Quantitative cell-adhesion assay for *Clostridium difficile* cytotoxin

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Summary. A quantitative assay for *Clostridium difficile* cytotoxin has been developed, based on the observation that suspended fibroblasts exposed to cytotoxin fail to adhere to plastic. A dye-binding technique was used to quantitate adherent cells, in order to obviate microscopy. Adherent BHK cells were fixed with glutaraldehyde and cell protein was stained with Coomassie blue R-250. Cell-bound dye was eluted and estimated spectrophotometrically. The amount of eluted dye was proportional to the number of adherent cells and cell staining was time dependent. Cytotoxin was purified by gel-permeation and ion-exchange chromatography and migrated as a single band on SDS-PAGE. After exposure of suspended BHK cells to purified cytotoxin, their adhesion to plastic was inhibited in a manner which depended on concentration of cytotoxin and on time and temperature of exposure. This study provides the basis for a *C. difficile* cytotoxin assay that is quantitative, rapid and reproducible and may have wider applicability in the study of other toxins or agents that inhibit cell adhesion.

Introduction

Clostridium difficile is responsible for antibiotic-associated colitis in man (Bartlett *et al.*, 1978; George *et al.*, 1978), and for caecitis in the antibiotic-treated hamster (Bartlett *et al.*, 1977). Two toxins mediating mucosal damage are produced by the organism, enterotoxin (toxin A) and cytotoxin (toxin B) (Taylor *et al.*, 1981). The relative contribution of each toxin to colitis in man is unknown but in the hamster model, caecitis appears to be dependent on the simultaneous activity of both toxins (Libby *et al.*, 1982).

Cytotoxin causes characteristic rounding up of many tissue-culture cell lines (Thelestam and Bronnegard, 1980), and this phenomenon is widely used in its detection. There appears to be little correlation between titre of cytotoxin in stool and clinical severity of disease (George *et al.*, 1982), so accurate quantitation of cytotoxin in clinical laboratories has been thought to be unhelpful, either diagnostically or prognostically. However, in a number of areas it is important to be able to quantitate cytotoxin, e.g., to investigate the stability and biological degradation of cytotoxin in different conditions. Immunoassays have been described (Yolken *et al.*, 1981; Aronsson *et al.*, 1983; Peterson *et al.*, 1986), but do not detect biological activity.

Conventional assays based on cell rounding are subject to errors of the order of ± 10 -fold (Pothoulakis *et al.*, 1986). *C. difficile* cytotoxin is conventionally detected by its cytopathic effect on fibroblasts. This test relies upon microscopy of cells and at limits of dilution is inherently subjective and imprecise. Therefore, we developed a quantitative bio-assay based on our observation that *C. difficile* cytotoxin inhibits adhesion of BHK cells to plastic tissue-culture plates.

Materials and methods

Cells

BHK cells (Flow Laboratories) were grown to at least 90% confluence in growth medium (GM) consisting of Ham's F10 medium (Flow Laboratories) supplemented with newborn calf serum (Gibco) 5% v/v, 2 mM glutamine, penicillin 50 U/ml and streptomycin 50 µg/ml. Cells were incubated at 37°C in a humid atmosphere containing CO₂, 5% v/v. All cell manipulations were performed in GM.

Cell adhesion

BHK cells were trypsinised with fresh trypsin EDTA solution (Flow Laboratories) at 37°C for 1 min and were then suspended in GM in polycarbonate screw-top vials. For adhesion assays, 0.5-ml volumes were transferred to Multi-well (Sterilin, Feltham) tissue-culture plates con-

sisting of 25 4-cm² wells, dispersed evenly by transient gentle rocking, and incubated at 37°C in CO₂ 5% v/v for 2 h to allow adhesion. During incubation, direct contact between Multi-well plates and metal incubator trays was prevented by interposition of a plastic flask, to avoid temperature-dependent differences in cell adhesion (Adler *et al.*, 1982). After incubation, plates were washed three times with phosphate-buffered saline (PBS) pH 7.2 containing (L) NaCl 8 g, KCl 0.2 g, Na₂HPO₄ 12H₂O 1.15 g, KH₂PO₄ 0.2 g, and adherent cells were quantitated as described below.

