Detection of clostridial toxins in stools from children with diarrhoea

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Summary. A cell-culture assay was used to detect toxins directly in stools from sporadic cases of infantile diarrhoea. Cytotoxins were revealed in 11 out of 58 samples from children with diarrhoea, nine of whom had no common enteric pathogens in their stools. A preliminary characterisation of the cytotoxins was obtained by neutralisation tests with clostridial antitoxins.

Introduction

Studies on clostridial toxins have been stimulated recently by the assessment of the role that clostridia can play in intestinal infections (Bartlett *et al.*, 1978; Taylor *et al.*, 1981; Borriello *et al.*, 1984). Direct detection of toxins in stools of patients is now the most rapid, convenient and reliable method for diagnosis of *Clostridium perfringens* food-poisoning (Giugliano *et al.*, 1983) and *C. difficile*-associated disease (Chang *et al.*, 1979). The enterotoxin of *C. perfringens* and the cytotoxin of *C. difficile* may be readily detected by cytotoxicity assays in tissue cultures (Chang *et al.*, 1979; Giugliano *et al.*, 1983).

In this study we assessed the possible role of cytotoxin-producing organisms in the aetiology of infantile diarrhoea, which still remains unknown in about half of all cases. A tissue-culture assay was used to detect bacterial toxins directly in stools from sporadic cases of infantile diarrhoea.

Materials and methods

Subjects

Fifty-eight children aged 0–3 years, consecutively admitted to a paediatric hospital in Naples, were examined during 1 year. All patients were admitted because of diarrhoea of various durations and unknown aetiology at the time of study. Stools were collected on admission and examined for the presence of enteric pathogens and for their ability to produce cytotoxic effects in tissue cultures. Stools from 27 asymptomatic children matched by age and season were also collected and examined for cytotoxicity as controls.

Identification of bacteria

Enterobacteria (Edwards and Ewing, 1972) and *Campylobacter jejuni* (Butzler and Skirrow, 1979) were isolated and identified by conventional techniques.

Enterotoxin assays

Isolates of *Escherichia coli* were tested for production of heat-labile (Guerrant *et al.*, 1974) and heat-stable (Dean *et al.*, 1972) enterotoxins.

Rotavirus detection

This was done by an immunoenzymatic assay (Rota-zyne; Abbot Laboratories, Chicago, IL, USA).

Cytotoxicity assay

Stool samples were diluted 1 in 10 in 0.01 M phosphate buffer (pH 7.4) and faecal extracts obtained by centrifugation (10 000 *g* for 15 min) and filtration of the supernate through 0.45- μ m membrane filters (Millipore Corp., Bedford, MA, USA). Chinese-hamster ovary (CHO), Vero and HEp2 cells were seeded, at a concentration of 2.5×10^4 cells/ml in Eagle's Minimal Essential Medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with fetal calf serum 1% v/v, into 96-well microtitration plates (0.2 ml in each well). Plates were inoculated with two-fold serial dilutions of faecal extracts (0.02 ml in each well) and scored for cytopathic effects after incubation for 48 h at 37°C in an atmosphere of air containing CO₂ 5% v/v. A positive result was recorded when $\geq 50\%$ of the cells in the tissue culture were affected; only faecal samples showing cytotoxicity at a dilution of 1 in 40 were considered positive.

Cytotoxic effects were characterised in preliminary neutralisation tests with three antisera: *C. difficile* antitoxin (kindly provided by Dr T. D. Wilkins, Virginia

Polytechnic Institute, Blacksburg, VA, USA); antiserum to enterotoxin of *C. perfringens* raised in rabbits by a method previously described by Stark and Duncan (1972); a polyvalent antiserum obtained by pooling monovalent antitoxins to *C. novyi* types A and B, *C. septicum*, *C. chauvoei*, *C. perfringens* types A, B, C, D and E (Wellcome Research Laboratories, Beckenham, Kent). Faecal extracts neutralised by the pool were further tested with the monovalent antitoxins.

