**Clostridium difficile enterotoxin (toxin A): new results**

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_Clostridium difficile_, the causative agent of antibiotic-associated or pseudomembranous colitis produces a plethora of extracellular antigens in vitro. These include two toxins, toxic A (cytotoxin), and the toxin B (cytotoxin), believed to be important bacterial determinants of pseudomembranous colitis.

**Purification of toxin A**

Crude culture filtrates were grown and concentrated as described previously (Burdon et al., 1981). Attempts to purify toxin A by published methods gave preparations which were impure (by immunological criteria, or as judged by silver-staining of SDS/polyacrylamide-gels), low-yielding, or not routinely reproducible (e.g. the final precipitation step described by Sullivan et al. (1982)). We can now routinely obtain pure toxin A by using the following two-step procedure. First, preparative electrophoresis in a discontinuous buffer system is carried out in flat bed gels. The active fraction is dialysed (fractions 1-5 from cathode end) by its mild cytotoxic activity towards L929 cells. The active fraction is dialysed against 0.05M-Tris/HCl pH 8.5, and loaded on to a DEAE-Sepharose CL 6B column (2.5 cm x 10 cm) which is eluted with the NaCl gradient described by Sullivan et al. (1982). Toxin A (detected by cytotoxic activity; total recovery 50% yield) is pure serologically, and immunologically as judged by two-dimensional immunoelectrophoresis using (a) pure toxin against antisera raised to crude toxin and (b) crude toxin against hyper-immune rabbit antisera raised to purified toxin. Only one polypeptide is observed on SDS/polyacrylamide-gels developed using silver stain.

**ELISA test**

Two ELISA tests have been described recently, one for antitoxin A (or B) (Aronson et al., 1982) and the other for assaying toxin A itself (Lyerly et al., 1983). The latter is a complicated test in which the anchor is rabbit antisera to crude toxin, followed by toxin-containing antigen samples. The second antibody is affinity-purified goat anti-toxin A antibody and the conjugate for detection is rabbit anti-goat IgG–alkaline phosphatase.

Using the reagents prepared as described above we have developed a simplified test in which the anchor antibody comprises IgG from the monoprecipitin rabbit antiserum raised to purified toxin A. The second layer consists of the antigen-containing antigen samples and the second antibody is the same IgG as used for the anchor coupled to horseradish peroxidase. This is now being used to screen clinical samples for toxin A.

**Production, location, composition of toxin A**

Toxin A production was followed over a period of 6 days. Hardy any toxin was released into the medium during log phase (duration 21h); maximum yields were found in culture filtrates at 96h after inoculation, by which time both total and viable counts were declining, the latter much more rapidly than the former. Peak production of toxin A coincided with peak production of toxin B. Attempts to extract toxin A from bacterial cells throughout the growth cycle by ultrasonication yielded only very small quantities of toxin A. However, we have shown (by fluorescence-antibody techniques) that the antigen reacting with the monoprecipitin anti-A serum is present in/on bacterial cells at 48h after inoculation, i.e. 2 days before maximum release (perhaps by autolysis) of functional toxin A into culture filtrates.

The amino acid composition of toxin A has been determined to be (molar ratios with methionine as 1): Asp (5), Thr (2), Ser (5), Glu (8), Pro (5), Gly (8), Arg (4), Val (3), Met (1), Ile (3), Leu (3), Tyr (2), Phe (2), His (2), Lys (2). The absence of cysteine may explain the lack of success in producing subunits by reductive cleavage methods. The full significance of this atypical amino acid composition has not been determined, nor have we as yet confirmed the presence or absence of carbohydrate in the toxin.

**Biological properties of toxin A**

There is general consensus that toxin A is an enterotoxin (i.e. causes fluid accumulation but with damage to mucosal epithelia, induction of an inflammatory response and haemorrhage) in several animal models; rabbit ileal loop (Burdon et al., 1981; Taylor et al., 1981), hamster caecal loop (Taylor et al., 1981; Lyerly et al., 1982). Toxin B, although vastly more potent than toxin A towards tissue culture cells, does not cause fluid accumulation in rabbit ileal loops or hamster caecal loops (Taylor et al., 1981; Libby et al., 1982). However, there are discrepancies as to its tissue damaging properties. Taylor et al. (1981) claim that only limited histological damage is caused to mucosal epithelium of hamster caeca whereas Libby et al. (1982) showed substantial damage. In our hands toxin B causes only very mild fluid accumulation in rabbit ileal loops and no histological damage. For this latter reason and to conserve purified toxin A required for numerous other studies, crude toxin was used in ligated rabbit ileal loops to examine the time course of fluid accumulation in relation to the extent and nature of tissue damage.

