Bacteroides fragilis Enterotoxin
Gene Sequences in Patients with Inflammatory Bowel Disease

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We identified enterotoxigenic Bacteroides fragilis in stool specimens of patients with inflammatory bowel disease and other gastrointestinal disorders. The organism was detected in 11 (13.2%) of 83 patients with inflammatory bowel disease. Of 57 patients with active disease, 19.3% were toxin positive; none of those with inactive disease had specimens positive for enterotoxigenic Bacteroides fragilis gene sequences.

Bacteroides fragilis, a gram-negative rod, constitutes 1% to 2% of the normal colonic bacterial microflora in humans (1,2). It is frequently associated with extraintestinal infections such as abscesses and soft tissue infections, as well as diarrhea in animals and humans (3-6). Enterotoxigenic B. fragilis (ETBF) is an emerging enteric pathogen associated with diarrheal diseases in children, adults, and animals (4,7-9). The pathogen is unusual in children during the first year of life, but diarrhea associated with it is common in children 1 to 5 years of age, which suggests early acquisition of the organism and maternal protection. The pathogenicity of B. fragilis is related to its production of a potent enterotoxin, a zinc-containing metalloprotease with a molecular weight of 20,000 (10). The enterotoxin is tight-junction specific; causes rounding, swelling, and pyknosis of cultured enterocytes; and induces a fluid response in ligated intestinal loops and a cytotoxic response in the HT-29 colon cell line (7).

Ulcerative colitis and Crohn disease are inflammatory diseases of the gastrointestinal tract characterized by spontaneous remissions and relapses. Many microbial pathogens, particularly Mycobacterium paratuberculosis, paramyxoviruses, and Listeria monocytogenes have been implicated in the etiology of inflammatory bowel disease (IBD) (11). In addition, enteric pathogens such as Campylobacter jejuni, Salmonella, Shigella, Yersinia, and Escherichia coli have also been associated with relapses of IBD (12). We investigated the prevalence of ETBF in 83 patients with idiopathic IBD, in 18 patients with routine culture-negative IBD, and in a control population of 69 outpatients (Table 1).

| Patient group | IBD (n = 83) | Diarrhea patients (n = 18) | Controls (n = 69) |
|---------------|-------------|---------------------------|------------------|
| Sex           |             |                           |                  |
| M             | 38          | 4                         | 40               |
| F             | 45          | 14                        | 29               |
| Age (yrs)     |            |                           |                  |
| Mean = 10-80  | 45.5        | 45.6                      | 58.2             |
| Duration of disease (yrs) |           |                            |                  |
| UC            | 1-13 (5.6)  | 1-6 (2.6)                 | NA               |
| CD            | 1-20 (10.5) |                            |                  |
| Therapeutic treatment |         |                            |                  |
| 5-ASA         | 58          | 0                         | 0                |
| 5-ASA+Steroids| 35          | 0                         | 0                |
| 6-MP/ Azathioprine | 20        | 0                         | 0                |
| MTX           | 1           | 0                         | 0                |
| Antibiotics   | 1           | 1                         | 0                |
| Antidiarrheals| 1           | 3                         | 8                |

The Study

The study protocol was approved by the Human Subjects Research Review Committee of...
the University of California, Davis. All samples were collected after informed consent was obtained. Of the 83 patients in the IBD group, 60 had Crohn disease, and 23 had ulcerative colitis (Table 2). Active disease was present in 68.6% of these patients on endoscopy (20 ulcerative colitis and 37 Crohn disease patients) in the form of mucosal erythema and ulceration. In the miscellaneous diarrhea group, 10 patients had irritable bowel syndrome; however, no mucosal erythema, edema, or ulceration was observed. None of the control patients had a history of diarrhea, and no erythema or ulceration was observed.

Table 2. Bacteroides fragilis enterotoxin gene amplification products in patients with inflammatory bowel disease (IBD)

| Patient group (no.) | ETBFa Gene (+) | p valueb |
|---------------------|----------------|-----------|
| IBD (83)            | 11 (13.25%)    | 0.023     |
| Active (57)         | 11 (19.3%)     | 0.0026    |
| Inactive (26)       | 0              |           |
| Crohn disease (60)  | 5              |           |
| Active (37)         | 5              |           |
| Inactive (23)       | 0              |           |
| Ulcerative colitis (23) | 6 |           |
| Active (20)         | 6              |           |
| Inactive (3)        | 0              |           |
| Diarrhea (18)       | 5 (27.8%)      | 0.0005    |
| Control (69)        | 2 (2.89%)      |           |

aETBF = enterotoxigenic Bacteroides fragilis
b p value in relation to control group

Fecal specimens from the IBD group were collected endoscopically from patients undergoing colonoscopy or flexible sigmoidoscopy for evaluation of symptoms of diarrhea or abdominal pain. The fecal specimens from the 18 diarrhea patients with negative routine stool cultures and the 69 controls were collected by endoscopy.

