Technique for measuring 50% end points in cytotoxicity assays for *Clostridium difficile* toxins

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SUMMARY Serial dilutions of *Clostridium difficile* culture filtrates were incubated overnight with HeLa cell monolayers. Cells were fixed in formalin, stained with crystal violet, rinsed, and drained. Cell rounding could be observed microscopically in the stained monolayers. Absorbance of the retained dye on monolayers in the drained wells was measured at 595 nm–405 nm. End points could also be estimated visually. The dilution at which dye absorbance was reduced by 50% agreed with that determined by microscopic observations. Five replicate dilution series showed high reproducibility. Specificity was verified by neutralisation with crude rabbit antibody to *C difficile* toxins. Cytotoxicity in faecal specimens was assayed in the same way, allowing reporting of titres, comparison with standard toxin preparations, and determination of the extent of neutralisation to be made. This novel assay technique has proved effective and reliable in a clinical setting and should allow the gathering of more information on the epidemiology of antibiotic associated colitis.

Determination of endpoints in the tissue culture assay currently used in clinical laboratories for recognising *Clostridium difficile* toxins in faecal specimens is cumbersome and subjective, entailing the laborious counting of cells with cytopathogenic effects.[1-5] Until an objective and accurate quantitative assay becomes available, the predictive value of the toxin titre in the clinical course of the disease will not be accurately assessed. Some laboratories have tried to substitute other assays, including counterimmunoelectrophoresis. The usefulness of this method for *C difficile* toxin assays is questionable.[6-8] Recently, an elegant enzyme linked immunosorbent assay (ELISA) for toxins A and B has been developed[9] and will certainly be the preferred method in the future. At present, its widespread use is restricted because monospecific antibodies for toxins A and B are available in only very few laboratories. The lack of specific antibodies results in a high percentage of false negative readings in the latex agglutination assay.[10] The tissue culture assay is still the most widely useful for detecting *C difficile* toxins as it can be carried out without the use of either purified toxins or monospecific antibody.

In purifying *C difficile* toxins A and B, I developed a technique for easily and accurately measuring endpoints in the tissue culture assay by reading absorb-

ance of stained monolayers in a microtitre plate reader.[11] I have now explored further the quantitation of this assay, the possibility of reproducible measurement of a 50% end point, and the usefulness of the technique for identifying and assaying *C difficile* toxins in clinical specimens.

Material and methods

TOXIN AND ANTITOXIN
Crude *C difficile* culture filtrates were used as toxin preparations. Preparation of filtrates and rabbit antitoxin were used as previously described.[11 12]

