cells were isolated by Ficol Hypaque (Lymphoprep, Nycomed, Norway) density-gradient centrifugation and resuspended in Lymphoquick T cell (VH Bio, UK) for 45 min at 37 °C to deplete B cells and monocytes. By comparing anti-CD45:anti-CD3 ratios using FACScan analysis the resulting populations were shown to be > 90% CD3+.

Table 1. Bacterial strains used in this study

Species	Strain no.	Isolated from	Source*
<i>C. upsaliensis</i>	ATCC 43954	Dog	ATCC
<i>C. upsaliensis</i>	17234	Human	LCDC
<i>C. upsaliensis</i>	17237	Human	LCDC
<i>C. upsaliensis</i>	17282	Human	LCDC
<i>C. upsaliensis</i>	17283	Human	LCDC
<i>C. jejuni</i>	ATCC 33291	Human	ATCC
<i>H. pylori</i>	PU3	Human	OLHSC

* ATCC, American Type Culture Collection, Manassas, VA, USA; LCDC, Laboratory Centre For Disease Control, Winnipeg, Canada; OLHSC, Our Lady's Hospital For Sick Children, Dublin.

Fig. 1. Effect of *C. upsaliensis* on morphology of HeLa cells. (a–d) Giemsa staining of HeLa cells after interaction with *C. upsaliensis* ATCC 43954 lysate for 24 h (a) and 72 h (c), compared with untreated control cells at 24 h, (b); and 72 h, (d) respectively. (e, f) Acridine orange/ethidium bromide staining of HeLa cells 72 h after interaction with *C. upsaliensis* 17234 lysate (e) and *H. pylori* PU3 lysate (f). Magnification $\times 170$ (a–d), $\times 425$ (e, f).

Two hundred microlitres of a 1×10^6 cells ml^{-1} suspension of cells were cultured in 96-well plates with phytohaemagglutinin (PHA; $10 \mu\text{g ml}^{-1}$, Sigma-Aldrich) in RPMI 1640 supple-

mented with glutamine, gentamicin and 10% fetal calf serum in the presence and absence of *C. upsaliensis* lysate. The cells were incubated for 72 h at 37°C in a humidified incubator

Table 2. CDT activity of lysates of *C. upsaliensis* isolates

Titre values represent the reciprocal of the highest dilution at which 50% or more cells exhibited morphological changes of enlargement and distension.

Day	Titre with lysate from:				
	17234	17237	17282	17283	ATCC 43954
1	8	4	16	8	32
2	128	6	128	64	256
3	256	128	256	128	1024
4	512	128	256	256	1024
5	512	128	256	256	1024

containing 5% CO₂, harvested and analysed by flow cytometry for cell cycle arrest using PI and CFSE. Individual flow cytometry experiments were repeated at least twice.

TUNEL assay and Annexin V/PI staining. For the TUNEL assay, HeLa cells treated with cell lysate were probed at 24 h intervals up to 5 d using BODIPY-FLX-14-dUTP probe (Molecular Probes), according to the manufacturer's instructions. Briefly, HeLa cells on mounted coverslips were overlaid with 200 µl reaction medium consisting of TdT buffer solution (1 M potassium cacodylate, 125 mM Tris/HCl, 1.26 ng BSA ml⁻¹), 2.5 mM CoCl₂, 50 U TdT (Boehringer) and 1

nM BODIPY-FLX-14-dUTP. Coverslips were incubated for 1 h at 37 °C in humidified chambers, washed with PBS, air-dried and viewed using UV microscopy. Untreated HeLa cells served as controls.

