ATYPICAL CYTOPHATIC ACTIVITY CAUSED BY *Clostridium difficile* A- B- CDT- STRAINS.

Walter Beata[1], Titball Richard[2], Goropevsek Ales[3], Rupnik Maja[1,4,5]

1Institute of Public Health, Maribor, Slovenia; 2University of Exeter, School of Biosciences, Exeter, Devon, United Kingdom; 3University Clinical Centre Maribor, Maribor, Slovenia; 4University of Maribor, Faculty of Medicine, Maribor, Slovenia; 5Centre of Excellence for integrated approaches in chemistry and biology of proteins, Ljubljana, Slovenia.

Introduction

C.difficile is known as a producer of toxin A, toxin B and binary toxin which contribute to the pathogenicity of the bacterium. Their activity in vitro can be seen as typical rounding of different cell lines [7,11]. Several non-toxinogenic strains (A-B-CDT-) have been observed to exhibit atypical cytotoxicity even though they are lacking the genes encoding known *C. difficile* toxins. The aim of this study was to characterize some properties of this new potentially toxic *C. difficile* molecule and to study its effect on eukaryotic cells.

Materials and methods:

The bacterial supernatant of selected A-B-CDT- *C.difficile* strains was prepared by growth in BHI, followed by low speed centrifugation and filtration with 0.45µm sterile filter.

The size determination was obtained by ultrafiltration with Vivaspin® centrifugal concentrators of the molecular weight cut off 10kDa and 3kDa.

The heat stability was tested by the sample supernatant exposure to 60 °C, 80 °C, 95 °C for 15 min, 30 min and 60 min respectively.

The susceptibility to proteinases (chymotrypsin, trypsin, protease K and pronase) was tested at concentrations of 1 mg ml^{-1}, 2 mg $ml^{-1}$and 3 mg ml^{-1} for 2 h or 4 h at the temperature of 25 °C. The Lipase A susceptibility was performed for 3 h at the final concentration of 8 mg ml^{-1}.

The cytotoxic activity was tested on HeLa and Vero cells examined under a phase contrast microscope and analysed by flow cytometry (BDFacsCantoII, Beckton Dickinson).

The cells intended for the cytometry analysis were grown in 6 well plates in total final volume of 2 ml per well. To obtain comparable results, cells were synchronised by serum starvation for 24h or 48h prior intoxication [2, 4, 13]. Subsequently, the cells were prepared for analysis by gentle trypsinisation and washing with ice cold, magnesium free phosphate buffered saline (PBS) [1, 3]. The cells for cell cycle analysis were fixed with ice cold 70% ethanol then washed 3 times with PBS [1, 3, 9]. The staining with Annexin-V-Fluos (Roche) was performed according to the manufacturer description. Propidium iodide (Sigma Aldrich) staining was performed at the final concentration of 0,0125 mg ml^{-1}.

Results

The size of the *C.difficile* culture supernatant fraction exhibiting atypical cytotoxicity, established using commonly available centrifugal concentrators was determined as less than 3KDa. The activity was found to be heat, proteinases and lipase A stable (data not shown).

The atypical cytotoxic activity of studied *C.difficile* A-B-CDT- strains was observed in vitro on various cell lines such as HeLa, McCoy and Vero. The cytotoxicity was primarily examined under a phase contrast microscope, and was defined as inhibition of cell division (observed as low number of cells in comparison to the untreated control) and also noticeable alterations were observed in the cell morphology (Fig 1).

Further analysis and confirmation of toxic action on eukaryotic cells was evaluated by flow cytometry. The analysis with propidium iodide and AnnexinV showed 8% higher number of early apoptotic cells (AnnexinV positive, propidium iodide negative) for *C.difficile* culture supernatant treated samples in comparison to non-treated samples (Fig 2).