Quantitation of cell protein of adherent BHK cells (dye-binding assay)

BHK cells adhering to plates after washing were fixed with glutaraldehyde (BDH) 3% w/v in PBS for 20 min at room temperature. A stock solution of Coomassie brilliant blue R-250 (C.I. 42660) (PAGE blue 83; BDH) 0.4 g in 880 ml of ethanol 30% v/v was mixed with glacial acetic acid immediately before use to make a final acid solution of 12% v/v. The solution was then filtered (Whatman's No. 1 filter paper). The cells were stained with Coomassie blue solution for 30 min at room temperature, then washed three times with an aqueous solution containing acetic acid 5% v/v and ethanol 10% v/v to remove unbound dye. Protein-bound dye was eluted at room temperature with 1.0 M potassium acetate (general purpose grade) in ethanol 70% v/v for 15 min. After gentle rocking to ensure homogeneity, the absorbance of the eluate was measured spectrophotometrically at 590 nm. Prolonged elution times were unreliable because of evaporation of ethanol. Background dye-binding by the newborn calf serum from GM was estimated in each plate by incubating GM alone in triplicate; the mean value was subtracted from the absorbance found with adherent cells.

Cytotoxin preparation

A single strain of *C. difficile* was isolated from a patient with pseudomembranous colitis and identified as described by Borriello and Honour (1981). The original isolate was maintained in cooked-meat medium. For cytotoxin preparation, a subculture from Cysteine Blood Agar (Oxoid) was cultured in Brain Heart Infusion Broth (Oxoid) in an anaerobic cabinet (N₂ 85%, H₂, 5%, CO₂ 10%) for 5 days at 37°C. All steps in the preparation of cytotoxin were performed at 4°C. Culture broth was centrifuged (10 000 *g* for 30 min), membrane filtered (0.45 µm; Millipore), and concentrated by ammonium sulphate precipitation by the procedure described by Pothoulakis *et al.* (1986), except that PBS was used instead of Tris-HCl solution. The precipitate obtained from the ammonium sulphate solution was dissolved in PBS and dialysed overnight against 100 volumes of PBS. This solution was fractionated by Sephacryl S-300 gel permeation chromatography with PBS as the eluant.

Volumes of 0.025 ml of each column fraction were added to 96-well plates (Costar, Cambridge, MA, USA) with each well containing confluent BHK cells in 0.2 ml of GM. Fractions causing characteristic cell rounding at 30 min were pooled and, after overnight dialysis against 0.05 M Tris-HCl pH 7.5, were further purified by ion-exchange chromatography through a DEAE-Sepharose CL-6B column (1.5 cm × 10 cm), eluting sequentially with 60-ml volumes of 0.05 M Tris buffer, pH 7.5, containing 0.05, 0.2, 0.25, 0.3, 0.32, 0.34 and 0.4 M NaCl respectively. The flow rate was 60 ml/h with fraction volumes of 5 ml. Fractions causing cell rounding at 30 min were filtered (0.45 µm; Millipore) and stored at 4°C. Control experiments showed that cytotoxicity was not influenced by the different NaCl concentrations. Filtered cytotoxin fractions were dialysed overnight against Ham's F10 medium and stored at 4°C before use within 48 h in the experiments described below.

Cytotoxic fractions purified by ion-exchange chromatography were concentrated with Aquacide Type I, mol. wt 70 000 (Calbiochem) and subjected to polyacrylamide gel electrophoresis with 0.2 M glycine, 0.025 M Tris running buffer, pH 8.3 in dissociating (SDS) and non-dissociating conditions (Laemmli, 1970). Solubilising buffer consisted of β-mercaptoethanol 0.5% v/v, SDS 1% w/v, glycerol 0.5% v/v, 0.1 M Tris pH 6.8. The sample buffer for non-dissociating conditions was 1.0 M Tris-HCl pH 6.7, glycerol 10% v/v and bromophenol blue 0.1% v/v. In non-dissociating and dissociating conditions, a single protein band was seen after Coomassie blue staining. Extrapolating from concurrent mol.-wt standards, this protein had a mol. wt of 54 (±2) × 10³.

Inhibition of cell adhesion by cytotoxin

Purified cytotoxin was added in the ratio of 1:20 (v:v) to cells incubated in suspension by rolling (Spiramix; Denley Instruments). After 30 min, 0.5-ml samples were added to Multi-well plates for adhesion assay. In some experiments, *C. sordelli* antiserum (Wellcome) sufficient to neutralise all cytotoxin present (Chang *et al.*, 1978) was added before plating. Cells exposed to cytotoxin were allowed to adhere to Multi-well plates for 2 h. Adhesion of cells exposed to cytotoxin was compared to that of non-exposed control cells and the ratio (cytotoxin exposed: non-exposed) expressed as a percentage.

