Microbiology (2005), **151**, 1395–1402 **DOI** 10.1099/mic.0.27671-0

Mechanism of internalization of the cytolethal distending toxin of *Actinobacillus actinomycetemcomitans*

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Received 1 October 2004
Revised 7 December 2004
Accepted 12 January 2005

Cytolethal distending toxin (CDT), which is encoded by three genes, *cdtA*, *cdtB* and *cdtC*, is now recognized to have a growing list of biological actions, including inhibition of cell cycle progression, promotion of apoptosis and stimulation of cytokine secretion. It appears that internalization of CDT is essential, at least for cell cycle blockade. Using purified recombinant CDT proteins from the periodontopathic bacterium *Actinobacillus actinomycetemcomitans*, the authors investigated which combination of toxin proteins produce cell cycle inhibition and which bound and/or entered into host cells. No evidence was found that CdtB bound to HEp-2 human epithelial cells. In contrast, both CdtA and CdtC bound to these cells. Induction of cell cycle arrest required that cells be exposed to both CdtB and CdtC. Pre-exposure of cells to CdtC for as little as 10 min, followed by removal of the free CdtC and addition of exogenous CdtB, resulted in the inhibition of cell cycle progression, suggesting that CdtB could bind to cell-surface-located CdtC. Using various methods to follow internalization of the CDT proteins it was concluded that CdtC acts to bind CdtB at the cell surface and transports it into the cell as a complex via an endosomal pathway blockable by monensin and brefeldin A.

INTRODUCTION

Actinobacillus actinomycetemcomitans, a Gram-negative, capnophilic, fermentative coccobacillus, has been implicated not only in the pathogenesis of serious periodontal disease (Slots & Ting, 1999), but also in a variety of human systemic diseases such as endocarditis, meningitis and osteomyelitis (Van Winkelhoff & Slots, 1999). The general pathogenic mechanisms and immune involvement in periodontitis, in particular the bacterial virulence factors responsible for the proposed immunosuppression in this disease, remain unclear. Studies suggest that bacterial virulence factors act to impair host defence mechanisms and play significant roles in infectious diseases associated with *A. actinomycetemcomitans* (Shenker, 1987; Wilson & Henderson, 1995; Henderson *et al.*, 2003). Some cytotoxic factors produced by *A. actinomycetemcomitans* have been studied at the molecular level, and cytolethal distending toxin (CDT) from this pathogen has been identified and cloned (Mayer *et al.*, 1999; Shenker *et al.*, 1999; Sugai *et al.*, 1998). CDT has also been isolated from various other pathogenic bacteria (Cope *et al.*, 1997; Okuda *et al.*, 1997; Pickett *et al.*, 1996; Scott & Kaper, 1994; Young *et al.*, 2000).

Abbreviation: CDT, cytolethal distending toxin.

With one exception, in all organisms studied, CDT is encoded by three contiguous genes, *cdtA*, *cdtB* and *cdtC*, which form an operon. The exception is *Salmonella typhi*, which encodes only CdtB (Parkhill *et al.*, 2001). The DNase activity of this protein depends on the internalization of the bacterium into the target cell (Haghjoo & Galan, 2004).

The best-described action of CDT is its ability to inhibit cell cycle progression by blocking intoxicated cells in G_2 (Comayras *et al.*, 1999; Cortes-Bratti *et al.*, 1999; Pickett & Whitehouse, 1999; Whitehouse *et al.*, 1999; reviewed by Henderson *et al.*, 1998). This cell cycle activity has been shown to occur in cells involved in the periodontal pathology that is assumed to be induced by *A. actinomycetemcomitans* (Belibasakis *et al.*, 2002; Yamamoto *et al.*, 2004). It has been suggested that CdtB is a DNase (Cortes-Bratti *et al.*, 2001; Elwell & Dreyfus, 2000; Lara-Tejero and Galan, 2000) and that cell cycle progression is blocked because of the activation of a DNA-damage-dependent checkpoint (Yamamoto *et al.*, 2004; Frisan *et al.*, 2003; Hassane *et al.*, 2003; Sato *et al.*, 2002) that eventually leads to apoptosis (Ohara *et al.*, 2004). The proposed role of CdtA and CdtC is to facilitate CdtB entry into the cell. CdtA has been found to localize to the plasma membrane (Mao & DiRienzo, 2002; Lee *et al.*, 2003), possibly through CdtA's

reported carbohydrate-binding domain. There is still controversy regarding the role of CdtC. There is evidence that this protein can also bind to the cell membrane (Lee *et al.*, 2003), which is confirmed in the present study as well as the fact that CdtC appears to enter the cell along with CdtB. Recently the crystal structure of the *Haemophilus ducreyi* CDT toxin has been elucidated and reveals that CdtC may have a modulatory effect on CdtB (Nesic *et al.*, 2004). Therefore CdtC's role may extend beyond the plasma membrane.

