PRODUCTION AND RELEASE OF TOXINS A AND B BY *CLOSTRIDIUM DIFFICILE*

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SUMMARY. The production and release of toxins A and B by *Clostridium difficile* during in-vitro culture was investigated. Cell-associated toxin A was detected by immunoelectrophoresis of bacterial extracts released by ultrasonication and by fluorescent antibody labelling of whole cells. Extracellular toxin A was detected by immunoelectrophoresis and by enzyme-linked immunosorbent assay; extracellular toxin B was detected by cytotoxin assay. Both toxins A and B were produced and released during the decline phase of the bacterial growth cycle. The possible significance of these results in relation to the pathogenesis of pseudomembranous colitis is discussed.

INTRODUCTION

Clostridium difficile is the major aetiological agent of colitis and pseudomembranous colitis associated with antimicrobial therapy (Bartlett *et al.*, 1978a; George *et al.*, 1978). This organism produces at least two separate toxins (Taylor, Thorne and Bartlett, 1980 and 1981; Burdon *et al.*, 1981), an enterotoxin (toxin A) and a cytotoxin (toxin B) (Lyerly *et al.*, 1982) as well as a myoelectric factor (Justus *et al.*, 1982). Some biological properties of the two toxins are known (Bartlett and Taylor, 1982; Stephen *et al.*, 1984) but less is known about bacteriological aspects of their production. Here, we describe experiments on the production and release of toxins A and B and discuss our findings in relation to the pathogenesis of the disease.

Measurement of low concentrations of toxin A in the presence of toxin B is difficult and only one preliminary report (Ketley *et al.*, 1983) has been published on toxin A production and release. The rabbit ileal loop assay is an insensitive method of detecting toxin A, and, without highly specific blocking antisera to neutralise toxin B, it is difficult to assay weakly cytotoxic A in the presence of highly cytotoxic B in standard cytotoxin assays. We have developed a sensitive enzyme-linked immunosorbent assay (ELISA) (Redmond *et al.*, in press), which is simpler than that described by Lyerly, Sullivan and Wilkins (1983). This ELISA and immunoelectrophoresis were used to

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monitor the appearance of toxin A in culture medium *in vitro*. The production of cell-associated toxin A was studied by immunoelectrophoresis and by fluorescent antibody (FA) techniques. In the FA technique the monoprecipitating antiserum to toxin A which is the basis of the ELISA was used.

There have been limited studies on toxin-B production and release *in vitro* (Bartlett *et al.*, 1978*b*; George *et al.*, 1980; Rolfe and Finegold, 1979). In initial experiments we used standard cytotoxin assays to follow the appearance of cell-associated and extracellular toxin B.

METHODS

Organism and growth conditions. *Clostridium difficile* strain 23289/78 (a clinical isolate) was grown in 100 ml of pre-reduced tryptic nitrate broth containing Bactotryptose (Difco) 20% w/v; glucose 0.1 % w/v; Na₂HPO₄ 0.2% w/v; KNO₃ 0.1% w/v; sodium formaldehyde sulfoxylate 0.03% w/v (TNB). Cultures were incubated overnight at 37°C in anaerobic conditions in anaerobic jars containing the GasPak system (BBL). These overnight cultures were used to inoculate 500 ml of TNB, in screw-capped bottles, to give an initial total count of *c.* 10⁷ cells/ml. The cultures were then maintained under nitrogen. Toxin-B production is enhanced by clindamycin (George *et al.*, 1980); empirical observation showed this also to be true for toxin A. The amounts used are strain (or substrain) dependent and must be below the minimum inhibitory concentration. Initially, clindamycin phosphate (Upjohn Ltd, Crawley, Sussex RH10 2NJ) 5.0 µg/ml was added to the 500 ml culture, but in subsequent experiments in which a new substrain of 23289/78 and clindamycin hydrochloride (Upjohn) were used, 0.5 µg/ml was found to be required for reproducible growth. Samples (5–20 ml as appropriate) were taken at regular intervals with a syringe and needle through a rubber diaphragm in the screw cap so as not to disturb anaerobic conditions.

Bacterial counts. Total counts were made in a Thoma chamber and viable counts were estimated by the method of Miles, Misra and Irwin (1938) on human-blood-agar plates incubated anaerobically at 37°C.

Concentration of culture supernates. Twenty-ml samples of bacterial cultures were: clarified by centrifugation at 50 000 *g* for 35 min at 4°C; concentrated 40 times by ammonium sulphate precipitation; resuspended in distilled water; dialysed against neutral phosphate-buffered saline (PBS) containing NaCl 4.25 g/L, KH₂PO₄ 2.44 g/L and Na₂HPO₄ 8.09 g/L (Redmond *et al.*, in press); filter sterilised; and stored at 4°C.

