Purification and characterisation of toxin B from a strain of *Clostridium difficile* that does not produce toxin A

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Summary. Most toxigenic strains of *Clostridium difficile* produce both toxin A and toxin B. The toxin produced by *C. difficile* strain 8864 was characterised and compared with those produced by *C. difficile* strain 10463. Toxin A was not detected by immunoassay in cultures from strain 8864 and all the cytotoxic activity produced by this strain was neutralised by antiserum to toxin B. Toxin B from strain 8864 was purified and compared with toxin B from strain 10463. The size of the purified subunits of toxin B from strain 8864 differed slightly from those of strain 10463 and there were small immunological differences. The effect on fibroblast cells was more like that of *C. sordellii* cytotoxin than of toxin B from strain 10463. These results suggest that *C. difficile* strain 8864 produces a modified toxin B and does not produce toxin A.

Introduction

Clostridium difficile causes pseudomembranous colitis and antibiotic-associated diarrhoea or colitis.^{1, 2} The exotoxins produced by this micro-organism seem to be responsible for the disease. It produces at least two toxins, A and B, which are high-mol.-wt proteins, sensitive to heat and highly hydrophobic.^{3, 4} Toxin B may exist in two immunologically related molecular forms: form 1 with subunits of 230–250 Kda and form 2 with subunits of 43 Kda.⁵ Experiments have shown that toxin A causes extensive mucosal destruction and fluid accumulation in the ileum and colon.^{6, 7} Toxin B appears to be inactive on the intestinal mucosa⁷ but is highly toxic to some cells in culture.⁸ The morphological changes in cells are very similar to those seen with cytochalasin and seem to involve alteration of the cytoskeleton.⁹ Epithelial cells become rounded whereas fibroblasts become actinomorphous with contraction of the cytoplasm around the nucleus and long processes radiating from the rounded cell body.¹⁰

Almost all strains of *C. difficile* tested produce both toxins A and B or are non-toxigenic.¹¹ The absence of certain amino acids seems to inhibit the production of one or the other toxin and one strain (no. 8864) produces toxin B only, even in complex media.¹² In this study, the toxin produced by strain 8864 was characterised and compared with toxins produced by *C. difficile* strain VPI 10463.

Materials and methods

Bacterial strains

C. difficile strain 8864 (CCUG 20309) was kindly provided by Dr E. Falsen, Culture Collection, University of Gothenburg, Sweden. This strain was originally isolated by Dr D. W. Burdon, Birmingham General Hospital, UK.¹² Strain VPI 10463 was used for production of toxins A and B.

Production and purification of the toxins

The toxins from strains 10463 and 8864 were prepared and purified as previously described.^{5, 13, 14} Briefly, the supernate obtained from 2-L cultures in brain heart infusion broth by centrifugation (6000 rpm, 20 min) and membrane filtration (0.45 µm) was concentrated on an XM100A ultrafiltration membrane (Amicon, USA) and washed three times with 0.1 M Tris (pH 8.0), 0.1 M NaCl. The final concentrate was applied to a DEAE-Sephadex A25 (Pharmacia, Sweden) column and proteins were eluted with a NaCl gradient. Toxin A eluted at 0.1–0.2 M and was purified by acid precipitation and by high resolution chromatography on a Mono Q column (Pharmacia); final elution was done in a fast protein liquid chromatography system (FPLC, Pharmacia) with a linear gradient from 0.1 to 0.3 M NaCl.¹⁴ Toxin B was eluted from DEAE-Sephadex at 0.4 M NaCl and, after concentration, was applied to a Sephacryl S400 (Pharmacia) column. Fractions with cytotoxicity were then applied to a Mono Q column, eluted with a gradient of 0.44–0.52 M NaCl⁵ and finally concentrated to contain about 1 mg of protein/ml and

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stored at 4°C for further analysis (no longer than a week).

Protein assay

Protein concentration was measured by a protein assay kit with Coomassie Brilliant Blue as the reagent (BioRad Laboratories, USA).

Assay of toxins on cells

Human lung fibroblast cells (kindly supplied by the Virology Laboratory, University of Gothenburg, Sweden) were used to measure the activity of the toxins, as described previously.¹⁵ Serial 10-fold dilutions of the test samples were applied (10 µl) to confluent cells in a 96-well microtitration plate containing 100 µl of Minimal Essential Medium (Gibco, USA) in each well. Plates were examined for cell damage after 24 h. Cytotoxic units (CU) were expressed as the maximum dilution that caused rounding of at least 50% of the cells. For neutralisation studies, samples were mixed with an equal volume of antiserum to toxin A or toxin B¹⁴ and incubated for 1 h before inoculation on to cells.

