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Evaluation of the Pathogenicity of the *Bacteroides fragilis* Toxin Gene Subtypes in Gnotobiotic Mice

Viviane Nakano,[1] Danielle A. Gomes,[2] Rosa M. E. Arantes,[3] Jacques R. Nicoli,[2] Mario J. Avila-Campos[1]

[1]Departamento de Microbiologia, Instituto de Ciências Biomédicas, Universidade de São Paulo São Paulo, SP, Brazil
[2]Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil
[3]Departamento de Patologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

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Abstract. Enterotoxigenic *Bacteroides fragilis* (ETBF) strains produce a metalloprotease toxin (BFT) related to diarrheal disease in animals, young children, and adults. Three different isoforms of the enterotoxin, designated BFT-1, BFT-2, and BFT-3, have been identified and sequenced. In the present study, the pathogenicity of the ETBF strains carrying *bft*-1 or *bft*-2 was evaluated. Each toxin gene subtype of ETBF (*bft*-1 or *bft*-2) was intragastrically monoassociated to germ-free mice during 10 days and histopathological data from intestines and liver compared with those from mice monoassociated to a non-enterotoxigenic *B. fragilis.* Histopathological alterations were observed in all groups of animals related to ETBF. These alterations were characterized mainly by ulceration, edema, and inflammatory infiltration in intestine. However, these lesions were slightly more severe in mice monoassociated with *bft*-2 subtype. No alteration or lesion was observed in animals associated with the non-enterotoxigenic *B. fragilis*. In conclusion, strains harboring *bft*-1 or *bft*-2 gene subtypes were able to induce histopathological alterations in intestine of a gnotobiotic mice model and it could explain the effect produced for the enterotoxin.

Bacteroides fragilis species is an obligatory anaerobic bacterium belonging to the indigenous intestinal microbiota. However, under certain conditions, this organism can cause a wide range of human diseases including abscesses, diabetic foot, and sepsis. Some of the strains belonging to this species are able to produce an enterotoxin that has been related to watery diarrhea disease in farm animals and young children and they are, therefore, termed enterotoxigenic *B. fragilis* (ETBF) [16].

B. fragilis toxin (BFT) has been characterized as a 20-kDa zinc-dependent metalloprotease belonging to the metzincin superfamily [14]. The target of BFT has been identified as the cell surface protein E-cadherin, which is the main structural component of the adherence area and it is responsible for cell-cell adhesions in eukaryotic cells [24]. The loss of intact E-cadherin may cause disruption of its linkage with β-catenin and secondarily α-catenin and actin, leading to a morphological change of epithelial cells [13].

Studies in vivo have shown that BFT induces both a fluid secretion and exfoliation of intestinal epithelial cells. In certain human intestinal carcinoma cell lines, particularly HT29/C1, this toxin causes the rounding-up of cells and the rearrangement of the F-actin cytoskeleton [3, 7]. In polarized cell monolayers, BFT alters the apical F-actin structure, resulting in disruption of the epithelial barrier function, which may consequently contribute to the diarrheal disease related to *B. fragilis* infection [2].

The *B. fragilis* toxin gene (*bft*) is contained in a 6-kb pathogenicity island or BfPAI, a region found in ETBF strains exclusively, together with a putative second metalloprotease (*mp* II gene), which has low identity (28%) with the enterotoxin gene [10, 15]. The presence of the 6-kb region only in ETBF strains suggested that this region might contain other important genes for the pathogenicity of these strains [23].

Correspondence to: Mario J. Avila-Campos; *email:* mariojac@usp.br

Moreover, the toxin gene has been cloned and sequenced from several ETBF strains and three different allelic forms have been identified. The *bft*-1 gene was found in the VPI 13784 strain isolated from lamb, while the *bft*-2 and the *bft*-3 genes were found in 86-5443-2-2 and Korea 419 strains, isolated from piglet and human subject, respectively [4, 9, 11]. Each of these alleles is unique but highly related (92–96% identical in their predicted aminoacid sequences) [23].

Studies have showed that, generally, the *bft*-1 is predominant in strains isolated from adults, while *bft*-2 is frequently found in strains from children with antibiotic-associated diarrhea as well as in cows. Nonetheless, further studies on epidemiology and on the clinical impact of ETBF are necessary [6,11].