Cortes-Bratti *et al.* (2000) reported that intoxication of cells by CDT follows the clathrin-coat-dependent internalization of this toxin and its transport via the Golgi complex. That report strongly suggested that CDT enters the cell by endocytosis to exert its effect on the target nuclei. However, it remained unclear which complex of CDT proteins is required for the uptake of the active species. In the present study we used homogeneous recombinant CDT proteins from *A. actinomycetemcomitans* to identify which combination of CDT proteins forms the active internalized CDT complex.

METHODS

Bacterial strains and growth conditions. *A. actinomycetemcomitans* Y4 (serotype b, ATCC 43718) was grown on brain–heart-infusion agar (Oxoid) supplemented with 5 % (v/v) horse blood (Oxoid) at 37 °C for 2 days in an atmosphere of 5 % CO_2 in air, harvested from the plates with sterile saline and centrifuged at 3000 ***g*** for 20 min (Henderson *et al.*, 2001). *Escherichia coli* strains HMS174(DE3) (Novagen) and M607 were used in this study. *E. coli* was routinely grown in Luria–Bertani (LB) broth (Oxoid).

Cloning of *cdt* genes into an N-terminal polyhistidine expression vector. The oligonucleotides 5′-GGATCCTGTTCGTCAAATCAACGA-3′ and 5′-CTGCAGTTAATTAACCGCTGTTGC-3′ were designed to amplify the 625 bp *cdtA* gene. The oligonucleotides 5′-GGATCCAACTTGAGTGATTTCAAA-3′ and 5′-CTGCAGTTAGCGATCATGAACAAA-3′ were designed to amplify the 785 bp *cdtB* gene. The oligonucleotides 5′-GGATCCCATGCAGAATCAAATCCT-3′ and 5′-CTGCAGTTAGCTACCCTGATTTCT-3′ were designed to amplify the 506 bp *cdtC* gene. These primers were designed to amplify each *cdt* gene without the DNA encoding the N-terminal signal peptide, and also contained recognition sequences (underlined) for restriction enzymes *Bam*HI and *Pst*I respectively. Purified chromosomal DNA from *A. actinomycetemcomitans*, prepared using standard methods (Henderson *et al.*, 2001), was used as the template for PCR. The PCR fragments were initially cloned into pCR4-TOPO (Invitrogen) and transformed into *E. coli* TOP10 to give PCR4cdtA, PCR4cdtB and PCR4cdtC. The *cdt* genes were cut from the corresponding pCR4cdt construct by digestion with *Bam*HI and *Not*I (the *Not*I site was present in vector sequence) and ligated to similarly digested pET-28a(+) (Novagen). The ligation mixtures were transformed into *E. coli* HMS174(DE3) and transformants were selected by growing at 30 °C on LB agar containing 30 μg kanamycin ml^{-1}.

Expression of *cdt* genes and purification of recombinant proteins. For *cdt* gene expression, positive clones were grown overnight in LB broth containing kanamycin (30 μg ml^{-1}) and rifampicin (200 μg ml^{-1}), diluted 1 : 20 in fresh broth, and incubated for a further 2 h at 37 °C. Gene expression was induced with 1 mM IPTG for 6 h at 30 °C. Cells were harvested by centrifugation at 6000 ***g*** for 20 min, then resuspended and lysed for 10 min with B-PER protein extraction reagent (Pierce & Warriner). The expressed proteins were contained in inclusion bodies. Purification of inclusion bodies was performed as described by the manufacturer of the B-PER reagent. Briefly, lysates were centrifuged and pellets were resuspended with the same volume of B-PER containing lysozyme (100 μg ml^{-1}), and then incubated for a further 5 min at room temperature. Ten volumes of 1 : 10 diluted B-PER was added to the lysates, then the inclusion bodies were collected by centrifugation at 15 000 ***g*** for 20 min. After washing twice with the same volume of 1 : 10 diluted B-PER, the pellets were treated with 8 M urea containing 300 mM NaCl/100 mM sodium phosphate buffer (pH 8·0). The recombinant proteins were purified using Ni-nitrilotriacetic-acid-agarose columns under denaturing conditions as specified by the manufacturer (Qiagen). The refolding of denatured proteins was performed as described by Takemura *et al.* (2000). These recombinant proteins were designated His-CdtA, His-CdtB and His-CdtC.

