PATHOGENIC MECHANISMS

Detection of intestinal and extra-intestinal strains of enterotoxigenic *Bacteroides fragilis* by the HT-29 cytotoxicity assay

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Summary. *Bacteroides fragilis* strains with enterotoxic activity can be isolated from the faeces of newborn farm animals with diarrhoea and are called enterotoxigenic *B. fragilis* (ETBF). These strains can now be detected in an in-vitro cytotoxicity assay with HT-29 cells. In this study, 146 *B. fragilis* strains (95 faecal and 40 extra-intestinal isolates) and 64 *Bacteroides* isolates belonging to species other than *B. fragilis* were tested for their ability to produce enterotoxin. Sixteen strains of ETBF were identified; all belonged to the *fragilis* species and represented 11% of all *B. fragilis* examined. The prevalence was similar among extra-intestinal and faecal strains, 11.5% and 10%, respectively. The production of enterotoxin in clinical isolates appeared to be associated with infections where tissue destruction was more prominent. Enterotoxigenicity was not associated with the presence of a plasmid and the plasmid profiles of ETBF strains that harboured plasmids were different. These results show that enterotoxin production by human isolates of *B. fragilis* is not uncommon and could represent a new virulence factor of *B. fragilis*.

Introduction

The role of anaerobic non-spore-forming bacteria in the ecology of the normal intestinal flora is considered to be mainly beneficial. Their metabolic activities are important for the health of the individual¹ and are thought to be responsible for resistance to colonisation by enteric pathogens.² However, some of these bacteria, notably those belonging to the genus *Bacteroides*, can cause severe infections at extra-intestinal sites.³ *B. fragilis* is the species most frequently isolated from infections in man,⁴ but it was not implicated as a cause of intestinal diseases until the last 10 years.

In 1984, Myers *et al.* isolated enterotoxigenic strains of *B. fragilis* (ETBF) from the faeces of neonatal lambs with diarrhoea.⁵ The enterotoxigenicity of these strains was demonstrated experimentally by their ability to elicit fluid accumulation in the lamb ligated intestinal loop test. Similar strains were isolated from the faeces of diarrhoeic calves,⁶ piglets⁷ and foals.⁸ Studies in man showed that individuals with or without diarrhoea can harbour ETBF strains in the intestine,⁹ but few data are available concerning the distribution of ETBF strains and their pathogenic potential in man.

The study of ETBF was stimulated by the demonstration that culture supernates of ETBF strains induce morphological alterations in a cultured cell-line from a human colon carcinoma, HT-29.¹⁰ Van Tassel *et al.* purified a protein of *c.* 20 kDa which was responsible for both enterotoxic and cytotoxic activities¹¹ and it became possible to use this in-vitro cytotoxicity test to detect ETBF instead of the cumbersome in-vivo procedure of the ligated intestinal loop. Moreover, as *B. fragilis* is primarily an extra-intestinal pathogen, the cytotoxic potential of the toxin suggested that it could participate in the invasive and destructive processes of extra-intestinal *B. fragilis* infections.

The aim of this study was to test *B. fragilis* isolates from faecal and extra-intestinal samples for their ability to produce cytotoxic changes in HT-29 cells.

Materials and methods

Bacterial strains

The bacterial strains used in this study comprised 146 *B. fragilis* strains and 64 strains of other *Bacteroides* spp. Of the *B. fragilis* strains, 95 were from clinical (i.e., extra-intestinal) samples and included the reference strains NCTC nos. 9343, 2429, 11295 and

11870 and ATCC 23745; 51 were from faecal samples of adults and children, with or without diarrhoea, and were isolated in different geographical areas of Italy. For *Bacteroides* spp. other than *fragilis*, 24 extra-intestinal and 40 faecal isolates were examined, comprising *B. vulgatus* (28 strains), *B. thetaiotaomicron* (15), *B. distasonis* (9), *B. uniformis* (5), *B. ovatus* (3), *B. merdae* (3) and *B. caccae* (1). The enterotoxigenic *B. fragilis* strain ATCC 43858 was used as a positive control. *Bacteroides* strains were identified by conventional methods¹² and, when necessary, identity was confirmed by a commercially available enzyme method, rapid ID32 A (bioMérieux sa, Marcy-l'Etoile, France).