The goal of this study was to evaluate the pathogenicity of the ETBF strains carrying *bft*-1 or *bft*-2 genes in gnotobiotic mice.

Materials and Methods

Bacteria. Four toxin-producing *Bacteroides fragilis* strains isolated from children with diarrhea and one non-toxigenic strain isolated from a healthy child were used in this study. These strains showed the following characteristics, according to the presence of toxin genes: *bft-1* subtype, *B. fragilis* AUS 0384; *bft-2* subtype, *B. fragilis* GAI 97124; and non-ETBF, *B. fragilis* AUS 0308. All the strains were stored in 10% skim-milk, at −80°C.

Mice. Germ-free 21-day-old NIH mice (Taconic, Germantown, NY, USA) were used. The animals were housed in flexible plastic isolators (Standard Safety Equipment Company, McHenry, IL, USA) and handled according to established procedures [21]. Experiments with gnotobiotic mice were carried out in micro-isolators (UNO Roestvaststaal B.V., Zevenaar, The Netherlands). Water and commercial autoclavable diet (Nuvital, Curitiba, PR, Brazil) were sterilized by steam and administered ad libitum in all animals. Controlled lighting (12 h light, 12 h dark) was used in the animals. All experimental procedures were carried out according to the standards set forth in the "Guide for the Care and Use of Laboratory Animals" [17] and approved by the Ethics Committee in Animal Experimentation of the Federal University of Minas Gerais (CETEA/UFMG).

Experimental Design. Experiments of colonization and anatomopathological examination were performed using three groups with five mice in each one. Each group was related to: group I, *B. fragilis* AUS 0384; group II, *B. fragilis* GAI 97124; group III, *B. fragilis* AUS 0308. Levels of the bacterial population in feces and clinical changes were noticed during the challenge experiments. At the end of the experiments, all mice were sacrificed by cervical dislocation.

Treatment. A single dose of 10^7 viable cells of each *B. fragilis* strain grown in BHI broth (anaerobiosis, at 37°C, for 24 hours) was administered in germ-free mice by intragastric intubation and the animals were maintained monoassociated for 10 days.

Fecal Microbial Counts. Feces freshly collected one day and 10 days after monoassociation were weighed and immediately introduced in an anaerobic chamber (Forma Scientific Company, Marietta, OH, USA), containing an atmosphere of N_2 85%, H_2 10% e CO_2 5%. Fecal samples were diluted in 100-fold of regenerated buffered saline. Serial 10-fold dilutions were made and 0.1 ml of serial dilutions was plated onto Brain Heart Infusion (BHI, Difco, Detroit, MI, USA) agar supplemented with hemine (0.1%) and menadione (0.1%) and incubated at 37°C, for 24 hours. Results were expressed as $\log_{10}$ of the colony-forming units (CFU) per gram of feces.

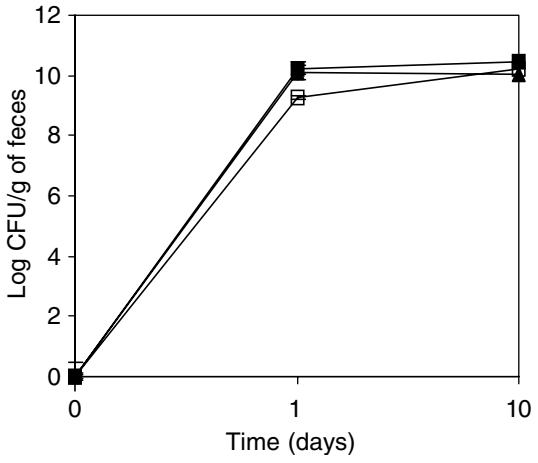

Fig. 1. Fecal population levels of *Bacteroides fragilis* strains in germ-free NIH mice associated intragastrically to subtype *bft-1* (■), *bft-2* (▲), and non-ETBF (□), for 10 days.

Histopathological Examination. Tissue samples from distal ileum, cecum, colon, and liver of all mice sacrificed were fixed in buffered 4% formaldehyde and processed for paraffin embedding. Briefly, the gastrointestinal tube was completely removed and the ileum, cecum, and colon were opened for the antimesenteric border, and the contents carefully removed. The intestinal segment stretched on filter paper was prefixed with Bouin's fluid containing 2.5% glacial acid for 90 to 120 minutes at room temperature. The prefixed segments were then rolled into a spiral with the mucosa facing inward, so as to form a "Swiss roll" [1]. The rolls were tied with string and fixed in Bouin's for an additional 18 to 20 hours. The material was routinely processed for paraffin embedding. Three non-consecutive histopathological sections (3–5 μm) were stained with hematoxylin-eosin. The slides were coded and examined by a single pathologist, who was unaware of the experimental conditions of each group.

