Enhancement of the cytotoxic activity of *Clostridium difficile* toxin A by surface-associated antigens

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Cell-surface antigens of *Clostridium difficile* and LPS from *Escherichia coli* were investigated for modulating effects on the activity of *C. difficile* toxin A on Vero and Caco2 cells. The antigens of *C. difficile* tested comprised: (i) an EDTA extract, which contained several major and minor cell-surface proteins and the membrane-associated lipocarbohydrate (LC); (ii) a guanidine hydrochloride extract, which mainly contained the surface-layer proteins; (iii) an aqueous phenol-extracted, protein-free LC. On their own, none of the antigens had a detrimental effect on the cells, with the EDTA extract and LC having a marginally protective effect. When these antigens were added to suboptimal levels of toxin A, there was significant enhancement of its cytotoxicity by the EDTA and LC preparations on both cell types. LPS showed some enhancement of the effect of toxin on Vero cells at the lowest levels of toxin investigated. It was concluded that this effect seen *in vitro* may have a role to play in the colon during infection with *C. difficile*.

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INTRODUCTION

The major virulence factors of most strains of pathogenic *Clostridium difficile* are accepted to be the two large clostridial toxins: toxin A (TcdA, an enterotoxin) and toxin B (TcdB, a cytotoxin), although certain other cell-associated molecules may have a secondary role in virulence, such as promoting adhesion or evading the immune response (Poxton *et al.*, 2001). Although there is a suggestion that the amount of toxins produced is related to virulence – mainly through the observations that the hypervirulent ribotype 027 strains produce more toxin than other types (Warny *et al.*, 2005) – there is not a strict correlation between the toxigenicity of the strain (the amount of toxin produced *in vitro*) and the degree of disease (Borriello *et al.*, 1987). The absence of both toxins leads to avirulence of the strain. Strains belonging to the A⁺B⁺ phenotype, which only produce toxin B, are as virulent as those producing both toxins (Drudy *et al.*, 2007). The ADP-ribosylating binary toxin CDT (cytolethal distending toxin), found in a minority of strains of *C. difficile*, can exert an effect on the cytoskeleton, but has no proven role in pathogenesis (Geric *et al.*, 2006). Other virulence factors, in particular putative adhesins, have been considered and, although the role of these factors is not fully understood, it is believed that they may contribute to pathogenesis (Péchiné *et al.*, 2007). However, as far as we

are aware, such components, either those belonging to *C. difficile* cells themselves or other bacterial components found in the colonic lumen, have not been considered as agents capable of modulating the action of toxins.

The aim of this study was to test the hypothesis that the cytotoxic effect of the *C. difficile* toxins can be enhanced by the presence of antigens associated with the cell surface of *C. difficile* and by LPS, a molecule likely to be found at high concentrations in the gastrointestinal tract. Two different cell lines were selected to test the activity of purified toxin A: the commonly used toxin-sensitive Vero cells and differentiating Caco2 cells.

METHODS

Production of crude toxin by dialysis culture. An overnight culture (1 ml) of a toxigenic strain of *C. difficile* (strain MPRL338a) was used to inoculate a sterilized, pre-reduced dialysis bag, containing before sterilization approximately 50 ml 0.85% NaCl, suspended in 500 ml proteose peptone yeast extract (PPY) medium (Deacon *et al.*, 1978), in a stoppered, vented 1 l conical flask. After 4 days of incubation at 37 °C in an anaerobic atmosphere (10% H₂, 10% CO₂, 80% N₂) in a DWS Mark III workstation (Don Whitley Scientific), the contents of the dialysis bag were harvested (10 000 g, 15 min) and the supernatant was dialysed overnight against Tris-buffered saline [TBS: 0.05 M Tris/HCl (pH 7.0), 0.15 M NaCl] at 4 °C. This supernatant, which contained the extracellular products but none of the culture medium components, was filter-sterilized and stored at 4 °C.

Purification of toxin A. This purification was based on methods developed by Krivan & Wilkins (1987) and Kamiya *et al.* (1989),

Abbreviations: GHCl, guanidine hydrochloride; LC, lipocarbohydrate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SLP, surface-layer protein.

which utilize the binding of toxin A to bovine thyroglobulin at 4 °C and its elution at 37 °C.