In order to prepare Cdt proteins without a polyhistidine tag, the *cdt* genes were extracted from the corresponding pCR4cdt on an *Eco*RI fragment (the *Eco*RI sites were present in the cloning vector) and ligated to *Bam*HI-digested OmpA vector (Ghrayeb *et al.*, 1984). These plasmids were transformed into *E. coli* M607. The periplasmic fraction containing the recombinant Cdt protein was isolated from induced cells by resuspending harvested bacteria in 30 mM Tris/HCl (pH 8·0), 20 % sucrose, then agitating for 10 min at 4 °C. The cells were collected by centrifugation at 8000 ***g*** for 20 min and treated with 5 mM $MgCl_2$ for 10 min at 4 °C with gentle agitation. The supernatants were collected after centrifugation at 8000 ***g*** for 20 min at 4 °C and dialysed against PBS. These recombinant proteins were designated Omp-CdtA, Omp-CdtB and Omp-CdtC. These methods of protein purification were previously used and sufficiently verified by Akifusa *et al.* (2001) using SDS-PAGE with these same recombinant His-Cdt proteins. All proteins were homogeneous and migrated with molecular masses of about 26, 32 and 22 kDa, respectively. Although refolding of the individual CDT His-tag proteins was not assessed, all proteins were active in subsequent cellular assays (Fig. 1).

Cells and culture conditions. The human epithelial cell line HEp-2 (HeLa, ATCC CCL-23) cells was grown in Dulbecco's minimal essential medium (DMEM; Gibco) containing L-glutamine (Sigma), 10 % fetal calf serum (Invitrogen), 100 μg streptomycin ml^{-1} (Sigma) and 100 IU penicillin ml^{-1} (Sigma) in an atmosphere containing 5 % CO_2.

Analysis of the cell cycle. To measure cell cycle arrest induced by Cdt proteins, 5 ml well plates of HEp-2 cells (2×10^5 cells ml^{-1}) were cultured in DMEM with 40, 200 or 1000 ng ml^{-1} of Cdt proteins for 1 to 4 days. HEp-2 cells were washed and fixed for 60 min with 80 % cold ethanol. After washing, the cells were stained in the dark at 4 °C for 1 h with propidium iodide (10 μg ml^{-1}) in PBS containing RNase (1 mg ml^{-1}). In some experiments CdtC was pre-incubated for 10, 20, 40, 60 or 120 min or for 12 h with cells and this recombinant protein was then washed out with three changes of PBS. CdtB was then added and the cells were incubated for a further 72 h before being used for cell cycle analysis. The data from 2×10^4 cells were collected and analysed on a FACScan flow cytometer (Becton Dickinson). Cell cycle analysis was performed using CELLQUEST software.

Binding analysis. HEp-2 cells (1×10^6 cells in each well of the 24-well plates) were incubated for 120 min with each of the recombinant His-Cdt proteins at a temperature of either 4 or 37 °C in DMEM. These cultures were then washed three times with PBS to remove unbound His-Cdt, and incubated with 10 % normal goat serum/0·1 % sodium azide in PBS to block non-specific binding. A murine anti-polyhistidine antibody (Sigma) was then added at a 1 : 20 000 dilution for 30 min to detect the 6 × His tag. The cultures

