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Acute appendicitis: the role of enterotoxigenic strains of Bacteroides fragilis and Clostridium difficile

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SUMMARY

Background: *The aim of this study was to investigate whether there is a relationship between enterotoxin-producing B. fragilis strains and toxigenic C. difficile strains and the pathogenesis of acute appendicitis.*
Material and methods: *Post-appendectomy tissues from 34 patients with histopathologically confirmed phlegmonous or gangrenous appendicitis were studied.*
Results: *Among 86 anaerobes isolated, the B. fragilis group was most frequently isolated: 34 B. fragilis strains were cultured from 21 post-appendectomy tissues. Two enterotoxin-producing B. fragilis strains were found. Enterotoxin titers (1:10 and 1:160, respectively) were measured on HT29/C cells. The presence of the enterotoxin gene was confirmed by PCR in DNA extracted from both strains. Among 21 DNA samples isolated from those post-appendectomy tissues from which B. fragilis strains were cultured, the presence of the enterotoxin gene was confirmed in only one case (the corresponding B. fragilis strain enterotoxin titer was 1:160). A unique toxigenic C. difficile strain was also cultured from the tissue of an adult patient with gangrenous non-perforated appendicitis. The presence of toxin A and toxin B genes was confirmed by PCR in DNA extracted from the C. difficile strain, but these genes were not found in the DNA extracted from the corresponding tissue.*
Conclusion: *The presence of enterotoxigenic B. fragilis and toxigenic C. difficile strains was shown in post-appendectomy tissue from patients with phlegmonous and gangrenous appendicitis, and the B. fragilis enterotoxin gene was detected directly in the corresponding tissue. Further investigations (including immunologic aspects) require to confirm the role of these toxins in pathogenesis of acute appendicitis.*

BACKGROUND

A high rate of aerobic-anaerobic microbial associations of microflora isolated from appendicitis tissue has been reported by many authors, and the leading role of endogenous microorganisms in the etiology and pathogenesis of appendicitis (autoinfection) has been demonstrated [1]. Bacteroides fragilis is the most common obligately anaerobic bacterial species isolated from serious human infections [2]. The capsule, lipopolysaccharide (LPS), outer membrane protein (OMP), pili, several enzymes, and short-chain fatty acids have been recognized as the most important virulence factors of B. fragilis [3–6]. In 1984 and 1985 Myers et al. described the enterotoxic activity of B. fragilis strains isolated from fecal samples collected from different animals with diarrhea The same group of authors in 1987 described enterotoxigenic B. fragilis strains (ETBF) isolated from humans with diarrhea [7]. Fur-

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ther investigations have shown that enterotoxin/fragilysin is an extracellular zinc metalloprotease, containing 1g-atom Zn per molecule, purified enterotoxin, hydrolyzed gelatin, azocoll, actin, tropomyosin, and fibrinogen. Optimal proteolytic activity occurred at 37°C and pH 6.5. The enzymatic activity was inhibited by metal chelators [8,9]. Since the early 1990s, the role of ETBF strains in human diseases has been studied in various countries. In Poland, ETBF strains have been isolated from fecal samples collected from diarrheic and non-diarrheic children [10], non-diarrheic adults [11], and extraintestinal sources [12]. The enterotoxigenic activity of B. fragilis strains isolated between 1976 and 1995 has also been identified [13]. C. difficile is the major causative agent of pseudomembranous colitis and antibiotic-associated diarrhea [14]. The role of two protein toxins, toxin A-enterotoxin and toxin B-cytotoxin, in the pathogenicity of this bacteria has been established, and other virulence factors have been described [15].

The aim of this study was to investigate whether there is a relationship between enterotoxin-producing B. fragilis strains and toxigenic C. difficile strains and the pathogenesis of acute appendicitis.