Multiplex-PCR. One colony of each tested bacteria was re-isolated from plates used for fecal bacterial count at day 10 and the *bft* gene subtypes were determined again by multiplex PCR, according to Kato et al. [11]. The bacterial DNA was obtained by using an Easy-DNA kit (Invitrogen). The reaction was carried out in a final volume of 25 μl containing 0.25 μl of Platinun *Taq* polymerase (0.5 U), 2.5 μl of 10 × PCR buffer, 1.25 μl of $MgCl_2$ (1.5 mM), 1 μl of dNTP mix (0.2 μM), 1 μl of each primer (0.4 μM), 6 μl of ultra-pure water, and 10 μl of template DNA. The amplification reaction was carried out in a thermocycler programmed to: 1 cycle of 94°C (5 min), followed by 35 cycles of 94°C (30 s), 62°C (1 min), and 72°C (2 min), and 1 cycle of 72°C (5 min). The amplified fragments were analyzed in agarose gel (1%) stained with ethidium bromide.

Results

All the inoculated *B. fragilis* strains became established in the digestive tracts of all gnotobiotic mice groups and

Table 1. Histological alterations produced by enterotoxiginic *B. fragilis* subtypes in 10 germ-free mice

Group subtypes	Organs		
	Cecum	Colon	Liver
I *(bft-1)*	Moderate edema and and an increase of the lamina propria Cells. Erosion slightly extensive with loss of he superficial epithelial layers, with loss of glandular architectural organization, exocytose, and nuclear hypercromasia of the glandular basal cells.	Slight edema and an increase of the lamina propria cells.	Preserved lobular structure without diffuse degenerative alterations, but with rare foci of mononuclear inflammatory cells.
II *(bft)*	Extensive and prominent ulcerations. Superficial erosion, congestion, and inflammatory infiltrate in the lamina propria.	Moderate edema and increase of the lamina propria cells.	A diffuse congestion with few mononuclear inflammatory cells.

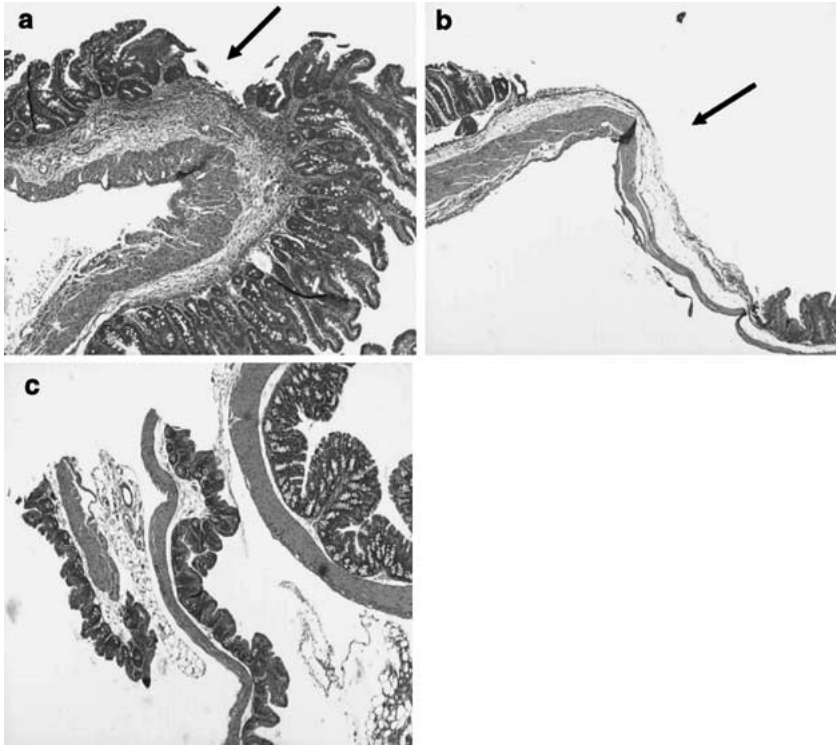

Fig. 2. Panoramic histopathologic aspects of the ileum-ceco colico transition region from gnotobiotic mice infected with *Bacteroides fragilis* subtype strains. (**a**) *bft-1*, distal ileum erosions affecting the superficial epithelium and submucosal inflammatory process; (**b**) *bft-2*, cecum with extensive ulceration; (**c**) non-ETBF; cecum and part of the proximal colon. Arrows: erosions and ulceration area. Hematoxylin-eosin. Original magnification × 64.