For the preparation of highly purified cytotoxin from the DEAE Sepharose columns, 400 ml of broth filtrate was used as the starting material. Even after concentration of fractions from the ion exchange column the amount of protein in solution was below the lower limit of detection of micro-Lowry and Coomassie blue spectrophotometric assays. Thus the actual mass of cytotoxin used in the cell binding assays was unknown. A dilution of the cytotoxin preparation causing c. 90% inhibition of adherence was used as the most concentrated sample (figs 2 and 3). At this concentration of cytotoxin, cell rounding was not detectable after incubation for 2 h, as used in adherence assays, but was apparent after incubation for 36 h.

Results

Validation of technique to quantitate adherent cells

Time dependence of cell adhesion. BHK cells incubated in suspension in polycarbonate screw-top vials with rolling for 30 min were plated out and allowed to adhere for 1.5, 2, 2.5 and 3 h, after which adherent cell protein was estimated by the dye-binding technique. Cell adhesion was maximal at 2 h after plating and remained constant thereafter.

Relationship of cell number to dye binding. Cells previously incubated in suspension with rolling for 30 min were counted with a Coulter counter, diluted and allowed to adhere in Multi-well chambers for 2 h. Adherent cell protein was estimated spectrophotometrically, as described. Cell number was plotted against absorbance of eluted Coomassie blue dye (fig. 1). For cell numbers of $(0.5-3) \times 10^5/\text{ml}$, there was an approximately linear relationship between number and absorbance. With cell numbers above $3 \times 10^5/\text{ml}$, the absorbance values began to plateau. In further experiments, cell suspensions containing about 1.5×10^5 cells/ml were used. This concentration was obtained by trypsinising cells grown to confluence in a 75-cm^2 flask and suspending these in 30 ml of GM.

Plate to plate variation. A suspension of BHK cells was rolled for 30 min and samples were allowed to adhere for 2 h in triplicate wells in each

of four different Multi-well plates. Adherent cell protein was estimated by the dye-binding technique and mean absorbance values (SEM) for each plate were; 0.597 (0.011), 0.615 (0.028), 0.560 (0.019) and 0.587 (0.024). Plate-to-plate variation in absorbance was comparable to intra-plate variation. Analysis of variance showed that inter-plate variation was not significant ($p = 0.16$). Thus, different plates could be compared directly.

Intra-plate variation. Two concentrations of cytotoxin, known to inhibit cell adhesion by around 30% and 50% respectively, were selected. Each sample of cytotoxin was assayed in duplicate on one Multi-well plate. One concentration resulted in adherence of 34.1 (SD 3.7)% and the other 48.2 (SD 5.7)%.

Effect of rolling cells on cell staining. Suspended BHK cells, rolled for 0, 1.5, 2.5 and 3.5 h at 37°C , were plated out in triplicate and allowed to adhere for 2 h. There was little difference in cell staining after rolling for up to 1.5 h, but after rolling for 3.5 h staining was reduced by 15%. Therefore, a rolling time of 30 min did not affect cell staining, and this time was used in quantitation experiments.

Time dependence of cell staining. Cells rolled for 30 min were plated out and allowed to adhere for 2 h. After fixation, cells were stained for 30 min or 120 min. Protein-bound dye increased by 43% between 30 min and 120 min of staining. We noted that prolonged staining (16 h) resulted in much higher dye-binding, but this was not quantitated. Thus a fixed time of 30 min staining was used in all quantitation experiments.

Inhibition of BHK cell adhesion by *C. difficile* cytotoxin

Time dependence. Cytotoxin was added to cells and *C. sordelli* antiserum added to samples taken after rolling for 5, 10, 15, 20, 30, 40, 50 and 60 min. A zero-time sample was prepared by incubating cytotoxin with antiserum for 5 min at room temperature before addition of cells (fig. 2). No inhibition of adherence was detected in cells exposed to the cytotoxin-antiserum mixture compared with adherence of non-exposed cells. Inhibition of cell adhesion was observed after exposure to cytotoxin for 5 min and increased with time.

