Studies on the production of enterotoxins by Bacillus cereus

P. C. B. TURNBULL

From the Food Hygiene Laboratory, Central Public Health Laboratory, Colindale Avenue, London

SYNOPSIS Evidence is presented for the existence of three distinct enterotoxins detected in concentrated cell-free culture filtrates of selected Bacillus cereus strains. The first was a product capable of stimulating the adenylate cyclase-cyclic-AMP system in intestinal epithelial cells and, possibly through this, causing fluid accumulation in ligated ileal sections ('loops') of young rabbits. This was elaborated by a strain isolated from an incident of diarrhoea and which caused diarrhoea in 6 of 10 monkey feedings. The second was tentatively identified as a factor which caused fluid accumulation in rabbit loops but not, apparently, through stimulation of the adenylate cyclase-cyclic-AMP system; this was elaborated by a strain isolated from raw rice which failed to produce symptoms in eight monkey feedings. Together, the behaviour of these two factors indicates that diarrhoea caused by B. cereus enterotoxin may be a cyclic-AMP-mediated event. The third, here referred to as 'pyrogenic toxin', caused severe tissue damage in the ileal mucosa and was elaborated by a strain isolated from a brain abscess. A factor produced by a strain isolated from an outbreak of vomiting which caused vomiting in 10 of 24 monkey feedings could not be detected in tests reported here but appears to be a fourth enterotoxin type.

Cytopathic effects in tissue cultures, suckling mouse tests, and assays of glycerol production by fat cells were not found to be of value in the detection of any of the enterotoxins.

Evidence has accumulated over the past five years that Bacillus cereus may be the aetiological agent of two distinct types of food poisoning characterized either by diarrhoea and abdominal pain 8 to 16 hours or by nausea and vomiting 1 to 5 hours after ingestion of contaminated food.

A wide range of foods has been implicated in the diarrhoeal-type syndrome including soups, cooked meat, poultry and vegetables, and dessert dishes and sauces, but B. cereus has not been isolated in large numbers from faecal specimens in these incidents (Hauge, 1950, 1955; Ormay and Novotony, 1969; Goepfert et al, 1972). In contrast, the vomiting-type illness has been associated almost exclusively with the consumption of cooked rice, usually fried, from Chinese restaurants. In most of the incidents, large numbers of B. cereus have been isolated from remnants of cooked rice, clinical specimens or both (Mortimer and McCann, 1974; Taylor and Gilbert, 1975; Gilbert and Taylor, 1976). The results of serotyping using a system developed in this laboratory (Taylor and Gilbert, 1975) have given further support to the theory that B. cereus may cause two distinct forms of food poisoning.

From this evidence it is reasonable to infer that some strains of B. cereus may elaborate a 'diarrhoeal' enterotoxin while others produce a 'vomiting' toxin; a third 'non-toxigenic' category may exist also.

Ezepecuk and Fluer (1973) described the purification of a B. cereus enterotoxin which they found to be a protein of molecular weight approximately 55 000-60 000. On parenteral administration this was lethal to mice and rabbits and caused vomiting in cats (Gorina et al, 1975).

Spira (1974) and Spira and Goepfert (1975) have determined some of the characteristics of a diarrhoeagenic factor or enterotoxin synthesized during exponential growth of B. cereus. This produced fluid accumulation in ligated rabbit ileal sections, altered vascular permeability in the skin of rabbits, and killed mice on intravenous injection. On Sephadex G-75 these activities were eluted simultaneously and separately from haemolytic and lecithinase activities; they survived 45°C for 30 minutes but not 56°C for 5 minutes and were destroyed by pronase and trypsin. They were present
to a far greater degree in 'culture fluid' (cell-free filtrate of brain-heart infusion broth containing 0.1% glucose) than in 'culture extract' (filtrates from washed cells disrupted ultrasonically), indicating that cell lysis was not the mechanism of enterotoxin production. Antisera to the culture fluid of one strain of *B. cereus* neutralized the fluid accumulation activities of five of six other strains, but antisera to cholera and *Clostridium perfringens* enterotoxins had no neutralizing effects. Unlike cholera toxin, the fluid accumulation effect of the *B. cereus* toxin could be removed by flushing.