MATERIAL AND METHODS

Patients: Thirty four patients (adults and children) with histopathologically confirmed phlegmonous or gangrenous appendicitis were investigated. The adult patients were treated by 2nd or 3rd-generation cephalosporines or by a combination of cephalosporine with metronidazole. The children were treated peri- or postoperatively with amoxicillin and clavulanic acid.

Post-appendectomy tissue was obtained perioperatively from the distal end of the appendix, so as to exclude the lumen. The tissue was inoculated into a Port A Cul (BBL) anaerobic transport container and transported to the laboratory. The material was prepared for microbiologic testing by thorough homogenization in an anaerobic chamber (Forma Glove box), and was kept frozen at –70°C until further investigation.

Bacterial culture was performed based on standard schemes for anaerobes. Briefly: the tissue was inoculated into Columbia blood agar (bioMerieux, France) , BBE (Bacteroides Bile Esculine agar; bioMerieux, France), CCCA (Columbia blood agar containing Cycloserine-Cefoxitin-Amphotericine B; bioMerieux, France) and BHI medium (bioMerieux, France) for incubation in an anaerobic chamber (Forma Glove box) at 37°C for at least 96 hours.

Identification of bacterial strains was done according to growth on selective media, colony morphology, Gram-staining, and biochemical characteristics, based on the API 20A and Rapid 32A tests (bioMerieux, France).

Determination of the enterotoxicity of B. fragilis strains was done using PCR for enterotoxin gene detection [16] and HT29/C cell line assay, which was performed according to the procedure of Welkel [17]. Briefly: 24-hour B. fragilis cultures in BHI medium were centrifugated. Supernatants were filtered with a 0.2 μm syringe filter (Corning, USA) and directly applied to the cell culture medium of HT29/C line in 96 microwell plates (Corning, USA). The results were read after 2 and 4 hours of incubation. Each experiment was compared with supernatants obtained from a known ETBF reference--type culture collection strain, ATCC 43858, and non-ETBF strain. Fresh BHI medium was also used as a negative control. For B. fragilis enterotoxin titer determination, double dilutions of supernatant filtrates in BHI were added to the cells. Incubation and observation was performed as above. The highest dilution with a positive cytotoxic effect was accepted as a titer.

Determination of the toxigenicity of C. difficile strains was done using the Tox A/B ELISA test, according to the manufacturer's instructions [18], and PCR for detection of toxin genes [19–21].

DNA extraction from post-appendectomy-tissue and bacterial strains was done according to the protocol of Genomic DNA PREP PLUS (A&A Biotechnology, Poland) and the phenol-chloroform--isoamyl extraction method [19]. DNA was extracted on minicolumns, followed by the cell lyzing procedure, using chaotropic salts, detergents (buffer LT) and proteinase K.

PCR for B. fragilis and C. difficile toxins detection was performed in a DNA thermal cycler (Techne, UK) using the following primer pairs and amplification profiles, respectively:

For detection of ETBF strains: 404/407 [16] primer pairs : 1 cycle for 4 min. at 94°C followed by 40 cycles: (94°C – 1 min; 52°C – 1 min; 74°C – 1 min).

Table 1. Anaerobes isolated from post-appendectomy tissue of patients with phlegmonous and gangrenous appendicitis.