Cyanogen bromide-activated Sepharose gel (4.4 g; Amersham Biosciences) was washed thoroughly in 1 mM HCl to remove any additives present. It was then allowed to react overnight (4 °C) with 60 ml 5 g bovine thyroglobulin l⁻¹ (Sigma) in 0.1 M MOPS buffer (pH 7.0) that had previously been centrifuged (8000 g, 10 min) and filtered (0.22 µm) to remove insoluble particles. The mixture was blocked with 40 ml 0.1 M ethanolamine for 30 min (4 °C) and then washed in 0.1 M MOPS buffer (pH 7.0).

The coupled beads were packed into a column (C10; Amersham Biosciences) and washed at 37 °C with 120 ml pre-warmed basic buffer [0.1 M glycine/NaOH (pH 10.0), 0.5 M NaCl] and 120 ml pre-warmed acidic buffer [0.1 M glycine/HCl (pH 2.0), 0.5 M NaCl]. The column was then equilibrated at 4 °C with 120 ml TBS. *C. difficile* culture filtrate (100 ml at 4 °C) was added. The column was washed with 25 ml TBS at 4 °C and toxin A was eluted with 50 ml TBS at 37 °C. The absorbance of the fractions was monitored at 280 nm, and fractions were separated by SDS-PAGE to check purity and tested for toxin activity with a Techlab kit for *C. difficile* toxin A/B detection. Cytotoxicity assays were also carried out in Vero and Caco2 cells. Fractions containing toxin were pooled, filter-sterilized, assayed for total protein by the Bradford method (Bio-Rad) and kept at 4 °C. For reuse, the column was washed with 60 ml acidic buffer at room temperature and equilibrated with 60 ml TBS at 4 °C.

Cell culture. The Vero cell line is a well-established cell line originally obtained from kidney epithelium of the African green monkey, and has been used extensively for testing the cytotoxicity of *C. difficile*. The Caco2 lineage was obtained from a human colon cancer and is used as a model for the human intestine. This lineage spontaneously differentiates after reaching confluency. Vero and Caco2 cells from our frozen stock were grown at 37 °C, 5 % CO₂, in 75 cm² flasks in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10 % fetal bovine serum (FBS; Sigma), 100 U penicillin G sodium ml⁻¹ (Gibco), 100 µg streptomycin sulphate ml⁻¹ (Gibco), 292 µg glutamine ml⁻¹ (Gibco) and 1 % non-essential amino acids (Sigma), and incubated at 37 °C, 5 % CO₂.

Proliferating cells were split when they reached 90 % confluency with 0.05 % trypsin in 0.53 mM EDTA and centrifuged at 1000 g for 10 min. A 10 % inoculum (10⁶ cells ml⁻¹) was used to inoculate new flasks.

Although it would have been preferable to use the Caco2 cells after they had differentiated, it was difficult to ensure that every layer of cells was exposed to the same concentration of toxin and/or antigens. Therefore, for both cell lines, the cells were used when they had just reached confluency.

EDTA extraction of surface proteins. An overnight culture of *C. difficile* in PPY medium was used as a starter to inoculate (1 %, v/v) 20 ml pre-reduced PPY. This was incubated at 37 °C under anaerobic conditions overnight or until a sufficient turbidity was achieved (OD₆₀₀=0.6).

Cells were harvested at room temperature (5000 g, 20 min) and washed twice in PBS. They were extracted with EDTA as originally described by Poxton & Byrne (1981). The washed pellet was resuspended in 1.5 ml 10 mM EDTA buffer and incubated at 45 °C for 90 min. Extractions were sonicated in a sonic bath for 1 min followed by a 10 s mix in a vortex mixer. Bacteria were removed by centrifugation twice (13 000 g, 2 min each). The extracts were stored at -20 °C.

Dialysis to remove EDTA was carried out in 1 l 10 mM Tris/HCl (pH 7.4) overnight at 4 °C on a magnetic stirrer. The buffer was

changed and dialysis was continued for a further 4 h. The dialysed samples were stored at -20 °C.

Extraction of surface-layer proteins (SLPs). SLPs from whole cells were extracted using 5 M guanidine hydrochloride (GHCl) using the method described by McCoubrey & Poxton (2001). A 1 % inoculum of a *C. difficile* overnight PPY culture was placed into 4 ml PPY and incubated anaerobically at 37 °C overnight. The overnight culture was harvested (3000 g, 20 min) and washed twice in the same volume of PBS. The pellet was then drained and resuspended in 0.3 ml 5 M GHCl solution and transferred into a screw-capped Eppendorf tube. The samples were shaken in a red blood cell shaker at room temperature for 2 h and then centrifuged twice at 13 000 g for 2 min to remove any remaining bacteria. The final supernatant was stored at -20 °C.