Results

Enteropathogens were isolated from 20 of the 58 children with diarrhoea examined (35%); rotavirus alone was found in six, *Salmonella* serovars in four, *Shigella flexneri* in three, *Camp. jejuni* in one; in six cases rotavirus was found as well as pathogenic bacteria (*Salmonella* serovars in three, *Camp. jejuni* in two and *Yersinia enterocolitica* in one).

Eleven of the 58 faecal extracts from cases (19%) and two of those from 27 control subjects (7.4%) produced cytotoxic effects in at least two of the cell lines tested (table). Nine of the positive samples were from children with diarrhoea from whom enteric pathogens were not isolated.

The cytotoxicity pattern of positive samples and the results of neutralisation tests are shown in the

table. The only two positive samples from control subjects and the four from patients that caused rounding of each of the three cell lines tested were neutralised by *C. difficile* antitoxin (table). Seven samples killed HEP2 and Vero cells but not CHO cells. The cytotoxic effects produced by two of the extracts were neutralised by antiserum to *C. perfringens* enterotoxin. The cytotoxic effects of another three of the samples were neutralised by the pooled clostridial antitoxins; two of these samples, tested further with the nine monovalent antitoxins, were neutralised by monovalent antitoxin to *C. perfringens* type B only. That finding, furthermore, was reproducible. The cytotoxic effects of two faecal extracts from children in whom rotavirus and *Camp. jejuni*, respectively, had been identified were not neutralised by the antisera available.

Discussion

With a cytotoxicity assay in tissue cultures, we have detected the presence of clostridial toxins in faecal samples from cases of infantile diarrhoea in which enteric pathogens were not found.

The cytotoxin of *C. difficile* was detected in the faeces of four children, three of whom had been

Table. Cytotoxic effects of faecal extracts for tissue-culture cells and neutralisation by clostridial antitoxins

Faecal sample	Cytotoxicity for cell types			Neutralisation by		
	HEP2	Vero	CHO	<i>C. difficile</i> antitoxin	<i>C. perfringens</i> anti(entero)-toxin	Pooled clostridial gas-gangrene serum*
<i>Cases</i>						
1	R	R	R	+	—	—
2	R	R	R	+	—	—
3	R	R	R	+	—	—
4	R	R	R	+	—	—
5	K	K	O	—	+	—
6	K	K	O	—	+	—
7	K	K	O	—	—	+
8	K	K	O	—	—	+
9	K	K	O	—	—	+
10	K	K	O	—	—	—
11	K	K	O	—	—	—
<i>Controls</i>						
1	R	R	R	+	—	—
2	R	R	R	+	—	—

+ = Neutralisation; — = no effect.

R = rounding of cells; K = killing of cells; O = no observed effect.

* This pooled serum contained nine monovalent clostridial antitoxins (see *Materials and methods*).

treated with antimicrobials before the onset of the diarrhoea. Because carriage of *C. difficile* and its cytotoxin is not rare in asymptomatic children (Thompson *et al.*, 1983; Torres *et al.*, 1984), it is difficult to assign a causative role for the toxin in symptomatic paediatric patients. It is equally difficult, however, to exclude an aetiological role for *C. difficile* in our patients because of the previous antibiotic treatment and the failure to identify any other pathogen. A close association between disease and toxic factors neutralised by antisera to *C. perfringens* toxins has been shown, these factors being detected only in faeces from children with diarrhoea who had none of the common enteric pathogens present. *C. perfringens* enterotoxin was found in the faeces of two children, neither of whom had been implicated in food-poisoning episodes, and the cytotoxicity of two faecal samples was neutralised by the monovalent antitoxin to *C. perfringens*

type B. This was an unexpected finding because the major toxins produced by *C. perfringens* type B should also be neutralised by at least one of the other antisera to the other types of *C. perfringens* (McDonel, 1980). Failure to obtain cross-neutralisation with other specific antisera may suggest the involvement of a toxic factor different from the classical toxins described for *C. perfringens*.

In conclusion, our results suggest that a screening of stools from diarrhoeic children for cytotoxicity might help to improve knowledge of the aetiology of diarrhoeal diseases. In this respect, a further step should be the identification of the organism responsible for cytotoxicity *in vivo* by its ability to produce the same putative toxin *in vitro*.

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