Polyacrylamide gel electrophoresis (PAGE)

Samples were analysed in precast gradient gels in accordance with the manufacturer's instructions (Pharmacia). Each well was loaded with 15–20 µg of protein. Non-denatured samples were run in a 4–30% gradient in 0.09 M Tris, 0.08 M boric acid, 0.0025 M NaEDTA (pH 8.4) at 150 V for 16 h. Some unstained gels were sliced into sections, eluted by dialysis and assayed for toxic activity. Denaturing PAGE was done in a 2–16% gradient gel in 0.04 M Tris, 0.02 M sodium acetate, 0.002 M EDTA, SDS 0.2% (pH 7.4). Samples were heated at 100°C for 10 min in 0.01 M Tris-HCl, 0.001 M EDTA, SDS 1% (pH 8.0) with or without β-mercaptoethanol 5%, applied to gels and run at 300 V for 10 min and then at 150 V for 2.5–3 h. The gels were stained with Coomassie Blue R-250.

Immunodiffusion

Immunodiffusion was done in 5 × 5 cm agar plates as described by Wadsworth;¹⁶ 10–20 µg of antigen and undiluted antiserum were added to each well and

left in a humidified chamber for 48 h until diffusion was complete. Plates were then washed with phosphate-buffered saline and stained with Coomassie Blue R-250. Antisera against the 230–250-Kda and the 43-Kda subunits of toxin B molecular forms 1 and 2 respectively from *C. difficile* strain 10463 were produced in rabbits as previously described.⁵

ELISA

Toxin A was measured by ELISA.¹⁷ Briefly, microtitration plates (Immulon type 2; Dynatech Industries, USA) were coated with affinity purified goat antitoxin A (provided by Dr D. M. Lyster, Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA) diluted 1 in 200. Bound antigen was detected with rabbit antitoxin A¹⁴ diluted 1 in 500 and horse-radish peroxidase-conjugated anti-rabbit immunoglobulin. Pure toxin A was used as a positive control, and culture filtrates from two non-toxicogenic strains of *C. difficile* as negative controls. The limit of detection of the assay was 5 ng/ml.

Results

Toxin production by *C. difficile* strain 8864

The crude culture filtrate from strain 8864 contained 4.46×10^8 CU/ml (table) which was specifically neutralised by antitoxin B. No cytotoxin which could be neutralised by antitoxin A was detected in any of the fractions from DEAE-Sephadex chromatography, nor was any toxin A detected by ELISA.

Purification of toxin B

Toxin B eluted from DEAE-Sephadex at 0.4 M NaCl, with a 7.4-fold increase in specific activity and a yield of 45% (table). Gel filtration through Sephacryl S400 increased the specific activity to about 13 times with a yield of 22%. The final step was FPLC on Mono Q; the cytotoxin eluted at 0.48–0.50 M NaCl. The three purification steps resulted in a 25-fold concentration of activity with an overall yield of about 8%. Toxin B was purified from strain 10463 by the same scheme. Pure form 1 had a specific activity of 4.2×10^3 CU/pg.

Table. Purification of the cytotoxin produced by *C. difficile* strain 8864

Purification step	Volume (ml)	Protein content (mg/ml)	Total protein (mg)	CU/ml	Total CU	CU/pg
Crude filtrate	15	9.0	135	4.46×10^8	6.7×10^9	0.05
0.4 M DEAE-Sephadex	6.7	1.22	8.17	4.47×10^8	3.0×10^9	0.37
Sephacryl S-400	3.0	0.77	2.31	5.00×10^8	1.5×10^9	0.65
Mono Q, FPLC	5.5	0.08	0.44	1.00×10^8	5.5×10^8	1.25

PAGE

Under non-denaturing conditions, cytotoxin from strain 8864 migrated in two bands (fig. 1a, lane 1), one migrating slightly faster than toxin B form 1 of strain 10463 (lane 2), and the other similar to toxin B form 2 of strain 10463 (lane 3). Cytotoxic activity was eluted from both bands; the third faint band had no cytotoxic activity. Results of SDS-PAGE are shown in fig. 1b. In the absence of reducing agents, the cytotoxin of strain 8864 appeared as two bands of about 220 Kda and 105 Kda respectively (lane 1). The 220-Kda subunit migrated slightly faster than the subunit from toxin B form 1 of strain 10463 (230–250 Kda, lane 3) whereas the 105-Kda subunit was similar to the subunit of toxin B form 2 from strain 10463 (not shown). There was no change in the 220-Kda band after treatment with a reducing agent

Fig. 1. PAGE of the purified cytotoxins from *C. difficile* strains 8864 and 10463. (a) PAGE in non-denaturing conditions: lane 1, cytotoxin from strain 8864; 2 and 3, toxin B from strain 10463, molecular forms 1 and 2 respectively. (b) SDS-PAGE; lanes 1 and 2, cytotoxin from strain 8864, without and with β -mercaptoethanol respectively; 3 and 4, toxin B molecular forms 1 and 2 respectively from *C. difficile* strain 10463 treated with β -mercaptoethanol.