Dialysis was carried out to remove the GHCl in 1 l of 6.25 mM Tris/HCl buffer (pH 6.8) overnight at 4 °C on a magnetic stirrer. The buffer was changed and dialysis continued for a further 4 h. The dialysed extracts were stored at -20 °C.

Surface lipocarbohydrate (LC). This method was based on those described by Poxton & Cartmill (1982) and Sharp & Poxton (1986). Six litres of pre-reduced PPY was inoculated with an overnight *C. difficile* culture and grown anaerobically overnight at 37 °C. Cells were harvested (10 000 g, 10 min, 4 °C), washed in PBS, suspended in a suitable volume of PBS and disrupted by sonication at 10 µm (MSE Soniprobe). The cell walls and any unbroken cells were removed by centrifugation (4000 g for 10 min, followed by 35 000 g, for 30 min) and the supernatants collected into a weighed container and lyophilized.

The lyophilized supernatant was delipidated twice with 200 ml chloroform/methanol (2:1) over 24 h, filtered through Whatman No. 1 paper and allowed to air dry. The dry delipidated cell contents and membrane fraction were resuspended in distilled water to 10 % (w/v) and mixed with an equal volume of 80 % (w/w) phenol solution. The mixture was stirred at room temperature for 30 min and then centrifuged at 2500 g for 20 min at 4 °C. The upper layer was collected and dialysed against tap water to remove the phenol. The dialysed material was then mixed with an equal volume of 0.2 M acetic acid/acetate buffer (pH 5.0) containing 0.02 M MgCl₂ and approximately 100 µg each of RNase and DNase (both from Sigma). The mixture was incubated overnight at 37 °C in the presence of toluene. The phenol extraction was repeated on the incubated mixture to remove nucleases and, after dialysis of the upper layer, lyophilized in a pre-weighed container. This was labelled crude membrane antigen and stored at room temperature.

Enhancement of cytotoxicity by antigen. Microtitre plates were inoculated with 100 µl of a 10 % FBS solution containing 10⁶ cells ml⁻¹ and incubated at 37 °C, 5 % CO₂, overnight until the cells were confluent. Blanks were inoculated with 20 µl DMEM medium, toxin controls were inoculated with 10 µl of a dilution of toxin in DMEM medium and 10 µl medium plus FBS, and tests were inoculated with 10 µl of the same toxin dilution and 10 µl of an appropriate concentration of the antigen (see Results). Each set of results (shown as individual columns in Figs 1–4) was the mean of 10–28 replicates for the Vero cells and 6–37 replicates for the Caco2 cells, and was normally 10–12 replicates. Plates were incubated at 37 °C, 5 % CO₂, and viability was measured using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay.

MTT assay. The MTT assay is a cell viability test. Viable cells are able to reduce yellow MTT into formazan, which is purple, and this reduction, which is carried out by a mitochondrial reductase, can easily be measured using a spectrophotometer. MTT solution (20 µl 5 mg l⁻¹ solution in sterile PBS) was added to each well. Plates were

incubated at 37 °C, 5% CO₂, for 4 h. The formazan produced was solubilized in DMSO (100 µl per well) and the colour change measured at A₅₇₀.

RESULTS AND DISCUSSION

Titration of toxin on cells

Initial experiments were performed to titrate the toxins against the two cell lines. Toxin was added in doubling dilutions ranging from 21 to 0.16 µg ml⁻¹ to the Vero cells. The first three dilutions drastically reduced cell viability; for example, the difference between 10.5 µg toxin ml⁻¹ and no toxin gave a *P* value of 0.0006. Although the reduction for the 5.25 µg ml⁻¹ toxin concentration also looked as significantly different as the previous concentration, only 6 replicates were used to calculate this mean (compared with up to 28 for other concentrations) and the difference was therefore not statistically significant. Lower concentrations did not significantly reduce cell viability (Fig. 1a). Pilot experiments with the Caco2 cells showed that they were more sensitive to toxin than the Vero cells, and therefore different concentrations of toxin were used in the initial titrations (Fig. 1b). The highest toxin concentration of 21 µg ml⁻¹ was diluted fivefold to 4.2 µg ml⁻¹ and further diluted tenfold. The 0.42 µg toxin ml⁻¹ concentration was

the lowest concentration that gave drastically reduced cell viability (*P*<0.0001 compared with no toxin).