CELLS AND CULTURE CONDITIONS
HeLa cells and maintenance conditions have been described previously.[13] To prepare assay plates HeLa cells were suspended from monolayers by treatment with trypsin and edetic acid, counted, and adjusted to a concentration of 160 000 cells/ml in growth medium consisting of Eagle's minimum essential medium with Earle's salts (MEM) (HEM Research, Rockville, Maryland) supplemented with 10% heat inactivated fetal bovine serum, 2 mM glutamine, 300 U of penicillin, and 300 μg streptomycin/ml. One tenth ml samples were pipetted into 96 well microtitre plates (Costar, Cambridge, Massachusetts). Monolayers were established by 24 hours' incubation at 35°C in a 5% carbon dioxide atmosphere.
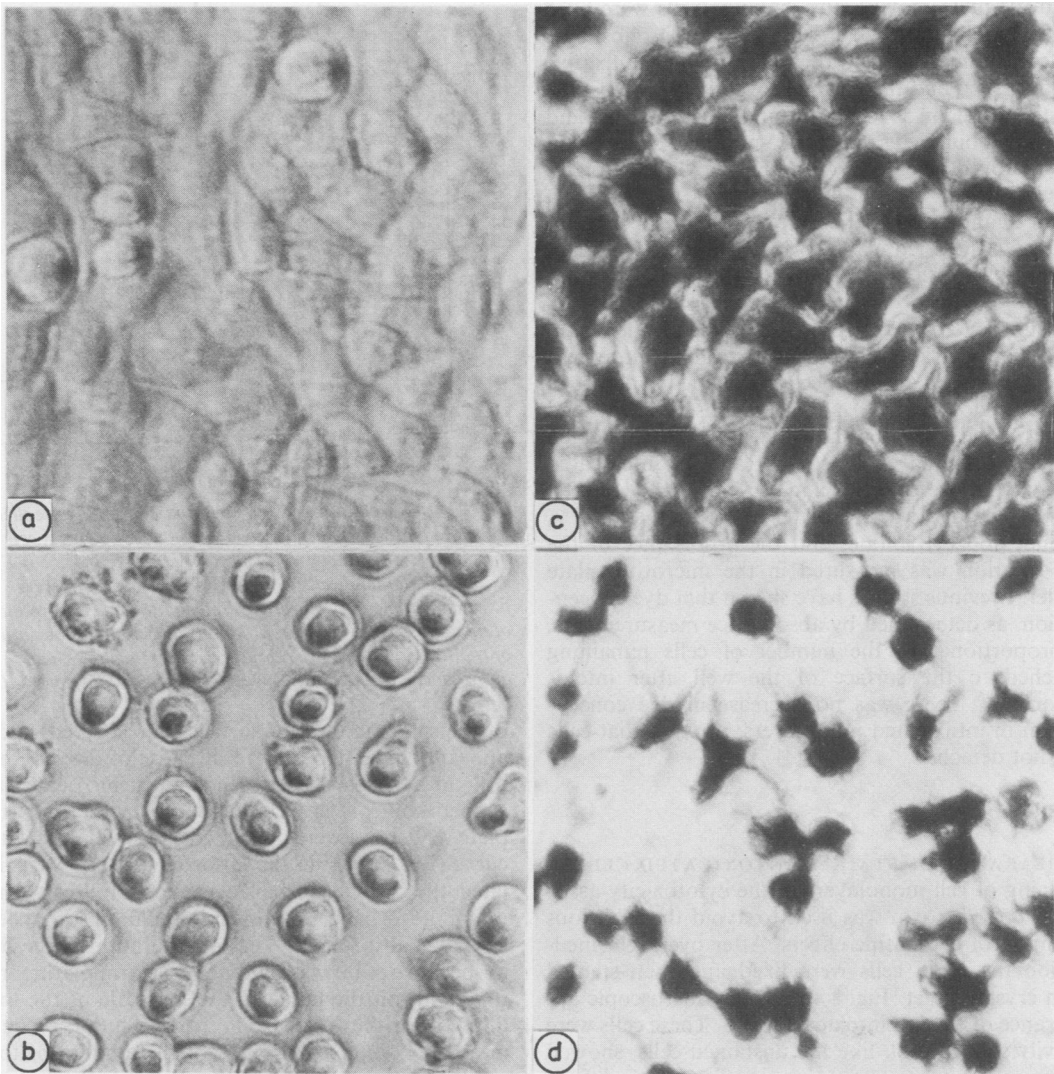
Fig. 1 *Microscopic appearances of stained intoxicated HeLa cell monolayers. HeLa cell monolayers were incubated overnight after addition of 0·1 ml of stool extract (b, d). One tenth ml of physiological saline was added to control monolayers (a, c). Cells were fixed and stained with crystal violet (c, d). Unfixed cells are shown in (a) and (b). Phase-contrast, original magnification × 160, Kodak No 11 filter (yellow-green).*

CYTOTOXICITY ASSAY

Four fold serial dilutions of toxin were prepared; MEM was used as diluent. One tenth ml sample of a dilution was added per well, and the plates were incubated overnight. Negative controls consisted of 0·1 ml MEM. For neutralisation serial twofold dilutions of rabbit antiserum in MEM were mixed with an equal volume of *C difficile* toxin at a 1/16 dilution. This dilution was chosen because in my experience positive patient specimens have always been cytotoxic at this dilution. Negative antibody controls were prepared by mixing antibody dilutions with equal volumes of MEM. The toxin dilution used in the neutralisation assay was mixed with an equal volume of MEM as a positive toxin control. One tenth ml samples of toxin-antitoxin mixtures and controls were incubated with cell monolayers.