Concentration dependence. Serial 1.5-fold dilutions of cytotoxin were incubated with suspended cells for 30 min at 37°C and antiserum added (fig. 3). There was an approximately linear relationship between cytotoxin concentration and inhibition of

Fig. 1. Coomassie blue staining of adherent BHK cells. Cells were allowed to adhere to Multi-well plates for 2 h and were washed, fixed and stained for 30 min with Coomassie blue. Stain was eluted and measured spectrophotometrically. The numbers of cells (mean $\pm$ SEM) initially in suspension are plotted against absorbance (mean $\pm$ SEM).

Fig. 2. Time course of inhibition of BHK cell adherence by *C. difficile* cytotoxin. BHK cells in suspension were exposed to a fixed concentration of purified *C. difficile* cytotoxin at 37°C. At the time shown, *C. sordelli* antiserum was added to cells which were allowed to adhere to Multi-well plates for 2 h in the presence of antiserum. Adherent cells were assayed by the Coomassie blue dye-binding technique. Results are expressed as mean percentage adherence compared to cells not exposed to cytotoxin (bar = SEM).

cell adherence in a given range. A two-fold increase in cytotoxin concentration resulted in a reduction of c. 20% in cell adherence, and a change from 20% to 80% cell adhesion represented a 5- to 6-fold concentration range. Below 20% and above 80% inhibition, results were non-linear. The same relationship was observed with a series of dilutions of crude *C. difficile* broth-culture filtrate containing cytotoxin.

Temperature dependence. Samples from a cell suspension were incubated at 4°, 20°, and 37°C in triplicate. Each sample was exposed to the same concentration of cytotoxin and *C. sordelli* antiserum was added 30 min after addition of cytotoxin. Cells were allowed to adhere to Multi-well plates at 37°C for 2 h. Cytotoxin reduced cell adherence by 60% at 37°C, 22% at 20°C, and 13% at 4°C. (These results are expressed as the percentage adherence when compared to control cells rolled for 30 min at

Fig. 3. Concentration dependence of inhibition of BHK cell adherence by *C. difficile* cytotoxin. BHK cells in suspension were exposed to a range of concentrations of purified *C. difficile* cytotoxin for 30 min at 37°C. Cells were allowed to adhere to Multi-well plates for 2 h at 37°C. Adherent cells were washed, fixed and assayed by the Coomassie blue dye-binding technique. Results (mean ± SEM) are expressed as percentage adherence compared to cells not exposed to cytotoxin.

the same temperature but not exposed to cytotoxin.) Thus, exposure of BHK cells to cytotoxin at different temperatures markedly affected the degree of subsequent inhibition of cell adherence.

Discussion

Previous assays of *C. difficile* cytotoxin have relied on observation of morphological changes in cells in tissue culture. These assays are subjective and prone to observer error and are thus inaccurate and poorly standardised. We set out to develop a sensitive quantitative bioassay for *C. difficile* cytotoxin, using our observation that cytotoxin inhibited adhesion of BHK cells to plastic tissue-culture plates. To quantitate adherent cells without direct cell counting, we assayed cell protein by a Coomassie blue dye-binding technique.

We have demonstrated that adherent BHK cell numbers can be estimated indirectly by measuring eluted Coomassie blue dye. A linear relationship was found between absorbance of eluted dye and cell concentration in the original suspension in the range $(0.5-3) \times 10^5/\text{ml}$. Although we did not count adherent cells directly, it has previously been reported that about 80% of suspended BHK cells adhered to plastic tissue-culture plates in conditions

similar to our own (Nath and Srere, 1977). Furthermore, we showed that in control conditions, in the absence of cytotoxin, BHK cell adhesion to plastic tissue-culture plates was maximal in 2 h. This was somewhat slower than previously observed by Nath and Srere (1977), but this may have been due to differences in several factors, including composition of serum (Knox, 1984), substrate surface (Curtis *et al.*, 1983), and the amount of shear applied when washing unbound cells from plates.

Staining of BHK cells with Coomassie blue R-250 is time dependent. This parallels the findings of Barer *et al.* (1986) and Laughton (1984) who pointed out that in each cell system, staining kinetics should be estimated. In the system employed in this study with BHK cells, exposure to Coomassie blue for 30 min gave a suitable range of absorbance values.