Evidence for the existence of a vomiting toxin produced by *B. cereus* has resulted from preliminary monkey feeding tests (Melling et al., 1976).

This paper describes studies undertaken (1) to establish whether *B. cereus* strains may be classified as diarrhoeal, vomiting or non-enterotoxigenic, and (2) in search of a reliable method for the detection and quantitation of the enterotoxin(s).

### Material and methods

**Organisms**

In preliminary tests, cell-free culture filtrates from 23 *B. cereus* strains isolated from (but not necessarily incriminated in) food poisoning investigations and a single clinical isolate (2141/74; serotype 11) from a brain abscess were screened for their abilities to induce fluid accumulation in ligated rabbit ileal ('rabbit loop') sections. Subsequently three strains were selected on the basis of their histories as possible prototypes of 'vomiting', 'diarrhoeal', and 'non-enterotoxigenic' strains (table I); these were also used in the monkey feeding tests of Melling et al. (1976). Strain 2141 was included to determine whether it possessed any unique activity whereby it could be distinguished from the food and faecal *B. cereus* isolates. Strain 4ac, being the principal strain used in the studies of Spira (1974) and Spira and Goepfert (1975), and strain 4096/73, on account of its relatively high rate of rabbit loop-positive results in the preliminary screening tests, were also included in part of the studies.

The organisms were grown in brain-heart infusion broth (BHIB; Difco); glucose was added to 0.1% when the effect of added glucose was under test. Cultures were shaken on a rotary shaker at approximately 110 rev/min for 18 to 20 hours at 36°C ± 1°C. In a study of the importance of incubation temperature, cultures were also grown at 32°C ± 1°C. Purity was checked on blood agar.

Culture filtrates of *Vibrio cholerae*, serotype Inaba (the National Collection of Type Cultures, 7254) grown in the media of Evans et al. (1973) and otherwise treated in the same manner as the *B. cereus* filtrates, were used for comparative purposes in the adenylate cyclase activity (ACA) assays.

### Ligated Rabbit Ileal ('Rabbit Loop') Assays

The basic procedure used was that of De and Chatterje (1953). Except where the importance of rabbit age was being verified, weaners weighing 850-1500 g were used. Ileal sections ('loops') were approximately 10-12 cm long, and adjacent loops were separated by 2-4 cm interloops. The holding period between inoculation of the loop and sacrifice of the animal was 3-7 hours, depending on the test. A final loop volume:length (V/L) ratio of >0.3 was regarded as evidence of net fluid secretion into the lumen and termed a 'positive loop'. The number of loops tied off in a single animal was usually five and never more than six after preliminary tests. Into each loop were injected 2 ml aliquots of cell-free filtrate, filtered ultrasonicated culture, concentrated cell-free filtrate or whole cells grown in rice which was subsequently homogenized by treatment with diastase for easy administration (Melling et al., 1976).

Filtrates were concentrated by dialysis at 4°C...
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against 70% Carbowax (Union Carbide) in water after the addition of 100 000 units of penicillin and 10 mg of streptomycin to approximately 80 ml of culture filtrate.

ADENYLATE CYCLASE ACTIVITY (ACA) ASSAYS

The procedure was based on those of Sharp et al (1973) and Flores et al (1974). Rabbits were held for 3 to 4½ hours between the injection of concentrated cell-free filtrates (CCFF) into the loops and sacrifice. After sacrifice, each loop was cut out and weighed and length and volume of accumulated fluid were measured. Loops were opened by a longitudinal incision and washed in ice-cold quarter-strength Ringer's solution. The mucosa was blotted, and epithelial cells were scraped off with a glass slide and transferred to ice-cold 75 mM Tris buffer (pH 8·0) containing 25 mM MgCl₂. This suspension was homogenized in a ground-glass tissue homogenizer. Protein content was determined by the method of Lowry et al (1951); when necessary, the concentration of the homogenate was adjusted to approximately 2 mg/ml and the exact protein content was redetermined.

ACA was determined by the method of Krishna et al (1968). The composition of the incubation mixture, the reaction-stopping mixture, and the precipitating compounds, the quantities used, and the procedures followed were as detailed by Flores et al (1974). ACA was measured in terms of pmoles of adenosine 3':5'-cyclic monophosphate (cAMP) formed per milligram of protein during a 20-minute incubation period and was determined for duplicate or triplicate samples of epithelial cells from any one rabbit loop.