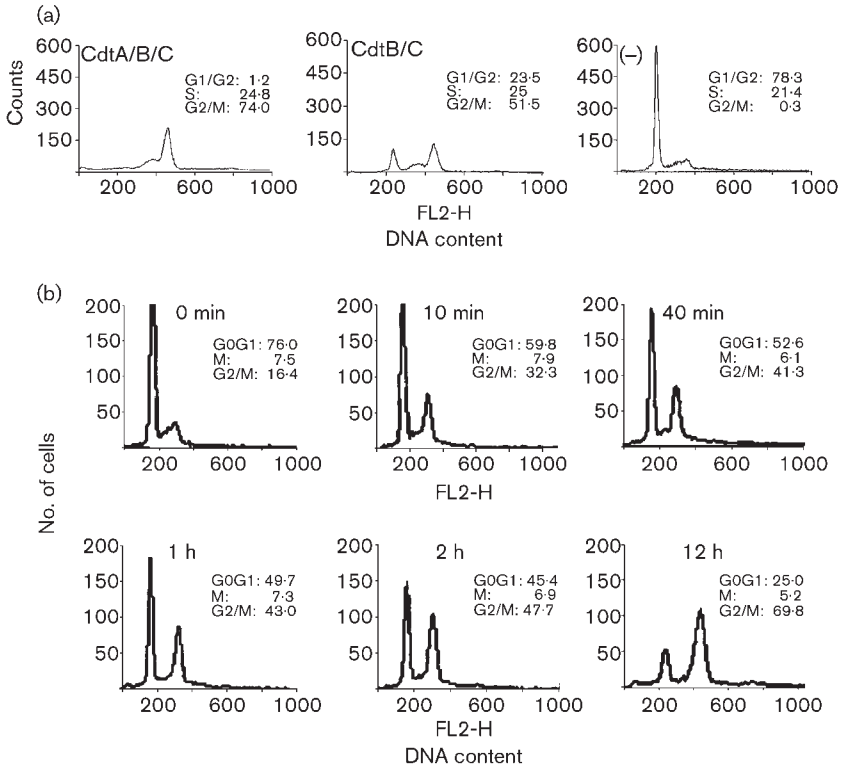

Fig. 1. DNA contents of HEp-2 cells treated with Cdt proteins. (a) HEp-2 cells were treated with recombinant His-tagged CdtA plus CdtB plus CdtC (denoted CdtA/B/C), or with CdtB plus CdtC (denoted CdtB/C) at a concentration of 1 μg ml^{-1}, or with medium alone (denoted –) for 3 days. (b) HEp-2 cells were treated with recombinant His-tagged CdtC (1 μg ml^{-1}) for 0, 10 or 40 min or for 1, 2 or 12 h, and then washed three times with PBS to remove unbound CdtC. CdtB (1 μg ml^{-1}) was then added to the cells, which were cultured for an additional 3 days. At the end of this period the cells were harvested, fixed with 80 % ethanol for 1 h, stained with 10 μg ml^{-1} propidium iodide and analysed by flow cytometry.

were washed three times to remove unbound mouse antibody and then incubated with a FITC-conjugated goat anti-mouse IgG F(ab′)$_2$ fragment (DAKO) for 30 min. After three washes with PBS, cells were removed mechanically with a rubber policeman and used for flow cytometry, with data from 2×10^4 cells being collected and analysed in each experiment.

Internalization assay. Flow cytometric analysis of the internalization of CDT was performed as described in the manufacturer's instruction for the DAKO IntraStain (DAKO). Briefly, HEp-2 cells (1×10^6 cells) were treated with various combinations of the recombinant Omp-Cdt and recombinant His-Cdt proteins at 37 °C for 12 h. To terminate the culture, cells were washed twice with PBS and removed from the plastic surface with trypsin/EDTA. Cells were resuspended in IntraStain Reagent A (fixation) and incubated at room temperature for 15 min. After washing twice in PBS, cells were resuspended with IntraStain Reagent B (permeabilization) and incubated with mouse anti-polyhistidine antibody (1 : 2000) at room temperature for 15 min. After washing twice in PBS, cells were incubated with FITC-conjugated goat anti-mouse IgG F(ab′)$_2$ fragment at room temperature for 30 min in the dark. Cells were washed twice and resuspended with 1 % paraformaldehyde in PBS. In each experiment the data from 2×10^4 cells were collected by flow cytometry and analysed.

Confocal microscopy. Fluorescent markers were monitored with a Leica TSC NT confocal laser scanning microscope (CSLM), using standard filter settings and sequential scanning to avoid overlap of emission from the fluorophores. Each His-Cdt protein was labelled, using standard methods, with FITC (Molecular Probes) and it was confirmed that the FITC-labelled proteins did not lose biological activity. Briefly, HEp-2 cells were incubated with FITC-labelled Cdt proteins (100 μg ml^{-1}) for 10 min, 2 h, 4 h or 6 h at 37 °C. At the end of this period, cultures were washed three times in PBS then fixed with 4 % paraformaldehyde in PBS for 20 min at room temperature. Rhodamine-conjugated phalloidin (Molecular Probes) was used to visualize the actin cytoskeleton when cells were observed by confocal microscopy. FITC fluorescence was detected by CSLM using standard fluorescein filter settings (488 nm argon laser line and BF530/30 filters and 568 nm krypton/argon laser line and BP600/30 filter). Rhodamine-phalloidin fluorescence was detected using standard rhodamine filter settings (568 nm krypton/argon laser line and BF600 filters).

To determine the effect of the inhibitors monensin and brefeldin A (both obtained from Sigma), these were added at concentrations ranging from 0·1 to 10 μM for 1 h prior to the addition of the toxin proteins and were present in the medium during the course of the experiment.

RESULTS

Which combination of CDT proteins induces cell cycle arrest at G_2/M?

The individual homogeneous recombinant *A. actinomycetemcomitans* CDT proteins failed, at all concentrations tested, to inhibit cell cycle progression. The only combinations to induce cell cycle arrest were CdtA/B/C and CdtB/C (Fig. 1a). This suggested that if CdtB was the active internalized component then CdtC may act to aid its internalization.

To determine if the binding of CdtC to the cell surface was sufficient to ensure the biological activity of CdtB, HEp-2 cells were incubated with CdtC (1 μg ml^{-1}) for various times ranging from 10 min to 12 h. After these times the CdtC was washed out with three changes of PBS. CdtB (1 μg ml^{-1}) was then added to the cells, which were

cultured for an additional 3 days before the DNA content was measured by flow cytometry. As shown in Fig. 1(b), even cells incubated for short periods of time with CdtC went into cell cycle arrest when incubated with CdtB.