For Annexin V/PI staining, cells treated with cell lysate were removed from tissue culture flasks by trypsinization, washed twice in cold PBS and resuspended in binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) at a concentration of 10⁶ cells ml⁻¹. One hundred microlitres of cells was transferred to a FACScan tube to which was added 10 µl of a 10 µg ml⁻¹ fluorescein-conjugated Annexin V solution (IQ Corp, Groningen, Netherlands) and 10 µl of a 50 µg ml⁻¹ PI solution. Annexin V is a phosphatidylserine-binding protein that detects phosphatidylserine on the surface of cells undergoing apoptosis, whereas PI associates with nuclear DNA, indicating necrotic cell death. Cells were vortexed gently and then incubated at room temperature for 15 min in the dark. Then 400 µl binding buffer was added to the cells and they were analysed by flow cytometry within 1 h of staining. The data from 10000 cells were collected and analysed using the LYSIS II software. The signals of green fluorescence (FL1; Annexin V) and orange fluorescence (FL2; PI) were measured by logarithmic amplification.

RESULTS

Effect of *C. upsaliensis* lysates on the cell cycle

Initially we examined the effect of a variety of *C. upsaliensis* isolates on HeLa cells. As shown in Fig. 1(a-d), *C. upsaliensis* ATCC 43954 lysates produced

Fig. 2. (a) Cell cycle analysis of HeLa cells following interaction with *C. upsaliensis* ATCC 43954 lysates (open peaks) compared with untreated control cells (shaded peaks) using CFSE staining, at the indicated times. HeLa cells exposed to bacterial lysates show unaltered fluorescence intensity throughout the observation period. In contrast, untreated HeLa cells show decreasing fluorescence intensity as the fluorescent dye is progressively partitioned into daughter cells over the 4 d time period. (b) Induction of G₂/M block in HeLa cells by *C. upsaliensis* ATCC 43954 lysates (shaded peaks) compared with uninfected control cells (open peaks) using PI staining. Flow cytometer settings were adjusted to have G₁ (2n DNA) and G₂/M (4n DNA) at approximately 250 and 500 fluorescence units, respectively. There is accumulation of lysate-exposed HeLa cells in G₂/M from day 2. In contrast, 98% of untreated cells remained in G₁ by day 3.

Fig. 3. (a) Human T cells labelled with CFSE 72 h after stimulation with PHA in the presence or absence of *C. upsaliensis* ATCC 43954 lysate. The open peak superimposed on the graphs of PHA-stimulated T cells (both lysate-treated and untreated) represents the unstimulated T cell population in each case. The percentages of the original population of T lymphocytes having undergone cell division (M1) and those remaining undivided (M2) are indicated for PHA-stimulated, lysate-untreated cells. Cells stimulated with PHA show a reduction in fluorescence intensity as cells divide, causing CFSE dye to be partitioned into daughter cells (top panel). In contrast, lysate-treated cells do not show stimulated cell division (bottom left) and cells treated with both lysate and PHA fail to divide, as evidenced by a single peak of fluorescence. (b) T cells stained with PI 72 h after exposure to *C. upsaliensis* ATCC 43954 lysates show an increased proportion of cells in G₂/M compared with untreated control cells. Flow cytometer settings were adjusted to have G₁ (2n DNA) and G₂/M (4n DNA) at approximately 70 and 140 fluorescence units, respectively. The percentage of T cells in cell cycle (M1) is indicated. The top right panel shows that PHA stimulation of non-lysate-treated T cells causes a proportion of the cells to enter cell cycle, with a small peak in the G₂/M (4n DNA) region. In contrast, PHA-stimulated lysate-treated T cells demonstrate a large accumulation of cells in the G₂/M (4n DNA) region (bottom right panel). The addition of lysate alone did not affect cell division (bottom left panel).

progressive distension of HeLa cells. *C. jejuni*-treated HeLa cells showed the same phenotypic changes, whereas HeLa cells exposed to lysates from *H. pylori* were unaffected (data not shown). In addition to the type strain, four clinical isolates of *C. upsaliensis* obtained from human stool specimens (LCDC strains 17234, 17237, 17282 and 17283) were tested for toxicity. Lysates from all five *C. upsaliensis* strains tested showed a cytodistending effect on HeLa cells, with some variation in titre (Table 2). Acridine orange/ethidium bromide staining of HeLa cells exposed to each of the five *C. upsaliensis* strains demonstrated progressive nuclear distension (Fig. 1e), characteristic of CDT (Whitehouse *et al.*, 1998; Sugai *et al.*, 1998).