Ultrasonication. Bacterial pellets from centrifuged samples were resuspended in 0.5 ml of PBS and disrupted by ultrasonication with a 3-mm diameter probe at 50 watts and 20 Hz for a total of 2 min. The sample was constantly cooled by ice. Sonicates were clarified by centrifugation at 11 000 *g* for 5 min at 4°C, filter sterilised and stored at 4°C.

Antisera. Antitoxin A and antisera raised against concentrated culture filtrates (here referred to as polyvalent sera) were prepared as previously described (Redmond *et al.*, in press).

Quantitative toxin analysis. Crossed immunoelectrophoresis, cytotoxin assay for toxin B and ELISA for toxin A were performed as previously described (Redmond *et al.*, in press). Cytotoxin units were defined as the dilution that caused 50% rounding of cells in cultures of the L929 mouse fibroblast cell line. For electrophoresis 20 µl of concentrated culture supernate were applied to each gel. The relative amount of toxin A was estimated by calculating the area under the appropriate precipitin arc; this was identified by comparison with gels in which pure toxin was run. An area of 1.0 mm² under the precipitin arc was arbitrarily defined as one unit of toxin A. Extracellular levels of toxin A were later determined in unconcentrated culture supernates by quantitative ELISA; the lowest limit of detection was defined as three standard deviations above the mean of the antigen-free blanks (equivalent to 0.15 µg of toxin A/ml).

Immunofluorescence of fixed bacteria. Bacterial pellets from 5-ml culture samples were washed once in PBS, recentrifuged at 11 000 *g* for 5 min at 4°C and smeared on to glass slides. Duplicate slides were fixed in cold dry acetone for 2 min, washed for 20 min in a phosphate-buffered saline used for fluorescent antibody work (FAPBS) which contained NaCl 8.5 g/L, Na₂HPO₄ 1.07 g/L and NaH₂PO₄·2H₂O 0.39 g/L (pH 7.1). Smears were then overlaid

with either rabbit antitoxin A, polyvalent serum or normal rabbit serum, each diluted 1 in 200 with FAPBS. Overlaid smears were incubated at room temperature in humid conditions for 2 h. Duplicate slides were washed separately in FAPBS for 10 min before being overlaid with 5% v/v sheep anti-rabbit fluorescein conjugate (Wellcome Reagents Ltd, Beckenham, Kent BR3 3BS) containing rhodamine 5% v/v (Difco) in FAPBS. The smears were incubated at room temperature for 30 min in a humid chamber. After washing separately for a further 20 min in FAPBS, the slides were mounted in alkaline buffered glycerol (NaHCO_3 0.175 g/L; Na_2CO_3 0.16 g/L; glycerol 90% v/v) and viewed with a Zeiss photomicroscope III.

Immunofluorescence of unfixed bacteria. Total numbers of bacteria in 20-ml samples were counted. The bacterial suspension was then centrifuged at 25 000 *g* for 30 min at 4°C, washed in FAPBS and recentrifuged. The resultant pellet was suspended in 3 ml of FAPBS, divided into 1 ml portions, centrifuged at 11 000 *g* for 5 min at 4°C and resuspended in 1 ml of either polyvalent serum, antitoxin A or normal rabbit serum, each diluted 1 in 100 in FAPBS. After incubation for 2 h at room temperature, the cells were centrifuged at 11 000 *g* for 5 min at 4°C and washed twice in FAPBS. Washed pellets were resuspended in 1 ml of 5% v/v sheep anti-rabbit fluorescein conjugate containing rhodamine 5% v/v diluted in FAPBS. After incubation at room temperature for 30 min the suspension was again washed twice in FAPBS. The pellet was resuspended in alkaline buffered glycerol and mounted on slides for microscopy.

RESULTS

Co-production of toxins A and B

In these experiments toxin A was determined by crossed immunoelectrophoresis. As judged by total cell count, the culture rapidly entered a phase of logarithmic growth of 12-h duration followed by a 12-h stationary phase. The ensuing decline phase was monitored over the next 4 days (fig. 1a). Viable counts showed a generally similar pattern to that of the total count. However, the results varied considerably, probably because these experiments had to be conducted in open bench conditions. Hence these data are not included in the results.

Cell-associated levels of toxin A rose during the decline phase to reach a peak at 72 h; by 144 h toxin A was undetectable. Extracellular levels of toxin A rose rapidly during early decline phase, reaching a peak at 96 h, after which levels fell (fig. 1b).

Concentrated culture supernates and cell sonicates were analysed for toxin-B activity (fig. 1c). During logarithmic growth, low levels of cell-associated toxin B were detected; thereafter levels of cell-associated toxin B rose. Extracellular toxin B levels remained low until the culture entered the decline phase, when levels rapidly rose to reach a maximum at 72 h and then slowly declined. Toxin B was not monitored in subsequent experiments.