Binding of CDT proteins to cells

The binding of the recombinant Cdt proteins to HEp-2 cells at both 4 and 37 °C was determined using flow cytometry. This study failed to demonstrate any binding of CdtB, even when added to cells at concentrations as high as 250 μg ml^{-1}. In contrast, the binding of both CdtA and CdtC at 37 °C was dose dependent (Fig. 2). Interestingly, CdtA bound to the cell surface at 4 °C, but CdtC failed to bind at this incubation temperature (data not shown). Monensin has been reported to inhibit the activity of CDT (Cortes-Bratti *et al.*, 2000) and we have confirmed these findings (results not shown). However, treatment of cells with monensin did not affect the ability of CdtA or CdtC to bind to the cell surface (data not shown).

Role of Cdt proteins in the internalization of CDT

To determine if individual Cdt proteins aid in the uptake of either themselves or other CDT proteins, recombinant non-His-tagged CDT proteins were individually incubated with other single-species His-CDTs in all possible permutations. This clearly showed that the only combination allowing the intracellular entry of a CDT protein was CdtC and CdtB (Fig. 3a). With this combination (His-CdtB with Omp-CdtC) a clear shift in fluorescence was observed, indicative of entry of CdtB into HEp-2 cells. This CdtC-mediated uptake of CdtB was completely inhibited by 10 μM monensin (Fig. 3b).

Direct microscopic assessment of CDT uptake

HEp-2 cells were incubated in the presence of individual FITC-labelled CDT proteins or various combinations of

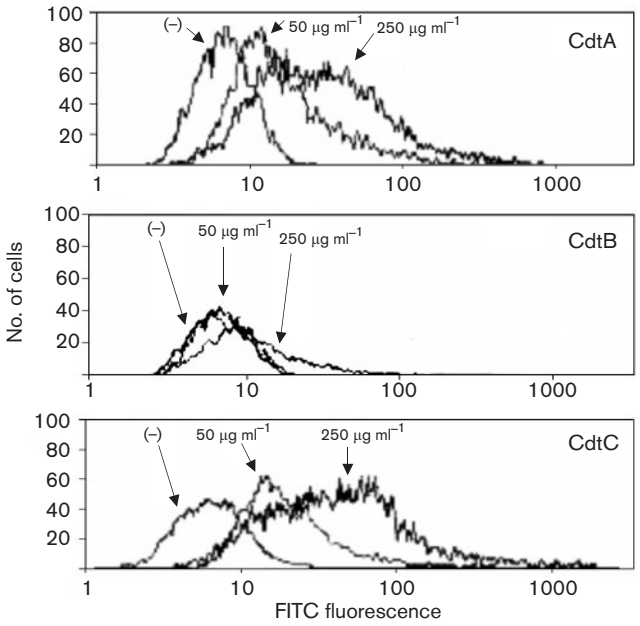

Fig. 2. Binding of CDT proteins to the surface of HEp-2 cells. Cells were treated with the individual His-Cdt proteins at concentrations of either 50 or 250 μg ml^{-1} at 37 °C for 10 min. Cells were washed three times with PBS and incubated with a murine antibody to polyhistidine for 30 min, and then after further washing the bound murine antibody was identified using a FITC-conjugated goat anti-murine F(ab′)$_2$. Cells were then analysed by flow cytometry to determine the binding of the fluorophore. The fluorescence output of cells that were not exposed to labelled Cdt is denoted (–).