Antigens and cells

It was important to know whether the cell-associated surface antigens themselves had toxic effects on the monolayers. For Vero cells, the antigens used for challenge were added to the monolayers as follows: 10 µl per well of a 380 µg antigen ml⁻¹ dialysed EDTA extract, 10 µl per well of a 770 µg antigen ml⁻¹ dialysed GHCl extraction, 10 µl per well of a 1000 µg antigen ml⁻¹ LC solution and 10 µl per well of a 1000 µg antigen ml⁻¹ *Escherichia coli* 018K⁻ LPS solution. Cell viability was tested using an MTT assay after 3 days of incubation and the results are shown in Fig. 2(a). No significant differences were found between the wells with no antigen or following the addition of GHCl extract (SLPs) or *E. coli* LPS. Cell viability was higher, however, after the addition of EDTA extract (*P*=0.0051) or LC (*P*=0.0003), presumably due to additional nutrient availability.

The Caco2 cells, being more sensitive to the toxin, were tested with half the concentrations of antigens that were added to the Vero cells. The results in Fig. 2(b) showed that there were no significant differences between the wells with no antigen and after the addition of any of the antigens,

Fig. 1. Effect of titration of toxin A on the viability of Vero (a) and Caco2 (b) cells determined by an MTT assay after toxin addition. Asterisks indicate results that were significantly different from the no-toxin control: *, *P*<0.05; ***, *P*<0.001.

Fig. 2. Effect of antigens on the viability of Vero (a) and Caco2 (b) cells determined by an MTT assay. Asterisks indicate results with a significant enhancement in cell number compared with the no-antigen control: **, *P*<0.01; ***, *P*<0.001. For Caco2 cells none of the antigens produced a significant difference compared with the no-antigen control.

suggesting that there was no detrimental effect produced by any of these four antigens when added on their own.

Effect of antigens on toxin activity

Once it had been determined that the extracts were not reducing cell viability on their own, they were tested to see whether they had any modulating influence on the activity of the toxin. The same concentrations of toxins and antigens employed above were added to the respective cell lines. For clarity, the results are shown in two separate graphs for each cell line (Figs 3 and 4), with the surface protein antigens (EDTA and GHCl; Figs 3a and 4a) separated from the carbohydrate antigens (LC and LPS; Fig. 3b and 4b).

In Fig. 3(a), the effect of the addition of protein antigens on Vero cells is shown. At the higher concentrations of toxin (21 and $10.5 \mu\text{g ml}^{-1}$), the decrease in cell viability with and without the presence of surface proteins was not significantly different. However, when the concentration of

Fig. 3. Effect of titration of toxin A on the viability of Vero cells determined by an MTT assay with or without the addition of surface-associated protein antigens (a) or surface-associated carbohydrate antigens (b). (a) White bars, cells + toxin; dark grey bars, cells + toxin + EDTA extract; light grey bars, cells + toxin + GHCl extract. (b) White bars, cells + toxin; dark grey bars, cells + toxin + LC extract; light grey bars, cells + toxin + LPS extract. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Fig. 4. Effect of titration of toxin A on viability of Caco 2 cells determined by MTT assay with or without the addition of surface-associated protein antigens (a) or surface-associated carbohydrate antigens (b). (a) White bars, cells + toxin; dark grey bars, cells + toxin + EDTA extract; light grey bars, cells + toxin + GHCl extract. (b) White bars, cells + toxin; dark grey bars, cells + toxin + LC extract; light grey bars, cells + toxin + LPS extract. **, $P < 0.01$.

toxin alone was not sufficient to reduce cell viability when compared with the controls (2.63 – $0.66 \mu\text{g ml}^{-1}$), a decrease in viability was observed after the addition of EDTA extract, although this was only significant at $2.63 \mu\text{g ml}^{-1}$ ($P = 0.0341$ compared with toxin alone). Lower concentrations were too low to affect cell viability before and after the addition of the EDTA extract. As was seen in Fig. 1(a), the dilution of $2.63 \mu\text{g ml}^{-1}$ was not enough to reduce viability, but after the addition of EDTA extract, the reduction in viability compared with the cells-only control was clearly significant ($P = 0.004$, asterisk not shown on figure). It should also be remembered that the EDTA extract on its own did not reduce cell viability; in fact, as seen in Fig. 2(a), cell viability was increased. The addition of the GHCl extracts, which contained only the SLPs, did not alter cell viability, suggesting that the SLPs are not responsible for this enhancement of toxin activity.