In vitro stimulation of ACA was attempted also by exposing rabbit ileum mucosal epithelial cells or HeLa tissue culture cells to the B. cereus extract in the presence of 10 mM adenosine triphosphate (ATP) and 10 mM nicotinamide-adenine dinucleotide (NAD). Subsequent assay of ACA was as described.

OTHER ASSAY SYSTEMS

Unconcentrated cell-free filtrates from B. cereus cultures which gave a high proportion of positive rabbit loop results were also tested in the suckling mouse model of Dean et al (1972), the glycerol production assay of Vaughan et al (1970), and for their effects on HeLa, Hep 2, VERO, and primary monkey kidney cell lines (kindly supplied by the Virus Reference Laboratory, Colindale).

HISTOPATHOLOGY

Periodically sections of positive and negative rabbit loops were fixed in 10% buffered formalin, processed histologically, stained with haemotoxylin and eosin, and examined for histopathological effects attributable to B. cereus enterotoxins.

Results

LIGATED RABBIT ILEAL ASSAYS

In terms of serotype or source of isolation, no meaningful pattern could be detected in screening tests on cell-free filtrates from the 24 B. cereus strains. Although just 11 of these strains were tested more than twice, only 2 of the 11 exhibited a >50% probability of being positive on repeated testing. One of these, strain 4096/74 (serotype 4), together with a loop-positive strain of Escherichia coli 0148 (NCTC 10664), was used to establish that maximum rabbit loop reliability was lost in this rabbit weight range when more than six loops were prepared in a single animal. Other preliminary indications from attempts to establish a procedure which gave consistent loop results were that incubation at 32°C ± 1°C, anaerobic incubation of cultures, ultrasonication before filtering, addition of 0·1% glucose to the culture broth, and the use of an alternative casamino acid-based growth broth were not of obvious value. Where investigated, no correlation was noted between the bacterial count and variability in rabbit loop results for a given culture. Consistent results were not obtained with the inocula of whole cells in rice.

Concentration by dialysis of the cell-free filtrate against 60-70% Carbowax gave the most satisfactory results; a 10-fold concentration appeared in retrospect to be optimal. The pH of CCFF consistently lay between 7·0 and 7·5. The accumulated results of tests using CCFF from cultures of the selected strains are shown in table I. By this method positive loops were fully developed by 3 to 4 hours after inoculation (fig 2a). Of note was the lower rate of positives given by the 4810 ('vomiting') and the 4ac strains.

The accumulated results revealed no difference in sensitivity of the response to the toxigenic filtrates along the length of the ileum used, and there was no indication that false results could arise from the use of cultures of different strains in the several loops of one rabbit.

ADENYLATE CYCLASE ACTIVITY (ACA)

The accumulated results of in vivo tests for altered ACA in response to CCFF of the selected B. cereus strains are given in table II; readings from individual tests and their corresponding rabbit loop results are shown diagrammatically in figure 1. Basal levels lay consistently within the range 19·34 pmoles cAMP/ mg/20 min with the exception of a single relatively high reading of 46 ± 4 pmoles/mg/20 min. Concen-
trated BHIB did not, on the four occasions tested, produce raised ACA levels. Boiling the mucosal cells resulted in a significant reduction (p < 0-001) from basal levels.

Evidence is provided for the existence of a product in the CCFF of strain 4433 (‘diarrhoeal strain’) which caused increased ACA. By a one-way analysis of variance, the mean of the 12 tests (51.1 pmoles/mg/20 min—table II) was significantly higher (p < 0-001) than the mean basal value (31.3 pmoles/mg/20 min) and mean values for BHIB and strains 4810, 2141, and 2532b.

A product was present in the CCFF of strain 2532b (‘non-toxigenic strain’) which produced fluid accumulation (positive rabbit loops) on seven of the nine occasions it was tested, but, with one exception, ACA readings were consistently of basal order (fig 1d); the single relatively high reading (46.9 ± 10.7 pmoles/mg/20 min) lay close to the single eccentric basal reading and was not considered significant.