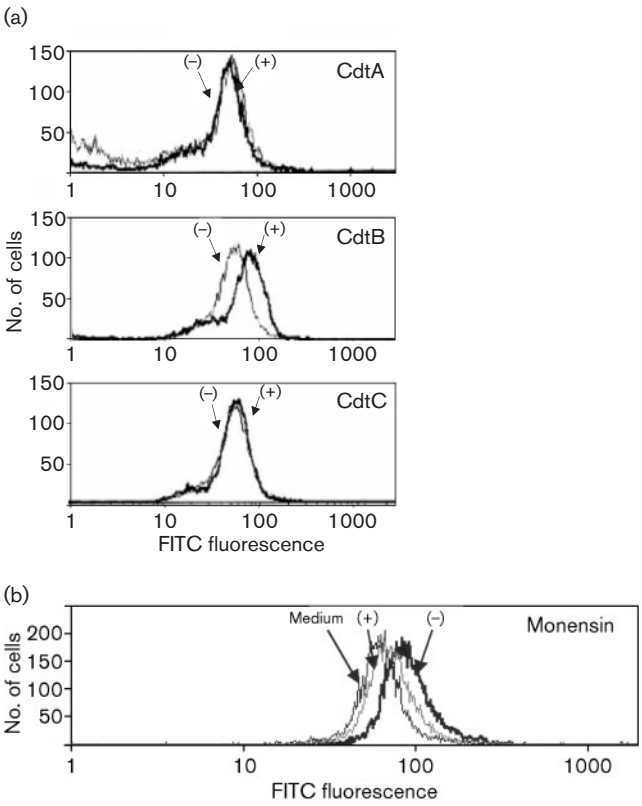

Fig. 3. (a) Internalization of Cdt proteins into HEp-2 cells. Cells were treated with the His-tagged CdtA, CdtB or CdtC (100 μg ml^{-1}) in the presence (+) or absence (–) of Omp-CdtC (100 μg ml^{-1}) at 37 °C for 6 h. At the end of the incubation period, cells were washed three times with PBS and detached from the plastic support. Then cells were stained with the IntraStain kit to disclose intracellular toxin proteins and analysed by flow cytometry as described in Methods. (b) Effect of monensin on endocytosis of CdtB by epithelial cells. HEp-2 cells were treated with CdtB and Omp-CdtC (100 μg ml^{-1}) in the presence (+) or absence (–) of monensin (10 μM) at 37 °C for 6 h. At the end of this period cells were washed three times with PBS and removed from the plastic and stained with the IntraStain kit for intracellular His-tagged protein and analysed by flow cytometry as described in Methods.

labelled and unlabelled proteins, and the uptake into cells was assessed by confocal microscopy. This confirmed the results from the flow cytometry that CdtB neither bound nor was taken up by cells (Fig. 4b). The medium control is shown in Fig. 4(d). CdtA (Fig. 4a) and CdtC (Fig. 4c), as expected, bound to the cell surface but binding did not appear to be uniform. There was no significant uptake of CdtC into cells.

Cells were incubated with various permutations of the CDT proteins using a combination of FITC-labelled and unlabelled His-tagged proteins. With all combinations, no significant fluorescence was detected until after 2 h of incubation. However, after 4 h incubation with a combination of FITC-labelled CdtB and non-labelled CdtC there was clear cytoplasmic localization (Fig. 4e). Likewise, when FITC-labelled CdtC was incubated with non-labelled CdtB there was also extensive cytoplasmic staining after 4 h (Fig. 4f). Interestingly, the fluorescence with these combinations was reduced after 6 h incubation (data not shown). These results strongly suggest that CdtB and CdtC in combination enter into the cytosol of the HEp-2 cells.

The combination of labelled CdtB with unlabelled CdtA showed some cell surface staining (Fig. 4g). Interestingly, the combination of FITC-labelled CdtA with non-labelled CdtB and CdtC revealed strong labelling at the cell surface and limited intracellular staining (Fig. 4h). In the presence of monensin, the combination of FITC-labelled CdtB with non-labelled CdtC or vice versa failed to enter into the cytoplasm and was located on the cell surface (Fig. 5a, b). Additionally, internalization of CdtB and CdtC was affected by brefeldin A (Fig. 5c, d).

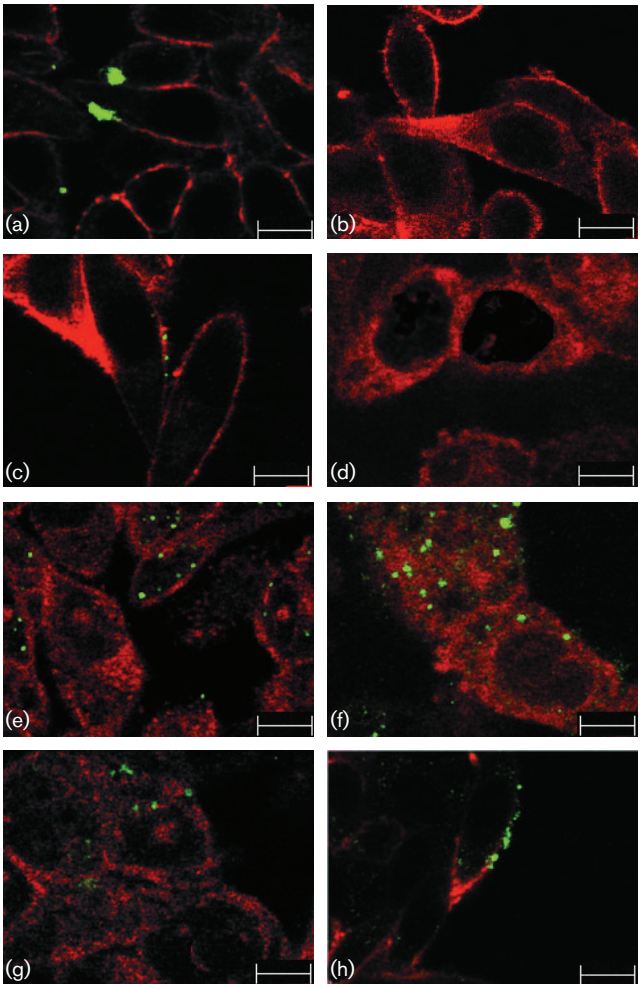

Fig. 4. Localization of Cdt proteins. HEp-2 cells were incubated with FITC-labelled CdtA (a), CdtB (b), CdtC (c), medium alone (d), FITC-labelled CdtB and unlabelled CdtC (e), FITC-labelled CdtC and unlabelled CdtB (f), FITC-labelled CdtB and unlabelled CdtA (g), and FITC-labelled CdtA and unlabelled CdtB/C (h). Cells were incubated for 4 h with the labelled toxins, fixed and stained with rhodamine-conjugated phalloidin before being analysed by confocal microscopy as described in Methods. Green, FITC-labelled toxin; red, filamentous actin. Bars, 10 μm.