After incubation medium and toxin were emptied

from the plate by vigorous shaking. Cells were fixed with a 2% solution of formalin in 0·067M phosphate buffered saline (pH 7·2) for one minute; the fixative was removed and the plates were stained with 0·13% crystal violet in 5% ethanol and 2% formalin and phosphate buffered saline for 15 minutes. Excess stain was removed by careful water rinsing, and the plates were air dried.

For quantitation of the endpoint the absorbance of the dye in the dried plates was measured at 595 nm in a microtitre plate reader (Multiskan MC; Flow Laboratories, McLean, Virginia). Absorbance at 405 nm was subtracted so that anomalies in the plastic would not affect accuracy. The toxin dilution, resulting in a 50% decrease in absorbance, was defined as the 50% cytotoxic dose (CD_{50}) and was chosen as the end point for the assay. To show that cells did not detach from the monolayer 12 control and 12 intoxicated monolayers were fixed and stained as described above. The dye was dissolved in 50% ethanol, 1% sodium dodecyl sulfate, and the absorbance of the dye solution was measured in the microtitre plate reader. Previous studies have shown that dye concentration, as determined by absorbance measurements, is proportional to the number of cells remaining attached to the surface of the well after intoxication.[14 15] There was no decrease in dye concentration in intoxicated monolayers, proving that cells had not detached.

Results

APPEARANCE OF STAINED INTOXICATED CELLS

Staining of cell monolayers in the cytotoxicity assay for *C difficile* toxin was used to avoid the laborious counting of cytopathic effects. After overnight incubation the toxin cells were fixed and then stained with crystal violet. Fig. 1 shows the microscopic appearance of stained intoxicated cells. These cells were heavily stained and, like the unstained cells, showed the rounding typical of cytotoxic effects of *C difficile* toxins. They had not detached, but they occupied a relatively small amount of the surface of the well. The control cells, on the other hand, were spread over most of the plastic surface and also became heavily stained. The assay prepared in this way could be stored indefinitely at room temperature if protected from light to prevent fading of the crystal violet.

QUANTITATION OF THE CYTOTOXICITY ASSAY

Reproducible 50% end points were quickly obtained after fixing and staining cells. Direct visual examination of fixed and stained plates permitted rapid screening for positive specimens and visual estimation of end points (Fig. 2 inset). The intoxicated cells allowed more light through than the controls because
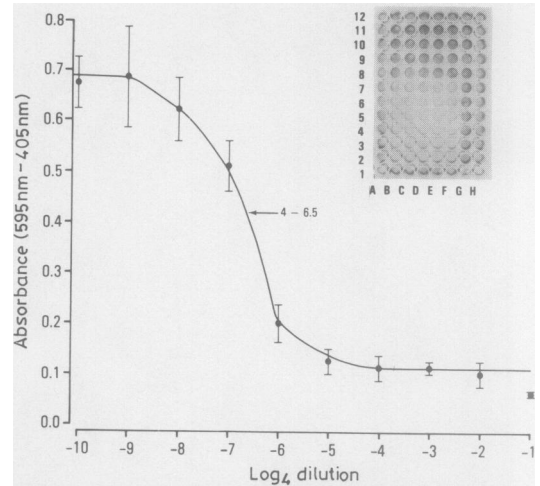

Fig. 2 *Determination of 50% cytotoxic dose. Five replicate four fold dilutions of crude toxin were added to HeLa cell monolayers, 0·1 ml per well (inset: Wells B-F 2, 4^{-1}; B-F11, 4^{-10}). Remaining wells were MEM controls. Cells were fixed and stained after overnight incubation and absorbance of monolayers was measured. Means and standard deviations of replicate dilutions are represented graphically. Mean absorbance of control wells was 0·54 ((SD) 0·04), n = 10.*

of the small amount of surface they covered, and so appeared lighter to the eye. Because of the difference in light absorbance between stained intoxicated and stained control cells, as measured in the microtitre plate reader, we were able to construct an absorbance curve (Fig. 2). From the absorbance curve we calculated the 50% absorbance value as follows: (maximum absorbance minus minimum absorbance) / 2 = 50% absorbance. The toxin dilution at the 50% absorbance point was obtained by extrapolating from this point on the curve to the log scale of the toxin dilution on the X axis. The toxin dilution so determined ($4^{-6.5}$) contains 50% of the amount of toxin needed to cause maximum rounding of all cells in the monolayer and is known as the 50% cytotoxic dose (CD_{50}). The reproducibility of this assay is shown by the small standard deviations apparent in the curve for the four fold serial dilution. Five separate five fold serial dilutions of the same toxin preparation were assayed on a different plate, and the mean 50% end point was exactly the same as that obtained with four fold dilutions.