Rabbit loops were positive on only 2 of 13 occasions using CCFF of strain 4810 (‘vomiting strain’) and ACA results were consistently of basal order (fig 1c).

CCFF from the brain abscess isolate, strain 2141, gave strongly positive loops (fig 2a) on eight of the nine occasions it was tested. A product in this filtrate caused considerable damage to the mucosa during the 3 to 4 hours holding period (fig 2b). In processing for ACA assay, the sloughed epithelium tended to be lost and the six low ACA readings (fig 1e) may have resulted from this. Two high ACA values suggested that this strain, like 4433, may elaborate a product which stimulates ACA.

By statistical analysis, the mean ACA levels of strains 4810, 2141, and 2532b were not significantly different (p > 0.1) from the mean basal level or from each other.

The mean ACA level obtained from the five assays on CCFF of V. cholerae NCTC 7254 was significantly higher (p < 0.001) than the mean basal level but was not significantly different (p > 0.1) from the mean ACA level obtained with B. cereus strain 4433.

By statistical analysis of the results obtained with each strain (including V. cholerae) taken separately and as a whole, ACA values and V/L ratios, considered either in absolute terms or by the criterion of positive = V/L > 0.3, were found to be independent.

No indication of raised ACA was found in the single test using CCFF of strain 4096 or in the two tests using CCFF from strain 4ac.

Increased ACA after in vitro incubation of CCFF from strains 4433, 4810, 2141, and 2532b with mucosal epithelial or rat liver cells was not successfully demonstrated; further work on this is under way.

OTHER ASSAY SYSTEMS

Unconcentrated cell-free filtrates from six B. cereus cultures, four of which had simultaneously produced strongly positive rabbit loops (V/L > 0.55), gave negative results in suckling mice as based on the criteria of Dean et al (1972). A single positive resulted from the administration of CCFF from strain 4096 which had given a massive V/L ratio of 2.0 at 7 hours.

Measurable stimulation of glycerol production by fat cells in the test of Vaughan et al (1970) was not found.

Tissue cultures were rapidly stripped from the sides of test-tubes or flats by an extracellular product or products of four B. cereus strains in BHIB; cytopathic effect, therefore, could not be looked for. No measurable increase in ACA levels was detected in an in vitro assay using HeLa cells and CCFF from strains 4433, 4810, 2141, 2532b, and V. cholerae.
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HISTOPATHOLOGY
Where sections were taken for histological examination, notable pathological changes were absent in positive rabbit loops of all B. cereus strains except the brain abscess isolate, strain 2141. Minor departures from normal, such as congestion of mucosal and submucosal vessels and shortening and thickening of villi, could be attributed to pressure from accumulated fluid.

CCFF of strain 2141, in contrast, consistently produced severe disruption of the mucosa. This was visible grossly as a loss of rugae and a formation of

Fig 1 Results of individual adenylate cyclase activity and rabbit loop assays for four strains of B. cereus and V. cholerae NCTC 7254. V/L = ratio of volumes of accumulated fluid; loop length; ACA = adenylate cyclase activity in pmoles/mg/20 min; basal = ACA of mucosal epithelial cells not exposed to any exogenous material in vivo.
patchy pseudomembranes on the luminal surface (fig 2b); the intestinal wall appeared abnormally thin and transparent (fig 2a; V/L = 0.95). A histological view of the distorted and disrupted mucosa with sloughed epithelium and extruded lamina propria cells is seen in figure 2d. The relatively normal gross and microscopic sections in figures 2b and c were from a rabbit loop injected with CCFF of strain 4433 which was also strongly loop-positive (V/L = 0.86).

Discussion

The results presented provide evidence for the existence of at least two distinct enterotoxins produced by *B. cereus*: (1) a diarrhoeal toxin, as produced by strain 4433, capable of stimulating the adenylate cyclase-cyclic-AMP system and, possibly through this (although the existence of a relationship between ACA and V/L ratios was not verified statistically), causing accumulation of fluid in ligated ileal sections of young rabbits. It is assumed that this toxin caused the diarrhoea in 6 of 10 monkey feeding tests (Melling et al., 1976), and it is probably related to or identical with the enterotoxin described by Spira and Goepfert (1975); (2) a 'pyogenic toxin' capable of rapidly causing severe tissue damage (fig 2) and probably involved in the formation of the abscess from which strain 2141 was isolated. Two other distinct enterotoxigenic entities may be represented in (3) the toxin of strain 4810 which caused vomiting in 10 of 24 monkey feeding tests but was not detected in tests reported here, and (4) the factor in the CCFF of strain 2532b which produced fluid accumulation in rabbit loops but failed measurably to raise ACA or cause diarrhoea or vomiting in eight monkey feedings.