No	Patient #	Adult/Child	Histopathologic diagnosis	Anaerobes isolated
1	6	A	Phlegmonous appendicitis	B. fragilis, B. ovatus, Prop. acnes
2	10	A	„	Act. naeslundii, B. fragilis, B. ovatus, B. uniformis, Streptococcus intermedius 1/2/3
3	16	A	„	Act. naeslundii, Prop. acnes
4	19	A	„	B. fragilis 1/2
5	23	A	„	Prop. acnes
6	24	A	„	Prevotella melaninogenica
7	25	A	„	B. fragilis 1/2/3, Prevotella rum.rum
8	31	A	„	Prop. acnes
9	38	A	„	B. fragilis, B. ovatus 1/ 2/ 3
10	39	A	„	B. fragilis 1/2/3, Prop. acnes
11	40	A	„	B. fragilis 1/2/3/4
12	59	A	„	B. fragilis, B. ovatus
13	62	A	„	B. distasonis, B. ovatus 1/2/3/4, B. thetaiotaomicron,
14	102	Ch	„	B. ovatus, B. thetaiotaomicron
15	106	Ch	„	B. fragilis 1/2
16	109	Ch	„	B. fragilis 1/2, B. ovatus
17	112	Ch	„	B. fragilis
18	116	Ch	„	B. vulgatus 1/2/3
19	117	Ch	„	B. distasonis, B. vulgatus
20	120	Ch	„	B. fragilis
21	12	A	Gangrenous appendicitis	B. distasonis, B. ovatus
22	13	A	„	B. fragilis
23	15	A	„	B.caccae, B. fragilis, Prev. rum. brev.
24	17	A	„	B. fragilis
25	30	A	„	B. fragilis, B. merdae, Prop. granul.
26	361	A	„ (Perforated)	*B. fragilis (1:10)
27	37	A	„	Act. naeslundii, Prev. oralis 1/2
28	572	A	„	¤Clostridium difficile
29	60	A	„	B. ovatus 1/2/3, B. thetaiotaomocron
30	101	Ch	„ (Perforated)	B. fragilis 1/2/3
31	1033	Ch	„ (Perforated)	*B. fragilis (1:160), B. distasonis, B. thetaiotaomicron,
32	107	Ch	„	B. distasonis, B. vulgatus
33	110	Ch	„	B. fragilis
34	113	Ch	„	B. fragilis 1/2, B. ovatus

Legend to table: # Numbers before 100 - adult patients; after 100 - children; * Enterotoxigenic *B. fragilis* strain .Toxigenicity of *B. fragilis* was determined by PCR and HT29/C cell line assay. In parenthesis - enterotoxin titers, obtained on cell line.; ¤ Toxigenic *C. difficile* strain. Toxigenicity was determined by PCR and TechLab A/B ELISA test.; 1 Tissue was *B. fragilis* enterotoxin gene - negative; 2 Tissue was *C. difficile* toxin A and B genes - negative; 3 Tissue was *B. fragilis* enterotoxin gene - positive

For detection of C. difficile toxin A and B genes: YT28/YT29 and YT17/YT18 [19–21] primer pairs: 1 cycle for 2 min at 94°C followed by 35 cycles: (94°C – 45 sec.; 55°C – 30 sec.; 70°C – 45 sec.). PCR-amplified products were electrophoresed in 1% agarose gels, stained with ethidium bromide and visualized by UV light. Molecular mass markers (123 bp) were run concurrently. The DNAs of ETBF reference strain ATCC 43858 and toxigenic C. difficile VPI 10463 strain were used as positive controls; in addition, the DNAs of non-toxigenic strains of B. fragilis and C. difficile were used as negative controls for each PCR assay, respectively.

RESULTS

From 34 post-appendectomy tissue samples, 86 different anaerobic bacteria were isolated. Among the cultured anaerobes only 12 strains of Gram-positive bacteria were isolated from 6 post-appendectomy tissues. The B. fragilis group was dominant among the 74 Gram- negative anaerobes isolated: 34 strains of B. fragilis were isolated from 21 post--appendectomy-tissue samples (13 adults and 8 children; cf. Table 1). B. fragilis strains were isolated alone from 11 post-appendectomy samples, or in association with Gram-negative (8 cases) or

Gram-positive and Gram-negative (2 cases) anaerobes.