Enterotoxin production

To assess enterotoxin production, each *Bacteroides* strain was grown overnight in Brain Heart Infusion (BHI) Broth (Oxoid, Basingstoke) supplemented with yeast extract (Oxoid) 0.5% and haemin 0.005% in an anaerobic cabinet (Microflow Anaerobic System, MDH Ltd, Andover, Hampshire) at 37°C. The cultures were centrifuged at 8000 *g* in an Eppendorf microfuge and the supernates were frozen immediately and kept at -20°C until used.

Cytotoxicity assay

HT-29 cells were grown in 50-ml flasks in RPMI 1640 medium with glutamine (Gibco, Life Technologies Ltd, Paisley) supplemented with penicillin 100 IU/ml, streptomycin 100 µg/ml and heat-inactivated fetal bovine serum (Hyclone Laboratories Inc., Logan, UT, USA) 15%, hereafter referred to as medium, at 37°C in air with CO₂ 5%. Cells were transferred to fresh medium with a 1 in 2 dilution three times a week. For the cytotoxicity assay, HT-29 cells from one flask were resuspended in 20 ml of medium, distributed (200 µl/well) into a 96-well microtitration plate (Falcon, Becton Dickinson, Lincoln Park, NJ, USA) and allowed to grow for 2-3 days until discrete clusters of cells were visible (fig. 1, panel A). Before the assay the medium was removed and 180 µl of fresh medium without serum was added to each well; 20 µl of two-fold dilutions of bacterial culture supernates were then inoculated into the wells in duplicate. The plate was incubated at 37°C in air with CO₂ 5% and then examined after 2 and 4 h for the presence of the typical toxin-induced cytopathic changes. The culture supernates were considered to contain *B. fragilis* enterotoxin if a cytopathic effect was visible that was neutralised by specific antiserum (see below). The cytotoxic titre was the highest dilution of the culture supernate that affected at least 50% of the cells after incubation for 4 h.

Production of antiserum to *B. fragilis* enterotoxin

A polyclonal antiserum against *B. fragilis* enterotoxin was produced in a New Zealand White rabbit

immunised with a crude enterotoxin antigen prepared by the methods of Van Tassel *et al.*¹¹ Briefly, the highly toxigenic strain NCTC 11295 was grown overnight in 1 L of BHI broth. Ammonium sulphate was added to the culture supernate to 70% saturation and left overnight with continuous stirring. The precipitate was harvested by centrifugation (10000 *g*), re-suspended in 25 ml of 50 mM Tris HCl, pH 7.5, containing the protease inhibitor TPCK (Sigma) 1 mg/ml, dialysed extensively against the same buffer and concentrated to a final volume of 10 ml by an Amicon ultrafiltration apparatus equipped with a YM 10 membrane (Amicon Division, W. R. Grace and Co., Danvers, MA, USA). Samples were applied to a PD-10 column (Pharmacia LKB Biotechnology, Uppsala, Sweden) and eluted with Tris-HCl buffer to complete desalting. This preparation was emulsified with an equal volume of Freund's complete adjuvant and 1 ml was injected subcutaneously in four different sites of the back of a New Zealand White rabbit. Three more doses of 0.5 ml of antigen preparation with an equal volume of Freund's incomplete adjuvant were injected intramuscularly at 2-week intervals. The rabbit was bled 10 days after the last injection.

Neutralisation test

The culture supernate from each strain was diluted to a concentration four-fold higher than the cytotoxic titre and then mixed with the same volume of doubling dilutions of anti-enterotoxin rabbit serum or normal rabbit serum. After incubation at 37°C for 30 min, 20 µl of each mixture was inoculated into HT-29 cells in microtitration wells containing 180 µl of medium. Neutralisation was indicated by the lack of any cytotoxic effect.

Plasmid fingerprinting

To assess the possible correlation between enterotoxin production and the presence of plasmids, extra-chromosomal DNA was extracted from the ETBF strains, including the reference strain ATCC 43858, by a modification of the method of Birnboim and Doly.¹³ Strains were grown overnight in 10 ml of pre-reduced BM broth¹⁴ at 37°C in the anaerobic cabinet. Cells were pelleted by centrifugation (4000 *g*) and washed once in TES buffer (50 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl). The pellets were then resuspended in 100 µl of 50 mM glucose with lysozyme 2 mg/ml in a buffer containing 25 mM Tris-HCl, 10 mM EDTA, pH 8.0, and kept at 37°C for 10 min; 200 µl of 0.2 N NaOH with SDS 1% and then 150 µl of 3 M potassium acetate (pH 4.8) were added. After centrifugation (10000 *g*) in an Eppendorf centrifuge, the supernates were treated twice with equal volumes of phenol:chloroform (1:1). The aqueous phases were precipitated with 2 volumes of absolute ethanol and kept at -80°C for 30 min. The pellets obtained by centrifugation (10000 *g*) were dried and resuspended