As CDT from other organisms (Whitehouse *et al.*, 1998; Comayras *et al.*, 1997; Sugai *et al.*, 1998) interferes with cell cycle progression, we analysed the effect of *C. upsaliensis* lysates on HeLa cell division using CFSE as a probe. Fig. 2(a) shows that control HeLa cells unexposed to *C. upsaliensis* lysates (shaded peaks) underwent cell division, as evidenced by the progressive diminution of CFSE fluorescence intensity during day 1 to day 4 of incubation and the appearance of a second peak as the CFSE dye was inherited by daughter cells. Conversely, lysate exposed HeLa cells (represented by the open peaks in Fig. 2a) showed fluorescence intensity unaltered from day 1 to day 4, indicating arrest of cell division.

In order to determine in what phase the cell cycle arrest was occurring we undertook flow cytometry analysis of the DNA content of HeLa cells after PI staining. The results, shown in Fig. 2(b), indicate that the cells were blocked in G₂/M. The majority of control, untreated HeLa cells (open peaks) had 2n DNA content, with a small peak at twice the fluorescence intensity representing dividing cells transiently in G₂/M phase (4n DNA content). In comparison, lysate-exposed HeLa cells (shaded peaks) showed progressive accumulation of cells with 4n DNA content. By 72 h, 70% of lysate-treated cells were in G₂/M, whereas less than 2% of untreated cells were in G₂/M.

A similar pattern of cell cycle arrest was also seen among human T cells exposed to *C. upsaliensis* lysates. After 72 h, unstimulated cells (without PHA) had a single well-defined peak of high fluorescence intensity. Stimulation with PHA resulted in the appearance of a second peak of lower fluorescence intensity, indicating partitioning of CFSE dye into daughter cells. In contrast, lysate-treated cells stimulated with PHA did not show evidence of change in CFSE intensity, indicating almost complete abrogation of cell division (Fig. 3a). PI staining demonstrated that, in the absence of lysate, a proportion (27%) of PHA-stimulated T cells enter into the cell cycle, as evidenced by the small peak of fluorescence in the 4n DNA region (G₂/M) (Fig. 3b, top panels).

Table 3. CDT activities of bacterial cultures, supernatants and lysates of *C. upsaliensis* ATCC 43954

Day	Bacteria*	Supernatant†	Lysate†
1	2.0×10^8	—	32
2	1.3×10^7	64	256
3	3.9×10^5	64	1024
4	2.0×10^5	64	1024
5	2.0×10^5	64	1024

* Lowest number of bacteria added to the cells at which 50% or more cells exhibited morphological changes of enlargement and distension.

† Titre values represent the reciprocal of the highest dilution at which 50% or more cells exhibited morphological changes of enlargement and distension.

However, cells stimulated to divide in the presence of bacterial lysate showed a larger population (43%) in cell cycle and a substantial proportion of these were accumulating in G₂/M phase (Fig. 3b, bottom right panel).

Toxin effect of *C. upsaliensis* cultures, lysates and supernatants

The toxic effect produced by bacterial lysates, whole bacteria and bacterial supernatants is shown in Table 3. Unconcentrated *C. upsaliensis* supernatants demon-

strated a very low titre of activity. However, a toxin effect was evident from both lysates and fresh cultures. The relative effects of lysates and cultures were similar after 96 h incubation for the type strain (Table 3) and equivalent results were seen among the four other *C. upsaliensis* isolates tested (data not shown).

Demonstration of host cell apoptosis

CDT-treated epithelial cells have been shown in previous studies to have morphological features suggestive of apoptotic cell death. In order to determine if cytodistended HeLa cells underwent apoptosis we used the TUNEL assay to detect nuclear DNA fragmentation, characteristic of this form of cell death. Increased host cell apoptosis was clearly evident 48 h after exposure of HeLa cells and the numbers of cells with apoptotic nuclear changes increased progressively up to 5 d after exposure. Immunofluorescence microscopy (Fig. 4a) clearly demonstrated strongly TUNEL-positive nuclei among lysate-treated HeLa cells. This contrasted with the background staining and occasional TUNEL-positive nucleus among control cells (Fig. 4b).