CLINICAL ASSAY FOR *C DIFFICILE* TOXINS

Fig. 3 shows the format of the microtitre plate assay for the identification and quantitation of *C difficile* toxins in clinical specimens. Four fold dilutions of crude toxin or stool filtrates permitted a visual estimation of the CD_{50}'s. In each case this was the 4^{-5}
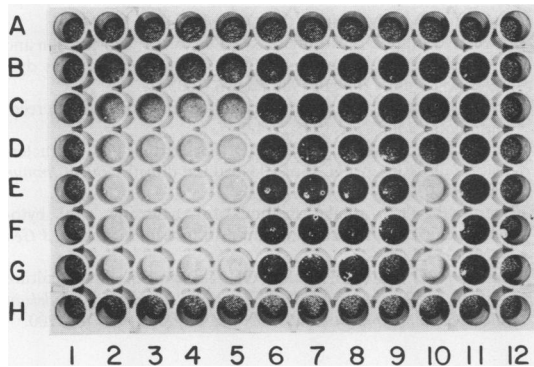
Fig. 3 *Cytotoxicity assay for detection and measurement of C difficile toxins in human faecal specimens. Columns 1, 11, and 12 are MEM controls; columns 2 and 3 faecal specimen titration; columns 4 and 5 C difficile standard toxin titration; both are four fold dilution series: row G, lowest dilution (4^{-1}); row B, highest dilution (4^{-6}). Columns 6 and 7 are C difficile antitoxin neutralisation assays; columns 8 and 9 antitoxin controls. Two fold dilutions of antitoxin were added to equal volumes of 1/16 dilution of faecal specimen (6, 7) or MEM (8, 9); row G lowest dilution, row B, highest dilution. Column 10 (E, F, G) is faecal specimen control; 1/16 dilution was added to equal volume of MEM. In all cases 0·1 ml volume was added per well.*

(1/1024) dilution (Fig. 3). This result agreed with the end point read from the absorbance curve (not shown). The medium control could easily be seen as negative and also had expected high absorbance. The effect of neutralisation by crude *C difficile* antitoxin was also obvious by rapid visual inspection, confirmed by absorbance readings. The antibody control was done to ensure that cytotoxicity by non-specific factors in the serum could not mask the neutralisation of toxin. For the specimen control in column 10 the dilution of stool prepared for the neutralisation assay was tested to ensure its toxicity and the validity of the assay. Using this assay method, stool extracts can be tested for toxicity, identified as having *C difficile* toxins, and a titre can be estimated immediately on inspection of the plate.

Discussion

In this report I have described a rapid, accurate, and reproducible technique for measuring 50% end points in *C difficile* toxin assays. The technique is useful in assaying clinical specimens, allowing measurement of toxin titres, and showing specificity through the use of neutralising antibody, without any need to count cells by light microscopy. Multiple determinations using two different dilution series showed that the end point was reproducible. As noted by Reed and Muench,[16] a 50% end point is more reproducible than the 100%

end point previously used.[11] [17] Accuracy is increased by bypassing the need for judgment in assessing cytopathic effects. The assays can be quickly observed by light microscopy to check that typical cell changes are present, or compared later with other specimens from the same patient. The assay can be used with crude antibody, thus making it available to any laboratory that can obtain tissue culture monolayers.

The neutral red vital stain method of Finter[18] has been used by Giugliano in assaying *C difficile* culture filtrates.[19] Damaged cells take up less neutral red than healthy ones, and determination of dye concentration allows an estimate of the extent of cell damage to be made. Unfortunately, this vital stain method is eight to 16 times less sensitive than microscopic examination, although it is more precise. The assay reported here is not based on a vital stain, nor is it a measure of dye concentration. The decrease in absorbance in intoxicated monolayers is due to the morphological change caused by the cytotoxin. The intoxicated cells round up and occupy a smaller surface area than the normal cells: this assay, therefore, directly measures the change in cell shape and is in agreement with microscopic observations.