Production of toxin A as determined by ELISA

Fig. 2 shows a typical result. The pattern of bacterial growth, judged by total count, was highly reproducible for *C. difficile* strain 23289/78 in the growth conditions used. Extracellular levels of toxin A were below the threshold of detection until 30 h, after which levels steadily rose to reach 12 µg/ml at 120 h. In five experiments in which the bacterial growth curve was monitored quantitatively, the maximum total count of bacteria always occurred around 20 h and ranged from 4×10^9 to 8×10^9 cells/ml. Concentrations of extracellular toxin A always remained below the limits of detection by ELISA until about 20–25 h (i.e., the beginning of the decline phase). At 96 h, levels

FIG. 1.—a) Total counts of *C. difficile* (●); (b) cell-associated (□) and extracellular (■) toxin A measured by crossed immunoelectrophoresis; c) cell-associated (Δ) and extracellular (▲) toxin B.

of extracellular toxin A were found to be 6.6, 9.2 and 10.0 $\mu\text{g}/\text{ml}$ in three experiments; in a fourth experiment a value of 2.4 $\mu\text{g}/\text{ml}$ was obtained.

Cell-associated toxin A detected by immunofluorescence of fixed bacteria

Bacterial smears were made of each sample and treated with either normal rabbit serum or antitoxin A and the fluorescent conjugate. All sample smears treated with normal rabbit serum showed rhodamine staining, but little or no fluorescence. Positive controls of post-stationary phase samples treated with polyvalent anti-serum revealed

FIG. 2.—Total counts of *C. difficile* (●) and concentration of extracellular toxin A measured by ELISA (■).

many brightly fluorescent cells. Smears of samples taken during the logarithmic and stationary phases of the growth cycle did not fluoresce when treated with antitoxin A. However, the proportion of stained cells continued to increase after 20 h as the culture entered the decline phase.

Cell-associated toxin A detected by immunofluorescence of unfixed bacteria

Culture samples treated with normal rabbit serum showed no fluorescence throughout the whole growth cycle. However, fluorescence was observed in post-stationary-phase bacteria treated with polyvalent serum. At no time during the logarithmic, stationary or decline phases was fluorescence observed in cells treated with antitoxin A, even though the normal pattern of extracellular toxin A release was detected by ELISA during the decline phase.

DISCUSSION

Detection of cell-associated toxin A was undertaken by means of crossed immunoelectrophoresis and fluorescent antibody techniques. Neither electrophoresis nor fluorescent antibody labelling of fixed organisms yielded evidence of cell associated toxin A in logarithmic and stationary phase cultures of *C. difficile in vitro*. Cell-associated toxin A was, however, detected with these techniques during the decline phase. Fluorescent labelling of unfixed cells failed to detect toxin A during any stage of culture. These results strongly suggest that toxin A is synthesised only during the decline phase and, furthermore, in its cell-associated form it appears to be

intracellular and not external to the bacterial cell wall. There remains the possibility that toxin A is synthesised before the decline phase, but in an antigenically different form. Alternatively, the toxin may not be released by sonication, or is not accessible to antibody. We are at present investigating these possibilities by immunoelectronmicroscopy.

Electrophoresis and ELISA both revealed that toxin A is released during the decline phase of in-vitro culture. In this paper, toxin which appears in the medium is referred to as extracellular toxin, whether it is secreted by the bacteria or released by bacterial lysis. As yet we have no evidence to distinguish between release by lysis or by active secretion, but the concomitant decline in bacterial numbers and rise in extracellular toxin strongly suggests that toxin accumulation in the medium is the result of lysis rather than secondary metabolism during the stationary phase. The possibility that toxin A is a spore component, as it is in the case of *C. perfringens* type A enterotoxin (McDonel, 1980) has been indirectly examined. Like most isolates of *C. difficile*, the strain used has lost the ability to produce many spores during laboratory storage, and this makes analysis of spores difficult. However, during the period of laboratory storage the strain has lost none of its potential to produce toxin Bn or its ability to cause dilatation of the rabbit ileal loop due to toxin A (Burdon *et al.*, 1981; Stephen *et al.*, 1984).

Production and release of toxin B during in-vitro culture appear to follow a pattern similar to that described for toxin A. Most cell-associated toxin B was found during the decline phase, accompanying a rise in extracellular levels of toxin B. The pattern of release agrees with previous published results (Bartlett *et al.*, 1978*b*; Rolfe and Finegold, 1979; George *et al.*, 1980). However cell-associated production of toxin B was found to decrease from the logarithmic to the decline phase by Rolfe and Finegold (1979) whereas Bartlett *et al.* (1978*b*) found maximum production at 24-48 h. Our results show a peak in cell-associated levels of toxin B at 72 h. These discrepancies may be explained by variations in the kinetics of toxin B production by different strains or in different media.

The nature of the production and release of toxins A and B may shed light on the characteristics of the lesion found in pseudomembranous colitis. The spreading, initially localised lesion, does not appear to contain a large population of bacteria as judged by histology (Sumner and Tedesco, 1975). This may be due in part to the fact that toxins are released by lysis of organisms in the microcolony by autolysis or as a result of interactions with host defence mechanisms.

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