Fig. 1. Effect of broth culture supernates of *B. fragilis* on HT-29 cells after exposure for 4 h (magnification 100 $\times$). Panel A, non-enterotoxigenic *B. fragilis*; B, ETBF reference strain ATCC 43858.

in 50 μ l of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing RNAase (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany) 20 μ g/ml and left at room temperature for 30 min. Each sample (10 μ l) was loaded on to an agarose 0.7% gel, run at 60 V for 2 h and viewed under UV light. *Hind*III-cleaved λ DNA (New England Biolabs, Beverly, MA, USA) was used as mol.-wt marker.

Results

Cytotoxicity test and detection of ETBF

HT-29 cells grown by this method appear as discrete clusters or "islets" of cells inside which the borders of the single cells are ill-defined (fig. 1, panel A). The

cytotoxic effect shown by cells exposed to the supernate of the reference enterotoxic strain ATCC 43858 was clearly visible after 2 h and reached a maximum at 4–5 h. The affected cells appeared rounded and more refringent, with clear outlines, and they tended to detach one from the other (fig. 1, panel B). The rounding effect was more prominent at the edges of the cell clusters. The supernates of *B. fragilis* strains considered to be ETBF induced identical cytotoxic changes.

Table I summarises the production of enterotoxin by the *Bacteroides* strains examined; 16 ETBF strains were found. The percentages of ETBF strains among clinical and faecal isolates were approximately the same (11.5 and 10%, respectively). In all cases, the cytotoxic effect was neutralised by the immune rabbit anti-enterotoxin antiserum to a dilution of 1 in 8. The non-immune rabbit serum neutralised the cytotoxic effect only when used undiluted. None of the other *Bacteroides* spp. produced cytotoxic changes in HT-29 cells.

Table II shows the cytotoxic titres of the ETBF strains; the range was 20–320, with a modal value of 80. Strains from extra-intestinal infections tended to have higher cytotoxic titres than strains from faeces. The source of isolation was known for six of the 11 ETBF strains from extra-intestinal infections: they were from relatively uncommon *B. fragilis*-associated

Table I. Enterotoxin production by *Bacteroides* strains from different sources

Species	Source	Number of strains		
		tested	toxin +ve	%
<i>B. fragilis</i>	Clinical	95	11	11.5
	Faeces	51	5	10
Other <i>Bacteroides</i> spp.	Clinical	24	0	0
	Faeces	40	0	0

Table II. Source and cytotoxic titre of 16 ETBF strains and reference strain ATCC 43858

Strain no.	Source	Cytotoxic titre
PR254	Abdominal abscess	20
13141	NR	40
B5	Osteomyelitis	80
B32	NR	80
CM3	Ascites	80
CM14	Biliary fistula	80
NCTC11295	Enteric fistula*	160
B52	NR	160
B45	Infection of hip prosthesis	160
B34	NR	160
B69	NR	320
PG148	Faeces†	80
AN209	Faeces†	40
PA5	Faeces‡	80
ANT1	Faeces‡	80
ANT2	Faeces‡	80
ATCC43858	Reference strain (faeces)	40

NR, not reported.

* From Ingham *et al.*¹⁸

† Children with diarrhoea.

‡ Healthy adults.

Fig. 2. Agarose gel electrophoresis of plasmid DNA preparations from ETBF strains. Lane 1, ATCC 43858; 2, B45; 3, B32; 4, B5; 5, B34; λ, *Hind*III-digested λ DNA; chr, chromosomal DNA. The 3.5- and 6.5-kb bands are indicated.

infections, such as bone infections and fistulas. Of the five ETBF strains from faeces, two were from 1-year-old children with diarrhoea and three from healthy adults. Specifically, two strains (ANT1 and ANT2) were isolated from the same stool sample of a student actively engaged in ETBF research in the laboratory. The two strains were isolated from different selective plates (data not shown) and were found to be different by plasmid fingerprinting (see below). The student was symptom-free and carried at least one of the two toxigenic strains after 1 month.

Fig. 3. Agarose gel electrophoresis of plasmid DNA preparations from two ETBF isolates from the same subject: λ, chr, as in fig. 2.