DISCUSSION

The biological activity of the CDT complex is due to the DNase activity of CdtB. Mutants of CdtB lacking DNase activity fail to induce cell cycle arrest (Elwell & Dreyfus, 2000; Lara-Tejero & Galan, 2000; Nesic *et al.*, 2004). The presence of a nuclear localization signal in the *A. actinomycetemcomitans* CdtB protein also supports the hypothesis that DNA is the molecular target (Nishikubo *et al.*, 2003). This evidence, as well as the proven nuclear localization of CdtB (McSweeney & Dreyfus, 2004), firmly establishes that DNA breakage as a result of CdtB activity, and not some

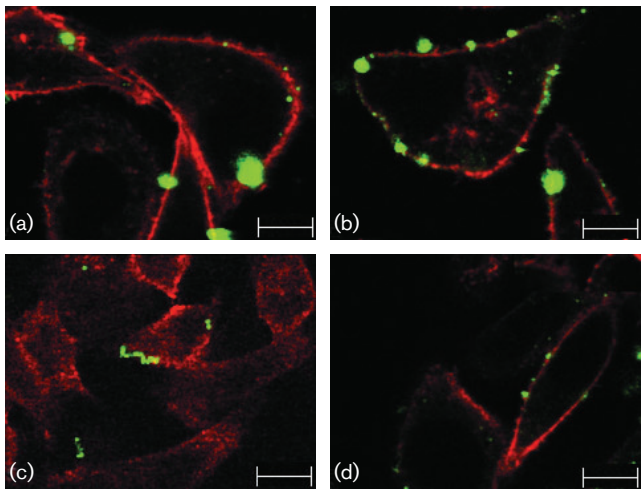

Fig. 5. Effect of monensin and brefeldin A on the localization of Cdt proteins. Cells were incubated with monensin (10 μM) (a, b) or brefeldin A (10 μM) (c, d). Treatments were performed in the presence of FITC-labelled CdtB and unlabelled CdtC (a, c) or FITC-labelled CdtC and unlabelled CdtB (b, d). Green, FITC-labelled toxin; red, filamentous actin. Bars, 10 μm.

modulation of signal transduction, is the mechanism of action of CdtB. However this does not rule out the possibility that CdtB activity may have an effect on other signalling pathways as well. If CdtB is the active moiety responsible for cell cycle arrest, it must enter the cell in order to damage the DNA. This raises the question of the role played by the CDT subunits in the internalization of CdtB. It has been suggested that cellular internalization of CDT is required for the activity of *H. ducreyi* CDT (Cortes-Bratti *et al.*, 2000). This has been deduced from experiments using a variety of reagents, including synthetic inhibitors, to block cell internalization pathways.

In the present study we cloned the *cdt* genes from the oral pathogen *A. actinomycetemcomitans* and expressed and purified to homogeneity the individual CDT proteins as 6 × His tag fusion proteins. This allowed us to compare the activity of each CDT protein and the possible interactions between them. None of the individual CDT proteins could induce cell cycle arrest. The minimum combination of proteins showing significant cell-cycle-arrest activity was CdtB and CdtC, which we reported previously (Akifusa *et al.*, 2001), and this is supported by the work of Lewis *et al.* (2001), Deng *et al.* (2001), Lee *et al.* (2003) and Shenker *et al.* (2004). We found that concentrations as low as 40 ng ml^{-1} of the combination of these two proteins were able to inhibit cell cycle progression.

The next question to address was whether all three CDT proteins bound to cells. Using the 6 × His tag as a means of assaying the binding of the CDT proteins to cells it was found that CdtB did not bind to HEp-2 cells at either 4 or 37 °C. CdtA bound to cells at both temperatures whereas CdtC only bound at 37 °C, indicating that the availability of CdtC-binding sites may be dependent on unrestricted cell activity. These results suggested that CdtA and/or CdtC might be required to bind CdtB to allow it to enter into cells. This is the classic mode of cell entry of many bacterial A/B toxins, where the B domain binds to the cell surface receptor allowing the uptake of the toxic A domain (Falnes & Sandvig, 2000).

To determine if CdtC bound to cells, and facilitated entry of CdtB, cells were exposed to His-CdtC for various times ranging from 10 min to 12 h. The cells were then washed extensively to remove all soluble CdtC and subsequently CdtB was added to the cells. This revealed that with as little as 10 min exposure of cells to CdtC there was a significant response to exogenous CdtB. With increasing time of exposure to CdtC the proportion of cells in G_2 increased. The simplest interpretation of these results is that CdtC, along with CdtA, can rapidly bind to cell membranes via a receptor where it can facilitate internalization of CdtB.