The technique reported here for assaying *C difficile* toxins can be easily used by any laboratory with a microtitre plate reader. The time needed to measure toxin titres can be considerably reduced and accuracy enhanced. The data from the plate reader can easily be transferred into a computer for analysis. This assay method should permit an accurate assessment of whether higher stool titres are associated with more severe disease and certain strains of *C difficile* in antibiotic associated colitis.

References

1 Aswell JE, Ehrich M, Van Tassell RL, Tsai C-C, Holdeman LV, Wilkins TD. Characterization and comparison of Clostridium difficile and other clostridial toxins. In: Schlessinger D, ed. *Microbiology*. Washington, DC: American Society for Microbiology, 1979:272–5.

2 Borriello SP. An evaluated micromethod for the detection of Clostridium difficile enterotoxin. *Microbios* 1978;7:25–8.

3 Chang T-W, Lauermann M, Bartlett JG. Cytotoxicity assay in antibiotic-associated colitis. *J Infect Dis* 1979;140:765–70.

4 George RH. A micro method for detecting toxins in pseudomembranous colitis. *J Clin Pathol* 1979;32:303–4.

5 Rifkin GD, Fekety FR, Silva J Jr, Sack RB. Antibiotic-induced colitis-implication of a toxin neutralized by Clostridium sordellii antitoxin. *Lancet* 1977;2:1103–6.

6 Sands M, Yungbluth M, Sommers HM. The non-value of counter-immunoelectrophoresis for the direct rapid detection of Clostridium difficile toxin in stool filtrates. *Am J Clin Pathol* 1983;79:375–7.

7 Welch DF, Menge SK, Matsen JM. Identification of toxigenic

Clostridium difficile by counterimmunoelectrophoresis. *J Clin Microbiol* 1980;11:470–3.

[8] West SEH, Wilkins TD. Problems associated with counterimmunoelectrophoresis assays for detecting Clostridium difficile toxin. *J Clin Microbiol* 1982;15:347–9.

[9] Laughon BE, Viscidi RP, Gdovin SL, Yolken RH, Bartlett JG. Enzyme immunoassays for detection of Clostridium difficile toxins A and B in fecal specimens. *J Infect Dis* 1984;149:781–8.

[10] Shahrabadi MS, Bryan LE, Gaffney D, Coderre SE, Gordon R, Pai CH. Latex agglutination test for detection of Clostridium difficile toxin in stool samples. *J Clin Microbiol* 1984;20:339–41.

[11] Rothman SW, Brown JE, Diecidue A, Foret DA. Differential cytotoxic effects of toxins A and B isolated from Clostridium difficile. *Infect Immun* 1984;46:324–31.

[12] Rothman SW, Brown JE. Inhibition of membrane functions in intact HeLa cells by Clostridium difficile cytotoxic culture filtrates. *Current Microbiology* 1981;6:221–4.

[13] Brown JE, Rothman SW, Doctor BP. Inhibition of protein synthesis in intact HeLa cells by Shigella dysenteriae 1 toxin. *Infect Immun* 1980;29:98–107.

[14] Gentry MK, Dalrymple JM. Quantitative microtiter cytotoxicity assay for Shigella toxin. *J Clin Microbiol* 1980;12:361–6.

[15] Brown JE, Griffin DE, Rothman SW, Doctor BP. Purification and biological characterization of Shiga toxin from Shigella dysenteriae 1. *Infect Immun* 1982;36:996–1005.

[16] Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. *Am J Hyg* 1938;27:493–7.

[17] Sullivan NM, Pellett S, Wilkins TD. Purification and characterization of toxins A and B of Clostridium difficile. *Infect Immun* 1982;35:1032–40.

[18] Finter NB. Dye uptake methods for assessing viral cytopathogenicity and their application to interferon assays. *J Gen Virol* 1969;5:419–27.

[19] Giugliano LG, Barer M, Mann GF, Drasar BS. Tissue culture systems for the examination of bacterial virulence. In: *Models of anaerobic infection*. Boston: Martinus Nijhoff, 1984:189–200.