To further investigate the nature of host cell death following exposure to *C. upsaliensis*, cells treated with bacterial lysate were stained with a combination of Annexin V and PI. Flow cytometry analysis of affected cells demonstrated increased surface expression of phosphatidylserine, as evidenced by increased Annexin V binding, compared to control untreated cells (Fig. 5). At the same time, these cells continued to exclude PI,

Fig. 4. (a) Fluorescence micrograph identifying apoptotic HeLa cells. At 96 h after exposure to *C. upsaliensis* ATCC 43954 lysates there are strongly fluorescent areas in the perinuclear region of cytodistended HeLa cells, indicating the presence of apoptotic (TUNEL-positive) DNA fragmentation. In contrast, untreated control cells (b) do not show TUNEL-positive nuclei. Magnification $\times 900$.

Fig. 5. Annexin V/PI staining of HeLa cells 48 h after exposure to lysates of *C. upsaliensis* ATCC 43954 (b) compared with uninfected control cells (a). Cells in the lower left of the cytogram are viable. Apoptotic cells in the lower right have increased Annexin V fluorescence without loss of viability. The percentages of cells in each quadrant are indicated.

which associates with the nuclear DNA of necrotic cells. Taken together these data clearly indicate that *C. upsaliensis*-lysate-treated, cytodistended HeLa cells undergo increased levels of apoptosis.

DISCUSSION

There exists considerable epidemiological evidence to support a substantial role for *C. upsaliensis* in human disease. However, partly because of the failure of commercially available *Campylobacter*-selective media to isolate this organism, *C. upsaliensis* is rarely isolated in clinical laboratories and is little known among clinicians (Bourke *et al.*, 1998). Consequently, the pathogenesis of *C. upsaliensis* infection is largely unexplored and data on potential virulence factors for this organism are scarce.

To date, no *C. upsaliensis* virulence determinants have been characterized in detail. This enteropathogen has been shown to adhere to tissue culture cells and lipids *in vitro* (Sylvester *et al.*, 1996). However, the bacterial factor(s) underlying cell adherence have not been ex-

plored. Preliminary studies suggested that this organism may harbour CDT activity (Pickett *et al.*, 1996; Musmanno *et al.*, 1998). Therefore, we evaluated the effect of whole-cell preparations and lysates of *C. upsaliensis* on tissue culture cells and human T lymphocytes. We have observed cytodistension, nuclear fragmentation, cell cycle arrest and apoptosis in affected host cells, suggestive of a CDT-like effect.

Direct proof that the observed host cell phenotypes are the result of CDT will rely on the construction of isogenic *cdt* mutant strains and comparison with wild-type organisms of their pathogenic effects. To date, despite intensive efforts, we have been unable to transform *C. upsaliensis* in order to construct isogenic mutant strains. Therefore, it is possible that the cytodistension, cell cycle arrest and cell death phenomena observed in this study are the result of bacterial factor(s) other than CDT. However, the characteristic nature of the *C. upsaliensis*-induced host cell events, coupled with the presence of *cdt* genes in all of 30 *C. upsaliensis* strains that we have tested (unpublished data), strongly support the notion that this organism harbours a CDT and that this virulence factor is probably common to most, if not all, *C. upsaliensis*.

Although the relevance *in vivo* of CDT is uncertain, Okuda *et al.* (1995) demonstrated watery diarrhoea following CDT administration in a suckling mouse model. Recently, Purdy *et al.* (2000) have shown that *C. jejuni* isogenic *cdt* mutants had reduced invasiveness in a mouse model of enteric infection. Furthermore, Albert *et al.* (1996) found CDT-positive *E. coli* strains more commonly among children with diarrhoea than among those without diarrhoea. *C. upsaliensis* produces self-limiting, watery diarrhoea in the majority of affected patients, whereas inflammatory diarrhoea with blood and leucocytes occurs infrequently (Jimenez *et al.*, 1999; Bourke *et al.*, 1998). Taken together these clinical and laboratory observations support the notion that *C. upsaliensis* diarrhoeal disease is caused, at least in part, by a toxin-induced increase in intestinal fluid secretion. CDT represents a potential candidate for this effect.