Adherent cells have been quantitated by several methods including direct microscopy (Patterson, 1979), assay of cell enzymes (Connolly *et al.*, 1986) or estimation of cell protein (Laughton, 1984; Shopsis and Mackay, 1984; Baumgarten, 1985). One method (Shopsis and Mackay, 1984) required initial lysis of cells before Lowry micro-assay of cell protein. Other Coomassie blue protein dye-binding assays have been described (Laughton, 1984; Baumgarten, 1985). These used a modification of the Bradford protein assay (Bradford, 1976), which depends on the colour change of Coomassie blue G-250 at low pH from brown-red to blue when bound to protein. The assay described in the current study relies on the fact that binding of Coomassie blue R-250 to protein is reversible in suitable conditions (Fazekas de St Groth *et al.*, 1963), and thus bound dye may be eluted from cells.

The purified *C. difficile* cytotoxin ran as a single band on SDS-PAGE gel. The estimated mol. wt of 54×10^3 was similar to that found by Pothoulakis *et al.* (1986). Using the Coomassie blue dye-binding method validated above, we examined the inhibition of BHK cell adhesion caused by this preparation. The time of cytotoxin exposure of suspended BHK cells was varied by adding *C. sordelli* antiserum to samples of a cell suspension at defined times to prevent further toxin activity. Cells were then allowed to adhere in the continued presence of antiserum. The first measurement of cell adherence after 5 min was less than the control value (obtained when cytotoxin was neutralised by antiserum for 5 min before addition of cells) and inhibition of cell adhesion increased with further exposure. Thus, cytotoxin decreased the ability of BHK cells to adhere after short exposures to

cytotoxin. Increasing concentrations of cytotoxin increased the rate of inhibition of cell adhesion (results not shown).

When cells were exposed for a fixed time (30 min) to a range of cytotoxin concentrations, inhibition of cell adhesion was proportional to the logarithm of the concentration. This relationship was found to be constant both for a crude broth-culture filtrate and for highly purified toxin. A six-fold increase in cytotoxin concentration decreased cell adhesion from 80% to 20%, and within this six-fold range cytotoxin concentrations could be quantitated.

Temperature of exposure markedly affected interaction between cells and cytotoxin; therefore, assays must be performed at controlled temperature. Temperature dependence of interaction of *C. difficile* cytotoxin and adherent cell morphology was demonstrated by Florin and Thelestam (1981). The molecular events following exposure of cells to *C. difficile* cytotoxin are still unknown, but temperature-dependent internalisation of other toxin molecules has been described (Montesano *et al.*, 1982).

Other biological effects of *C. difficile* cytotoxin on cultured fibroblasts have been demonstrated. Thelestam and Bronnegard (1980) used a fluorescent antibody to label actin, and described disruption of straight actin bundles in human lung fibroblasts exposed to semi-purified cytotoxin. Wedel *et al.* (1983), using electronmicroscopy, demonstrated that partially-purified cytotoxin caused disruption of actin-containing myofilaments in smooth muscle cells from rabbit aorta. Pothoulakis *et al.* (1986), using a highly purified cytotoxin preparation, showed that cell rounding of IMR-90 fibroblasts was related to the ratio of globular to filamentous actin. The change in actin configuration, first demonstrated at 15 min, was nearly complete at the onset of detectable cell rounding (30–60 min). This is thought to be an important intracellular event preceding morphological change. Using the Coomassie blue dye-binding technique, we demonstrated that brief exposure (5 min) of BHK cells to purified cytotoxin caused inhibition of cell adhesion.

Other actions of the cytotoxin that have unknown pathophysiological significance include inhibition of protein and nucleic acid synthesis (Florin and Thelestam, 1981) and electrolyte leakage from affected cells (Rothman *et al.*, 1984). The stimulation of guanylate cyclase and inhibition of adenylate cyclase were reported by Vesely *et al.* (1981), but have yet to be confirmed. Biochemical studies on the biological target of the toxins of *C. difficile*, namely, the colonocyte, are still to be performed.

Other toxins inhibit the adhesion of fibroblasts to plastic wells. For example, the enterotoxin of *C. perfringens* has been shown to be active in this respect. McClane and McDonel (1979) demonstrated inhibition of cell adhesion after exposure of Vero cells to *C. perfringens* enterotoxin by use of direct microscopy to quantitate cells. We confirmed that *C. perfringens* enterotoxin inhibited cell adhesion by the dye-binding technique; we found that concentrations of the order of 1–100 µg/ml were necessary to obtain measurable inhibition of BHK cell adhesion after exposure for 30 min. Some toxins however, may have no effect on cell adhesion and

therefore this assay would not be applicable to these toxins. For example, exposure of BHK cells to cholera toxin, even at high concentration (10 nM), did not inhibit adhesion (unpublished observation).

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