Fecal specimens were cultured for B. fragilis in the selective medium Bacteroides Bile Esculin agar. Positive cultures were identified by using the RapID ANA II Panel (REMEL, Inc, Lenexa, KS). Plates were incubated anaerobically at 37°C for 48 hours. The presence of B. fragilis enterotoxin in the isolates was detected in the HT-29 colon cell line (13,14). HT-29 cells were grown and maintained in RPMI medium with glutamine (Gibco, Life Technologies, Inc., Grand Island, NY) supplemented with penicillin (100 IU/ml), streptomycin (100 g/ml) (Sigma, Saint Louis, MO), and heat-inactivated fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT) at 12% in 25-ml flasks at 37°C in 5% CO2. For the cytotoxicity assay, HT-29 cells were suspended in 20 ml of medium for each plate, and 180 l/well were placed into 96-well tissue culture plates (Corning Glass Works, Corning, NY). The cells were allowed to attach and grow for 2 to 3 days. Supernatants from B. fragilis isolates grown on Brain Heart Infusion Broth were filtered through a 0.45- m Acrodisk syringe filter (Gelman Sciences, Ann Arbor, MI), and 20- l serial dilutions were placed into the wells in duplicate. The plates were incubated at 37°C for 3 to 4 hours under 5% CO2 and examined for typical cytopathic changes. Cultures were considered positive for B. fragilis enterotoxin if a visible cytopathic effect was neutralized by specific antiserum. The highest dilution of the culture supernatant producing cytopathic changes in at least 50% of the cells after 3 to 4 hours of incubation was considered the cytotoxic titer. For the neutralization assay, dilutions of 1:25 anti-enterotoxin rabbit antiserum in phosphate-buffered saline were mixed with culture supernatants positive for enterotoxin. After incubation for 30 minutes at 37°C, 20 l of each mixture was inoculated into HT-29 cells as in the cytotoxicity assay. Neutralization was indicated by the lack of cytotoxic effect.

DNA was extracted from the fecal specimens and amplified by using specific primers (14) to detect B. fragilis enterotoxin gene sequences. Chi-square was used to determine statistical significance.

1 A 100-ml sample of stool was suspended in 400 l of TES buffer (50 mM Tris [pH 8], 5 mM EDTA, 50 mM NaCl) and centrifuged at 1,000 x g for 3 min to remove large particles. The supernatant was then centrifuged at 5,000 x g for 7 min. The pellet was washed once in 200 l of TES, and centrifuged at 5,000 x g for 3 min, and the supernatant was discarded. The pellet was suspended in 100 l of sterile H2O and boiled for 10 min, then centrifuged at 1,000 x g for 2 min and the supernatant containing the DNA extracted twice with phenol: chloroform: isooamyl alcohol (25:24:1) and precipitated with ethanol. The sequences of the primers and used PCR conditions were as described (14). Amplification with the outer primers RS-3 (5’ TGAAGTATAGCCGACGATGACCG 3’) and RS-4 (5’ GCTCAAGCGGCCCCAGTATA TGACC 3’) yielded a 367-bp product. Amplification of this product targeting the 16S rRNA gene of enteric bacteria was used as described by Kato et al. (15).
ETBF was cultured from four patients with IBD and two patients with diarrhea. However, B. fragilis enterotoxin gene sequences were detected in the stools of 11 (13.25%) of 83 patients with idiopathic IBD (five with Crohn disease and six with ulcerative colitis). All 11 patients positive for ETBF had active disease by endoscopic and histologic tests (Table 2). No ETBF was found in patients with inactive disease. The Crohn disease patients who were positive for enterotoxin gene sequences had superficial mucosal disease. In the control group, 2 (2.9%) of 69 patients were positive for B. fragilis enterotoxin gene sequences. Enterotoxin amplification products were also detected in the specimens of 5 (27.7%) of 18 patients with diarrhea due to miscellaneous causes (Table 2). All the specimens were positive when amplified with primers specific for the 16S rRNA gene of enteric bacteria.

Conclusions

The normal colonic microflora of humans is a complex ecosystem of approximately 500 species of aerobic and anaerobic microorganisms. Although the gut of the newborn infant is sterile, Bacteroides species—the predominant anaerobic constituent of the colonic flora—appear at approximately 10 days, are established by 2 weeks, and usually remain constant lifelong (1,2). In breast-fed infants, Bifidobacterium are the predominant population, and Bacteroides group organisms remain undetectable. However, after weaning, the Bacteroides group organisms increase and Bifidobacterium organisms decrease substantially (16).

The exact etiology of idiopathic IBD is still unknown, although a potential role for infectious agents or toxins that may stimulate an inflammatory response has been suggested (16,17). For example, Peptostreptococcus, Coprococcus, and Bacteroides sp. have been reported in patients with Crohn disease (17). A role for these microorganisms in the disease process is also suggested by the clinical responses of some patients to antibiotics (18). Observations of B. thetaiotaomicron in patients with ulcerative colitis (16) and B. vulgatus in guinea pigs with experimentally induced ulcerative colitis (19) suggest that microorganisms may influence the development or maintenance of intestinal inflammation in IBD.

In this study, B. fragilis enterotoxin gene sequences were detected by nested polymerase chain reaction (PCR) in the stools of 13.2% of patients with inflammatory bowel disease and 2.9% controls. The low recovery of ETBF in culture may be due to the length of time from specimen collection to processing in the laboratory (most specimens were kept frozen for at least 2 weeks before culturing). Similar results have been reported by Sack et al. (9), who found a marked reduction in the recovery of B. fragilis with time. Amplification of enterotoxin gene directly in the stools of these patients appears to be a more sensitive detection method. In a previous study (14), we found 100% correlation between PCR and enterotoxin production in isolates; we therefore did not routinely perform the HT-29 cell assay in specimens of these patients.

In the IBD group, all the patients positive for ETBF had active disease, which suggests an association with disease activation or flare-up. ETBF was also found in patients with ulcerative proctitis, collagenous colitis, and microscopic colitis. In patients with Crohn disease, ETBF was usually seen in the colonic superficial inflammatory disease type. The presence of ETBF in IBD patients may represent alterations of endogenous bacterial flora, which may be related to either the etiology or flare-up of the disease or both. Colonization with ETBF may be acquired early in life or may be a de novo infection related to flare-ups of the disease.

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