(lane 2) whereas the 105-Kda subunit dissociated into peptides of about 43 Kda (lane 2), similar to those formed after reducing toxin B form 2 of strain 10463 (lane 4).

Immunodiffusion

The two precipitin lines formed between toxin B form 2 of *C. difficile* strain 10463 and its own antiserum fused in identity with the single precipitin formed with toxin B form 1 (fig. 2a) as previously described.⁵ Cytotoxin B from strain 8864 formed a single precipitin line with antiserum to toxin B form 1 of strain 10463, which showed partial identity with the precipitin formed with toxin B form 1 of strain 10463 (fig. 2b); cytotoxin from strain 8864 formed two precipitin lines with antiserum to toxin B form 2 of strain 10463, which showed identity with one of the precipitin lines formed with purified toxin B form 2 from strain 10463 (fig. 2b).

Cytotoxic effects on lung fibroblast

In fig. 3, the cytotoxic effect of purified toxin B from *C. difficile* strain 8864 is compared with that of toxin B molecular form 1 from *C. difficile* strain 10463; 8×10^4 CU of toxin B form 1 of strain 10463 rounded the cells and many of them showed actinomorphous changes

Fig. 2. Immunodiffusion test. (a) Well 1, antiserum to *C. difficile* strain 10463 toxin B molecular form 1; 2, antiserum to *C. difficile* strain 10463 toxin B molecular form 2; 3, pure 10463-toxin B molecular form 1; 4 and 5, pure 10463-toxin B molecular form 2. (b) Well 1, antiserum to strain 10463 toxin B molecular form 1; 2, antiserum to strain 10463 toxin B molecular form 2; 3, pure 10463 toxin B molecular form 1; 4, strain 8864-cytotoxin; 5, pure 10463 toxin B molecular form 2.

Fig. 3. Effect of cytotoxin from *C. difficile* strain 8864 and toxin B molecular form 1 from *C. difficile* strain 10463 on human lung fibroblast cells: (a) untreated cells; (b), (d) and (f) cells incubated with cytotoxin from strain 8864, (b) 1×10^4 CU ($\times 50$), (d) 1×10^3 CU ($\times 100$) and (f) 1×10^2 CU ($\times 100$); (c) and (e), cells treated with toxin B molecular form 1 from strain 10463 ($\times 100$): (c) 8×10^4 CU and (e) 8×10^2 CU.

(fig. 3c), whereas with 8×10^2 CU all the cells became actinomorphic (fig. 3e). Although 8000 pg (1×10^4 CU) of cytotoxin B from strain 8864 caused extensive detachment of cells (fig. 3b), 8000 pg (3×10^7 CU) of toxin B form 1 from strain 10463 did not cause detachment of the cells; 1×10^3 CU of toxin from strain 8864 rounded the cells (fig. 3d) and 1×10^2 CU caused rounding and elongation of the cells. Actinomorphic changes were not seen (fig. 3f).

Discussion

It is thought that the production of toxins A and B by *C. difficile* is co-regulated¹¹ because most strains

which have been described produce both toxins or are non-toxigenic. However, *C. difficile* strain 8864 appears to produce toxin B only. Although this strain was grown under optimal conditions for toxin production,¹⁸⁻²⁰ its cytotoxic activity was completely neutralised by antitoxin B, whereas antitoxin A had no effect. Furthermore, no toxin A activity was detected during the purification procedures or by ELISA. These results strongly suggest that *C. difficile* strain 8864 does not produce toxin A even in an inactive form.

Analysis of toxin B from strain 8864 in SDS-PAGE after treatment with β -mercaptoethanol demonstrated two subunits of 220 Kda and 43 Kda whereas toxin B from strain 10463 had subunits of 230-250 Kda (form 1) or 43 Kda (form 2). In gel immunodiffusion,

the cytotoxin from strain 8864 showed partial identity with the 230-Kda subunit of toxin B from strain 10463 indicating that it lacked some toxin B epitopes; these missing epitopes might be responsible for the difference in size observed in PAGE.

The effect on fibroblast cells was also different; with toxin B from strain 10463, cells became rounded and highly arborised, giving the characteristic actinomorphic appearance. In contrast, with the toxin B from strain 8864, no arborisation of cells occurred but there was rounding and significant detachment.

Arborisation seemed to occur because the cytoplasm collapsed around the nucleus and projections remained attached to the supporting matrix by the adhesion plaque proteins.²¹ The cytotoxin from strain 8864 may have an effect on vinculin or talin or on related components of the adhesion plaque proteins. *C. sordelli* toxin also causes rounding of fibroblast cells without arborisation.²²

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