Plasmid fingerprinting

Of the 16 ETBF strains, eight harboured plasmids, as did the reference strain ATCC 43858. However, the plasmid patterns were different: one, two or more plasmids were present, of both high and low mol. wt (fig. 2). Most frequently, two extra-chromosomal DNA bands were visible, of *c.* 6.5 and 3.5 kb. When two strains from the same subject were examined for their plasmid contents, they were different (fig. 3); only one of the two contained several extra-chromosomal DNA bands.

Discussion

The description 10 years ago of an enterotoxin produced by *B. fragilis* suggested new hypotheses about the role of this micro-organism in various infections. As the first recognition of this toxin was in strains isolated from the faeces of diarrhoeic farm animals, its involvement in the aetiology of diarrhoea was assumed. Studies in human subjects demonstrated the presence of ETBF,^{8,15} but did not corroborate a role for these strains in diarrhoeal diseases. Apart from sporadic isolations, two large prospective controlled studies were performed, one in a defined Apache population,¹⁶ and the second in a large number of children.¹⁷ The only significant association of ETBF with diarrhoea was limited to a subpopulation in the first study (children aged 13–36 months).

The weak association of ETBF with diarrhoea does

not exclude the hypothesis that ETBF strains, being producers of an enterotoxin that also has cytotoxic activity, could be at an advantage in causing extra-intestinal infections. As an initial investigation of this hypothesis, the frequency with which ETBF strains are isolated from extra-intestinal *versus* intestinal samples needed to be assessed and the present study aimed to identify ETBF strains among *B. fragilis* isolates from both intestinal and extra-intestinal samples. The HT-29 cytotoxicity assay was easy to perform and reproducible; the cytotoxic effect was clearly recognisable with minimal experience and showed a clear-cut endpoint. By this assay, with a reference enterotoxigenic strain as control and an anti-enterotoxin rabbit serum to neutralise the cytotoxic effect, several ETBF strains were identified. This is the first report of ETBF strains outside the USA; most of the strains examined were isolated in Italy, although the reference strain NCTC 11295, which proved to be toxigenic, was a metronidazole-resistant strain isolated in the UK.¹⁸ Therefore, ETBF strains are probably distributed world-wide.

Enterotoxin production was found only in the species *B. fragilis*; no strains of the related *Bacteroides* spp. produced changes of any kind in HT-29 cells. Moreover, approximately the same ratio of ETBF to total strains was found for strains from both faecal and extra-intestinal sources. This observation might imply that enterotoxin production is a "neutral" characteristic that does not provide any selective advantage over non-enterotoxic strains. However, a closer examination of the sources of ETBF demonstrates that an appreciable proportion of high-titre toxin-producing strains were isolated from uncommon pathological conditions, e.g., osteomyelitis and fistulas, where destructive processes are prominent. In terms of the pathogenesis of *B. fragilis*-induced infection, it is well established that the polysaccharide capsule of this species is essential in abscess formation through the presence of oppositely charged groups on

the bacterial surface.^{19, 20} It could be speculated that the enterotoxin might play a role in those invasive *B. fragilis* infections where an abscess is not the sole or principal manifestation. However, further studies of this hypothesis are needed.

ETBF strains were found among unselected faecal isolates, from children with diarrhoea and adults without intestinal symptoms. The existence of healthy carriers is common for all enteropathogenic microorganisms and does not rule out the possibility that such strains can cause diarrhoea when they colonise "susceptible" intestines where the bacterial flora or the mucosal defences are immature, as in infants and young children.

Another important issue is whether ETBF strains belong to a single clone or cluster. All attempts to find phenotypic or genotypic similarities have failed; serology,²¹ restriction fragment length polymorphism of rRNA²² and restriction endonuclease analysis of DNA¹⁶ have demonstrated that ETBF strains comprise a heterogeneous population. In the present study, plasmid profiles of ETBF strains were examined to determine whether the heterogeneity of ETBF was reflected in the plasmid fingerprinting and whether toxin production was associated with the presence of a particular plasmid. Only half of the ETBF strains had extra-chromosomal bands and the plasmid profiles of those with such bands were different, confirming the heterogeneity of the strains examined. Plasmid fingerprinting could be a useful marker for epidemiological studies; two isolates from the same healthy carrier were distinguished by their different plasmid profiles.

In conclusion, ETBF strains were isolated at an appreciable and similar frequency from faeces and from extra-intestinal samples. The data indicate that the role played by these *B. fragilis* strains could be important and deserves further investigation.

We are grateful to Fabiana Superti for help with cell culture and to Rita Cardines and Antonella Frate for performing plasmid preparations.

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