The cell cycle analysis revealed accumulation of cells in G2/M phase for the cell synchronisation time up to 24h, with no significant difference for various intoxication times (24 h or 48 h). However, the extended cell synchronisation facilitated apoptosis showing significant number of dead cells in *C.difficile* treated samples (Fig 3).

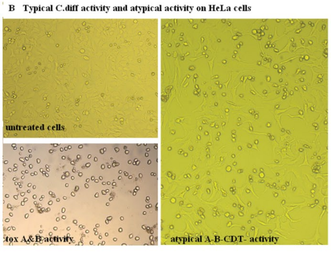


Figure 1. *C.difficile* typical and atypical activity on HeLa Cells observed 24h after intoxication.
untreated cells showing healthy HeLa cells,
typical cytotoxic effect caused by *C.difficile* toxinotype 0 (A+B+CDT-) showing typical rounding of the cells,
atypical *C.difficile* A-B-CDT- activity showing decrease in cell number and alteration to the shape and size. In comparison to the control there was no multiplication of the cells.

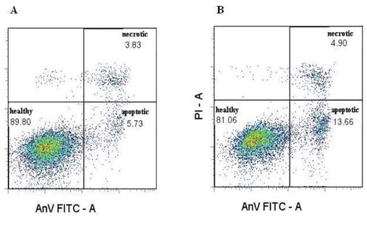
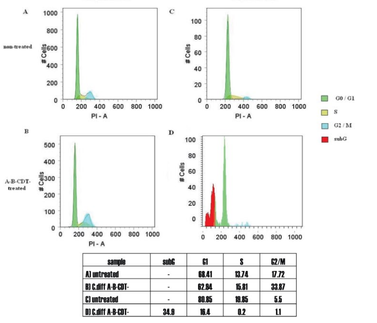


Figure 2. Flow Cytometry dot plots showing proportion of healthy, apoptotic and necrotic cells.
A) negative control, untreated HeLa cells,
B) *C.difficile* A-B-CDT- treated HeLa cells.
Necrotic cells: AnnexinV positive, PI positive, **Healthy cells:** AnnexinV negative, PI negative, **Apoptotic cells:** AnnexinV positive, PI negative



sample	subG	G1	S	G2/M
A) untreated	-	68.41	12.74	17.71
B) C.diff A-B-CDT-	-	67.04	15.81	23.07
C) untreated	-	80.95	10.05	5.5
D) C.diff A-B-CDT-	34.9	10.4	8.2	1.1

Figure 3. Flow Cytometry histograms of cell cycle analysis
A) negative control for B, untreated Vero cells,
B) *C.difficile* A-B-CDT- treated Vero cells, 24 h synchronisation, 48 h intoxication,
C) negative control for D, untreated Vero cells,
D) *C.difficile* A-B-CDT- treated Vero cells, 48 h synchronisation, 48 h intoxication.
The population in red stands for DNA of apoptotic cells, the population in green stands for DNA of cells in G0/G1 cell cycle phase, the population in yellow stands for DNA of cells in S phase the population in blue stands for cells G2/M phase.

Conclusions

Heat and proteinases stability of new atypical *C. difficile* cytotoxic activity significantly decreases the possibility of protein-like structure; however, it does not exclude it completely as small peptides can be resistant to the proteolytic activity. Moreover, small size of the compound makes further purification particularly difficult. Cell cycle analysis with flow cytometry revealed significant accumulation of cells in G2/M phase after treatment with culture supernatant. Interestingly, several other bacterial toxins have been found to inhibit cell cycle or block mitosis [5, 6, 8, 12]. Furthermore, among many other pathogenic bacteria such as *Pseudomonas spp., Yersinia, E.coli or H.pylori, Clostridium botulinum* C3 exoenzyme was found to be a cyclomodulin blocking cell cycle in G1 phase [8].
The microscopic evaluation of *C.difficile* compound treated eukaryotic cells confirmed by cytotoxic assays established on flow cytometry, provide strong evidences for the presence of yet another toxin-like compound that might play a role in *C.difficile* pathogenicity.

References

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