We found that fluid accumulation became significant by about 6h, and was maximal by 10-12h after injection; by 12h macroscopic haemorrhagic damage was evident. Histological examination showed that incipient damage to villus tips was evident by 2h; villus architecture was progressively destroyed, with villus core material extruded. Inflammatory responses occurred later and by 12h massive haemorrhage had occurred. In this study tissue damage was shown to precede fluid accumulation.
Ussing chambers were mounted with stripped rabbit ileal mucosa and either crude or pure toxin A added to the mucosal side of the chamber to final concentrations of 800 and 30 μg/ml respectively; increases in short-circuit currents of the order of 25 μA were observed in both preparations over a period of 90 min, at which point the tissue was responsive to theophylline but not glucose. Histology showed that damage to villi was had occurred by 90 min in contrast to Hughes et al. (1983) who claimed no damage was induced in similar experiments. Unidirectional flux experiments have not yet been carried out and may be of equivocal value in analysing perturbations in active transport systems until and unless effective means are found for controlling the effects due to cell damage.


**Clostridium perfringens** enterotoxin: a brief review

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The enterotoxin produced by *Clostridium perfringens* is responsible for one of the most common types of food poisoning in this country. During the period 1970–1981, there were 116030 reported cases of food poisoning in England and Wales: *Salmonella* accounted for 91953 (79%) cases and *C. perfringens* was responsible for 19888 (17%) cases. Illness often follows the preparation of meat or poultry dishes to be served to large numbers of people. The food is frequently pre-cooked the day before serving and then inadequately stored, so that spores surviving the cooking process may germinate and multiply to hazardous levels. Ingestion of large numbers of vegetative cells of *C. perfringens* in food leads to multiplication and sporulation in the intestine. When the mature spores are released from the sporangia there is an accompanying release of enterotoxin, leading rise to the symptoms of diarrhoea and abdominal cramps.

Between 1970 and 1981, 438 (71%) of the 617 *C. perfringens* outbreaks occurred in mass-catering situations. Fatal cases are relatively rare, most people recovering within 12–24 h, but those which do occur are associated with elderly or debilitated persons. Thus the lethal potential must be recognized, especially since such a high proportion of outbreaks occurs in hospitals and old people's homes.

Duncan et al. (1972) isolated mutants of *C. perfringens* with altered abilities to sporulate, and used them to show that enterotoxin was a sporulation-specific gene product. About 3 h after inoculation of vegetative cells into sporulation medium, heat-resistant spores develop, followed closely by the accumulation of intracellular enterotoxin. Maximum numbers of spores are obtained after 10 h, and at this time free spores can be detected, which reach a maximum after 14–15 h. With the liberation of mature spores enterotoxin is released and hence intracellular toxin decreases whilst extracellular toxin increases in parallel with the increase in numbers of free spores (Duncan, 1973).

The role of enterotoxin at sporulation is still not clear, but it is possible that its accumulation may result from over-production of a protein synthesized for some specific function.

Abbreviation used: ELISA, enzyme-linked immunosorbent assay.

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Frieben & Duncan (1973) extracted a component of the spore coat protein which was identical in biological and serological activity to enterotoxin. It was also observed that some toxigenic strains develop large paracrystalline inclusions at the same stage that deposition of fragments of spore coat material occurs (Duncan et al., 1973). Studies with mutants suggested that a single gene mutation was responsible for lack of spores, enterotoxin and paracrystalline inclusion. Although enterotoxin is only produced by sporulating organisms, not all strains which sporulate produce the toxin, and even amongst toxigenic strains there is not always a direct relationship between good sporulators and good toxin producers.

Various sporulation media have been developed in attempts to obtain maximum toxin production in vitro but there is apparently no single medium which is suitable for all strains (Skjelkvale et al., 1979). Heat-resistant strains (i.e. those which withstand heating at 100°C for 60 min) produce more spores and toxin when raffinose is a component of the medium, whereas heat-sensitive strains prefer starch (Table 1). Essential importance to the expression of the medium are the culture conditions employed. For example, heating the cultures at 75°C for 20 min before transferring to sporulation medium greatly increases the yield of spores and toxin (Tsai & Riemann, 1974). The mechanism of this heat activation is unknown but it has been suggested that heat could induce dormant spores to germinate or that it could select for those cells with an increased inherent ability to sporulate.

Various procedures have been developed for the purification of enterotoxin from sonicated cell extracts, including affinity chromatography (Scott & Duncan, 1975; Barnhart et al., 1976) and preparative polyacrylamide-gel electrophoresis (Enders & Duncan, 1977). Hauschild & Hilseheimer (1971) and Stark & Duncan (1972) employed procedures involving several chromatographic steps. In 1973, Sakaguchi and co-workers published details of a simplified method involving ammonium sulphate precipitation, differential solubilization and gel filtration on Sephadex G-200 (Sakaguchi et al., 1973). This method was improved (Granum & Whitaker, 1980) by replacing the differential solubilization with a second ammonium sulphate precipitation step. This procedure works well providing that the enterotoxin in the sonicated cell extract represents at least 8–10% of the total protein (Bartholmew & Stringer, 1983). Thus great care must be taken in choosing the correct strain and sporulation conditions to provide maximum toxin production.