Although the evidence is indicative rather than conclusive that the factor produced by strain 2532b could not cause diarrhoea, it suggests that the ability per se of CCFF of a *B. cereus* strain to give positive rabbit loops should be interpreted with caution. Without concentration, the ability of a potentially loop-positive strain to produce positive loops was very variable; concentration reduced this
variability greatly but not completely, and positive loops were obtained in 3 to 4 hours. The failure of concentrated BHIB and of 11 of 13 CCFF of strain 4810 to produce positive loops appears to rule out osmotic or other effects of the concentration process as the cause of positive loops. The parameters investigated in an attempt to establish a procedure giving consistent results have been listed; factors to be of significance were rabbit age (weaners, 850-1500 g), a maximum of six loops per rabbit, the culture medium used, and the concentration of cell-free filtrate.

The ACA assay is useful as a research tool but even the in vitro system, if successfully established, would be costly and time consuming. Final comparative values depend on accurate pipetting and protein determination of microlitre quantities of suspended cells. Absolute values are further dependent on accurate weighing of milligram quantities of ATP. Several repeat assays on a single toxin are necessary to overcome the relatively large probable error of, and the variations found between, single ACA readings (fig 1). However, as an accurate indicator of the production of diarrhoeal toxin by a B. cereus strain, this test system recommends itself for use in conjunction with steps in the purification of diarrhoeal toxins, and it is hoped that this, in turn, would make possible the development of immunological methods for routine detection of these toxins.

Positive loops were not obtained on any occasion with strain 4ac used by Spira and Goepfert (1975) for isolation of an enterotoxin, and its CCFF did not produce raised ACA levels in two trials; a fresh culture of this strain is being requested for further tests.

The enterotoxins of Gram-negative organisms, such as V. cholerae, E. coli, and toxigenic non-invasive Shigella dysenteriae, do not in general produce major pathological changes in the mucosa of ligated ileum although, after 8 to 12 hours, ischaemic damage may occur from fluid pressure (Formal et al, 1961; Leitch et al, 1966; Formal et al, 1972; Gemski et al, 1972; Keusch et al, 1972; Levine et al, 1973). Exceptions are found in the reports of Sakazaki et al (1974) of haemorrhages in the lamina propria and submucosa of rabbit loops in response to V. parahaemolyticus enterotoxin and of Keusch et al (1972) of epithelial disarray and cell extrusion and necrosis in response to partially purified enterotoxin of Sh. dysenteriae 1. Among Gram-positive organisms, Hauschild et al (1967) and Niilo (1974) reported that no significant lesions developed in lambs or chickens respectively in response to C. perfringens enterotoxin, but McDonel (1974) reported slight to severe epithelial disruption in rat ileum, and McDonel and Duncan (1975) recorded changes ranging with increasing doses of C. perfringens enterotoxin from slight epithelial disruption to intense desquamation with lymphocytic infiltration and congestion in rabbit ileum. Prohaska (1963) reported that acute ileitis could be induced in dogs by direct infusion of Staphylococcus aureus enterotoxin into the ileum.

In the case of B. cereus, production of enterotoxins causing severe pathological changes in rabbit ileum is clearly not common to all strains; other B. cereus isolates from cases other than gastroenteritis are being screened for production of similar toxins.

These results provide a better understanding of the B. cereus enterotoxin which causes diarrhoea and reveal the existence of another hitherto undetected 'pyogenic' toxin.

The rabbit loop and ACA assays are clearly of no value for the detection and measurement of the B. cereus toxin which causes vomiting. A more convenient and reliable system than monkey feeding must now be found with a view to the purification of this toxin and hence to the development of simple immunological methods for its routine detection in food and other specimens.

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