reached similar high intestinal levels of about 10^{10} CFU/g of feces (Fig. 1) within one day. Diarrheic episodes were observed in group II at the beginning of the monoassociation. Histopathological alterations were observed in all the mice groups inoculated with strains harboring different gene subtypes (Table 1) and they were mostly seen in the distal ileum-cecum-colon transition area documented in Figure 2.

The cecum and colon walls showed moderate edema, and an increase of the lamina propria cells was noticed, mainly in erosion areas of the cecal mucosa. These erosion areas were few, extensive, and characterized by local loss of the superficial epithelial layers, with intense reactivity of basal layers, characterized by loss of glandular architectural organization, exocytose, and nuclear hypercromasia of the glandular basal cells (Fig. 2a).

Moreover, in the cecum of at least two animals of group II, superficial erosion and inflammatory infiltrate in the lamina propria were observed, but they were more extensive and prominent (Fig. 2b).

In group III, inoculated with a non-ETBF strain, neither lesions nor alterations were observed (Fig. 2c). Cecum length and intestinal wall thickness were typical

in germ-free mice, despite the bacterial monoassociation. Although Figure 2 shows the ileum-cecum-colonic transition, the proximal colon was affected as well in a similar pattern.

All the strains showed the initial genetic profiles (*bft* gene subtypes) after recuperation at the end of the experiments (data not shown).

Discussion

Metalloprotease toxins may act as virulence factors in microorganisms. In some instances, they directly damage the tissue during the infection or inactivate endogenous factors that normally are involved in the host response regulation to infections. *B. fragilis* typically resides in the colon and it strengthens the potential role of this metalloprotease as a virulence factor in colonic disease [18].

The BFT toxin stimulates morphological changes in intestinal epithelial cells lines, such as HT29, HT29/C1, Caco-2, T84, MDCK, and HCT-8, which are capable of forming tight junctions but not on epithelial cell lines, such as HEp-2 and HeLa, lacking this property. Moreover, this toxin causes cell rounding and dissolution of tight clusters, characteristically related to F-actin redistribution [3, 7, 12, 24].

The biological activity of each of the three BFT alleles has been characterized and the toxin produced by *bft*-2 subtype (BFT-2) exhibits a higher biology activity than BFT-1, whereas BFT-3 may be less active than either BFT-1 or BFT-2 [23]. However, the in vivo activity of the three BFT alleles has not been evaluated. Rielgler et al. [22] demonstrated that BFT-2 produced electrophysiological alterations, morphologic damage, and increased epithelial permeability to mannitol in human colonic mucosa.

Biologically active BFT has been detected in different amounts in feces of animals and humans, but its role in diarrhea disease has not been determined [19, 20]. Histological changes including rounding and exfoliation of the intestinal epithelial cells as well as submucosal inflammation were observed in vivo after the colonization by ETBF strains. These histological alterations are related to secretion in ileal and colonic ligated intestinal segment in rats, rabbits, and lambs [18]. Myers et al. [16] observed that BFT toxin produces a light intestinal inflammation in lambs with epithelial cell exfoliation and crypt hyperplasia in cecum and colon, and with few heterophils in lamina propria.

The intestinal lesions described here were similar to those described in gnotobiotic and conventional piglets colonized with enterotoxigenic *B. fragilis*, [5, 8]. We clearly showed a correlation between qualitative structural damage of the intestine wall and colonization with the two studied subtypes, since the group infected with the non-ETBF strain was not affected. These induced morphological changes in the intestine wall may explain the physiopathology of the signals and symptoms associated with the previously described effects of the enterotoxin.

Additionally, our results suggest that strains carrying different *bft* gene subtypes may have a different pathogenic potential, and it indicates the need for more studies to evaluate the pathogenesis of each subtype in different animal hosts. Certainly, it could provide a better understanding of these organisms in ecological and pathogenic terms.

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