The same experiments were performed with the surface carbohydrate of *C. difficile* (LC), which is analogous to lipoteichoic acid (Poxton & Cartmill, 1982), and *E. coli*

LPS. The results are shown in Fig. 3(b). As noted previously, neither agent on its own had a detrimental effect on the cells. However, when added in combination with the higher concentrations of toxin (dilutions of 21 and $10.5 \mu\text{g ml}^{-1}$), which already affected cell viability, the addition of LC significantly reduced the cell viability further ($P=0.0016$ and $P=0.0362$, respectively). This was not observed after the addition of *E. coli* LPS. At lower concentrations of toxin (2.63 and $0.66 \mu\text{g ml}^{-1}$) where cell viability was not affected in the presence of toxin alone, the addition of either LC or *E. coli* LPS significantly enhanced the toxic activity. The reduction in cell viability was highly significant at the two concentrations for both LC ($P<0.0001$ and $P=0.0003$) and LPS ($P=0.0071$ and $P=0.0004$).

Caco2 cells were used for similar experiments, but using half the concentrations of the potential modulating antigens. The results of the protein surface extracts (EDTA and GHCl) are shown in Fig. 4(a). As observed with Vero cells, at the higher concentrations of toxin (21 and $4.2 \mu\text{g ml}^{-1}$), there were no significant differences in the reduction in cell viability with and without the presence of surface proteins. In fact, the addition to toxin at $0.42 \mu\text{g ml}^{-1}$ of either of the protein extracts seemed to counteract the effect of the toxin ($P=0.0020$ for EDTA and $P<0.0001$ for GHCl, asterisk not shown on figure). However, when the concentration of toxin was insufficient to reduce cell viability when compared with the controls ($0.04 \mu\text{g ml}^{-1}$), a reduction in viability was observed after the addition of EDTA extract ($P=0.0061$ compared with toxin alone). As seen in Fig. 1(b), this concentration was not enough to reduce viability, but after the addition of the EDTA extract, the reduction in viability compared with the cells-only control was clearly significant ($P=0.0027$; asterisk not shown on figure). The addition of the GHCl extract, which only contained the SLPs, did not appear to reduce cell viability further.

Similar experiments were performed with the surface carbohydrate of *C. difficile* (LC) and *E. coli* LPS (Fig. 4b). As stated earlier and seen in Fig. 2(b), the addition of these concentrations of LC and LPS were not detrimental to the cells. However, when they were added in combination with the higher concentrations of toxin (21 and $0.42 \mu\text{g ml}^{-1}$), which already affected cell viability, the addition of either LC or LPS significantly increased cell viability ($P=0.0141$ and $P=0.0011$, asterisks not shown on figure) suggesting a protective effect. At lower concentrations of toxin ($4.2 \times 10^{-3} \mu\text{g ml}^{-1}$) where cell viability was not affected in the presence of toxin alone, the addition of LC significantly enhanced the toxic activity ($P=0.0029$). This enhancement was not seen after the addition of *E. coli* LPS.

The most significant effects were seen with LC alone and the EDTA extract, which represents the surface-associated proteins (including the SLPs) and LC of the cytoplasmic membrane (Poxton & Cartmill, 1982). This suggests that LC may be the key factor in enhancing the cytotoxic effect.

The cell wall of *C. difficile* is a complex structure containing several proteins and carbohydrates and, for most of them, their function remains uncertain. Some show variability in different strains, such as the SLPs, Cwp66 or Cwp84, and the flagellar proteins FliC and FliD (McCoubrey & Poxton, 2001; Péchiné *et al.*, 2005). These proteins are highly immunogenic and have adhesive and/or protease activities, but their exact role in pathogenesis has not been established (Péchiné *et al.*, 2005). LC is common to all *C. difficile* strains investigated and is also shared with *Clostridium sordellii* (Poxton & Cartmill, 1982). Its role is uncertain but it is analogous to the lipoteichoic acids of other Gram-positive bacteria, and shares structural and physical properties with the LPSs of Gram-negative bacteria (Sharp & Poxton, 1986), so it would not be surprising if it had a biological activity. The LPS from *E. coli* only affected Vero cells to any great extent and then only at low levels of toxin.

The aim of this study was to determine whether the surface antigens of *C. difficile* and LPS might have a role in the pathogenesis of *C. difficile* infection by modulating toxin activity. Enhancement of toxin activity was demonstrated by some agents, but only at suboptimal levels of toxin and with monolayers of cultured cells. Whether this increase in the efficiency of the toxin is significant *in vivo* remains to be determined.

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