An antibody to the 6 × His tag was also used to assess the uptake of the CDT proteins into cells. In this study a crude periplasmic preparation of the three individual CDT proteins lacking the 6 × His tag was used to determine which combinations of CDT proteins were required to ensure entry of proteins into cells. CdtA and CdtB showed no ability to enhance the uptake of either their 6 × His-tagged counterparts or other 6 × His-tagged CDT proteins. CdtC did not enhance the uptake of His-CdtA or of His-CdtC. However, it did promote the uptake into cells of His-CdtB, as determined by flow cytometric analysis. This enhanced uptake of CdtB was blocked by the ionophore monensin, which increases membrane permeability to H^+ and Na^+. Monensin inhibits the acidification of intracellular compartments including endosomes and is believed to inhibit receptor-mediated endocytosis because the rise in intraendosomal pH blocks receptor–ligand dissociation (Tartakoff, 1983). This confirms that the blockade of CDT activity by monensin reported by Cortes-Bratti *et al.* (2000) is due to the failure of the cells to endocytose the toxin and not due to non-specific cell cycle effects of this ionophore.

To look at the uptake of the CDT proteins in more detail they were fluoresceinated and various permutations of labelled and unlabelled proteins were incubated with cells to determine what molecular species actually entered cells. The fluorophore did not block the biological activity of the CDT proteins. Direct visual examination of cells by confocal microscopy confirmed the previous results using other cell culture methods that CdtB neither bound nor was taken up by cells. CdtA and CdtC showed what appeared to be binding to the cell surface but this was not uniform. When fluoresceinated CdtB and unlabelled CdtC or unlabelled CdtB and fluoresceinated CdtC were added to cells, a vesicular-type fluorescent staining inside cells was first observed after 2 h of incubation, reaching a peak at 4 h and diminishing in fluorescent intensity by 6 h. The toxin appeared to be in vesicles. When cells were incubated with FITC-labelled CdtA together with unlabelled CdtB and CdtC, fluorescent staining inside cells was less intense than that observed with the combination of CdtB and CdtC, indicating a slower uptake of the triple complex compared to the latter combination. The FITC-labelled CdtB did not show obvious nuclear localized staining at 4 h. This probably reflects the small amount of fluorophore taken up by cells, which is only detected when it is within a vacuole. In studies where nuclear localization has been identified, some form of signal amplification has been employed (McSweeney & Dreyfus, 2004). Both Golgi-perturbing agents tested, monensin and brefeldin A, inhibited internalization of fluorescein-labelled CdtB/CdtC. However, binding to the cell surface was not perturbed, indicating that the block in intracellular trafficking did not affect the localization of the CDT receptor but rather that internalization of the toxin itself was blocked. This finding can be explained by the profound effect both drugs have on the endosomal system. As pointed out above, the collapse of the pH gradient in endosomes caused by monensin inhibits receptor-mediated endocytosis (Dinter & Berger, 1998). Brefeldin A, on the other hand, causes tubulation of endosomes and interferes with the redistribution of proteins out of the endosomal system (Klausner *et al.*, 1992).

Thus it appears, at least for HEp-2 cells, that CdtC binds to the host cell membrane and allows entry of CdtB into the cell where it can interact with DNA and block cell cycle progression. Our data support those of Lee *et al.* (2003), who also reported that CdtC of *Campylobacter jejuni* bound to mammalian cells. However a previous study by Mao & DiRienzo (2002), using Cdt from *A. actinomycetemcomitans*, could not find cell surface binding of CdtC by fluorescent microscopy. This may have been because they used too little protein, as our data show that even at a high concentration of protein (100 μg) there is limited binding of CdtC compared with CdtA; therefore lower levels of protein may not have been sufficient to detect binding using microscopy. The fact that CdtC enters the cell with CdtB but CdtA does not (Fig. 4h) is interesting, and may suggest that there is more to the role of CdtC than just to facilitate entry for CdtB. As revealed by the recently published crystal structure of the *H. ducreyi* CDT, the N-terminus of the CdtC peptide can block the active site of CdtB, thereby reducing its activity. The crystal structure of the CDT holotoxin also shows that CdtA and CdtC form a potential peptide-binding cleft quite possibly for a receptor. Absence of either one of these subunits diminishes CDT activity. This may explain our observation that the toxic activity of the holotoxin was greater than that of the toxin composed of only CdtB and CdtC subunits. However there was no toxic activity with CdtA and CdtB in the absence of the CdtC subunit, implying that CdtC is the essential component responsible for entry of CdtB into the cell, and has an additional role over and above receptor binding.

Although CdtA appears not to be essential for activity it is not clear what role this component plays in the final activity of CDT, and further work is required to fully identify the role of CdtA and its mechanism of host cell binding.

ACKNOWLEDGEMENTS

This work was supported by BBSRC grant 31/C16749.

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