All the cultured B. fragilis strains were investigated for the presence of enterotoxin gene in PCR using 404/407 primer pairs. Two enterotoxin-producing (ETBF) strains were isolated from the tissue samples numbered 36 and 103, collected from an adult and a pediatric patient with gangrenous and perforated appendicitis (Table 1). Enterotoxin production by these strains was confirmed in the HT29/C cell line assay. The titers of enterotoxin were 1:10 and 1:160, respectively. All 21 post-appendectomy tissue samples that were B. fragilis culture-positive were studied in PCR for the presence of the B. fragilis enterotoxin gene. A positive result was obtained in one tissue: no.103 , in which an enterotoxigenic B. fragilis strain with enterotoxin titer 1:160 was present (Table 1). The B. fragilis enterotoxin gene was not found in the DNA extracted from the second tissue sample (no. 36), where there was a corresponding ETBF strain with enterotoxin titer 1:10.

In one case of gangrenous appendicitis (nonperforated) a unique C. difficile strain was cultured. The ELISA A/B test for C. difficile toxin detection gave a positive result. This result was in full accordance with PCR results, detecting the C. difficile toxin A and B genes in DNA extracted from this strain. In the DNA extracted from the corresponding tissue of this adult patient, the C. difficile toxin A and B genes were not found.

DISCUSSION

The role of members of the B. fragilis group in the pathogenesis of appendicitis, especially phlegmonous and gangrenous (perforated), has been discussed since the 1970s [22–24]. Scandinavian authors [23,24] described this group as the most commonly isolated species from excised appendix samples. Further research by other authors in the late 1980s and 90s confirmed this observation [25,26]. The role of encapsulated strains of B. fragilis in intraabdominal abscess formation is well known [19,27], while the relation of enterotoxigenic B. fragilis strains to diarrhea has been discussed in the literature by many authors [28,29]. ETBF strains have been cultured from both intestinal and extraintestinal sources [16,30,31], but not from post-appendectomy tissue.

In our study, the B. fragilis group was dominant among the 86 anaerobes isolated from 34 investigated post-appendectomy tissue samples taken from patients with gangrenous and phlegmonous appendicitis. The most commonly isolated strain was B. fragilis. Our results are in accordance with those obtained by other authors [23–26]. It is very difficult to reach an answer to the question whether there is a relationship between enterotoxin production by B. fragilis and the pathogenesis of acute appendicitis. We cultured 2 ETBF strains from 2 out of 21 post-appendectomy tissue samples in which B. fragilis strains were found. Both patients (one adult and one child) had gangrenous perforated appendicitis. It is possible that the presence of ETBF strains in these post-appendectomy tissues was a result of endogenous transmission from another part of the intestine. In these cases data about the intestinal flora of patients before surgery would be very helpful.

Many authors have reported the detection of the B. fragilis enterotoxin gene by PCR directly in stool samples [8,29,32,33]. We used this method to search 21 post-appendectomy tissues for the presence of B. fragilis enterotoxin gene, but detected it in only one case (the enterotoxin titer of the corresponding strain was 1:160). To our knowledge this is the first observation of the B. fragilis enterotoxin gene directly in post-appendectomy tissue (in vivo). The detection of the B. fragilis enterotoxin gene directly in post-appendectomy tissue would appear to be a useful tool for the confirmation of diagnosis in some complicated cases of appendicitis.

We also cultured a unique toxigenic C. difficile strain from the tissue of an adult patient with gangrenous (non-perforated) appendicitis. However, we did not find the C. difficile toxins A and B genes in the corresponding post-appendectomy tissue, obtained in such a way as to exclude the lumen. The C. difficile toxins act in concert to create intestinal wall damage [14,34]. The effect of the C. difficile toxin A (enterotoxin) on human colonic lamina propria cells and its capability to suppress human mucosal immune responses by inducing the early loss of macrophages, followed by T-cell apoptosis, was recently described by Borriello's group [34]. The same mechanism would seem to be possible in case of acute appendicitis as well. Further research (including immunological aspects) on a larger number of patients would be required to confirm the possible involvement of enterotoxigenic B. fragilis and C. difficile strains in the pathogenesis of acute appendicitis.

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