Compared with bacterial lysates *C. upsaliensis* supernatants had low or absent toxic effects in this assay (Pickett *et al.*, 1996). However, we have shown that cytodistending toxin effects are also produced when HeLa cells are exposed to fresh cultures of *C. upsaliensis*. It is difficult to draw meaningful conclusions from a direct comparison of the toxin activity induced by *C. upsaliensis* bacterial cultures and those from sonicated lysates. However, our data are consistent with the notion that the CDT activity is localized in the bacterial outer membrane, a site recently implicated for the CDT of *C. jejuni* (Hickey *et al.*, 1999). An alternative explanation is that toxin secretion can be induced on contact with host cells. Current experiments in our laboratory are aimed at addressing this question.

CDT has been reported previously to induce cell cycle arrest in cells of non-epithelial origin. For instance the periodontal pathogen *A. actinomycetemcomitans* pro-

duces a cell-cycle-inhibitory factor encoded by a *cdt* gene cluster (Sugai *et al.*, 1998) and this CDT effect appears to be responsible for G₂M arrest in T cells (Shenker *et al.*, 1999). More recently, *H. ducreyi* CDT has been implicated as a cause of T cell cycle inhibition and apoptosis (Gelfanova *et al.*, 1999). Our present report shows that *C. upsaliensis* lysates can induce G₂M phase arrest in T cells. These preliminary observations require further study in order to confirm the nature of the cell-cycle-inhibitory factor involved and the precise subpopulation of T cells targeted by this effect. However, these data suggest that *C. upsaliensis*, and perhaps other enteric campylobacters, may be able to modulate the local immune response in the intestine during the infection process. To our knowledge, modulation of the immune response by an enterobacterial toxin effect has not previously been reported.

The precise mechanism underlying CDT-mediated cell death is not defined. Recently, DeRycke *et al.* (2000) described multi-polar abortive mitosis and a micro-nucleation process as lethal events in CDT-exposed HeLa cells. Previously, immunofluorescence studies showed that abnormal chromatin condensation and nuclear fragmentation occurs during cell death following exposure to CDT (Whitehouse *et al.*, 1998). This appearance, also observed in HeLa cells following treatment with other bacterial CDTs (Sugai *et al.*, 1998), is reminiscent of apoptotic cell death. However, whether CDT-treated epithelial cells ultimately die through an apoptotic mechanism remains to be proven. A cytotoxic-necrotizing-factor-producing strain of *E. coli* also with CDT activity, BM2-1, induces apoptotic cell death (De Rycke *et al.*, 1996). It is also noteworthy that *C. jejuni* outer-membrane proteins cause apoptosis of chicken lymphocytes (Zhu *et al.*, 1999). Furthermore, *H. ducreyi* CDT is responsible for apoptosis in Jurkat cells (Gelfanova *et al.*, 1999), and *A. actinomycetemcomitans* causes murine macrophage apoptosis (Kato *et al.*, 1995). In the present study we examined HeLa cells treated with *C. upsaliensis* lysate for apoptosis using the TUNEL assay. The presence of apoptotic DNA fragmentation among affected cells was clearly evident among cytodistended, lysate-exposed HeLa cells after 48 h. Dual Annexin V/PI staining further confirmed that cytodistended cells predominantly undergo apoptosis rather than necrosis (Fig. 5). Given the importance of programmed cell death as a homeostatic mechanism for maintaining the balance of intestinal epithelial cells, the host cell mechanism(s) underlying *C. upsaliensis*-induced apoptosis and its relevance *in vivo* will be an important area for future investigation.

In summary, we have shown that *C. upsaliensis* cell lysates produce epithelial cell cytodistension and cell cycle arrest occurs both in tissue culture cells and in human T cells, effects characteristic of CDT. We also have confirmed that cytodistended epithelial cells undergo apoptosis. These data provide the first insights into the potentially complex interactions that underly the aetiopathogenesis of enteric infection by this emerging bacterial pathogen.

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