EFFECT OF PROBIOTICS ON VISCERAL PAIN THRESHOLD
IN AN EXPERIMENTAL MODEL OF IRRITABLE BOWEL SYNDROME

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ABSTRACT: Irritable bowel syndrome (IBS) is a chronic functional disorder characterized by abdominal pain and abnormal bowel function. Probiotics are used in clinical practice to treat bowel movement disturbances in IBS patients. However, it is not clear if probiotics are useful for treatment of the abdominal pain associated with visceral hypersensitivity. The aim of this study was to investigate the effect and mechanism of action of a probiotic bacterium, Streptococcus faecalis 129 BIO 3B (SF3B) on visceral pain threshold using a 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced rat visceral hypersensitivity model. In TNBS-treated rats, a decrease in the visceral pain threshold of the distal colon was observed, and improvement in the threshold reduction was observed by repeated oral administration of SF3B. SF3B dose-dependently suppressed the degranulation of rat peritoneal mast cells induced by compound 48/80. These results suggest that oral administration of SF3B may prevent the abdominal pain associated with colonic pain threshold by inhibiting degranulation of mast cells in the distal colon of IBS patients.

KEYWORDS: Irritable bowel syndrome, Mast cell, Probiotics, SF3B, Visceral hypersensitivity

INTRODUCTION

Irritable bowel syndrome (IBS) is one of the most common functional disorders. It is characterized by chronically abnormal bowel habits and, in most cases, accompanied by abdominal pain (Drossman et al., 2006), affecting an increasing number of people worldwide (Sperber et al., 2016).

The pathophysiology of IBS is complex and multifactorial. It has been suggested that the pathophysiological features of IBS are alterations in gut motility (Horikawa et al., 1999), small-bowel bacterial overgrowth (Posserud et al., 2007), microscopic inflammation (Chadwick et al., 2002), and visceral hypersensitivity (Mertz et al., 1995). In addition, studies of colonoscopic biopsy specimens have demonstrated that the number of mast cells is increased in the colonic mucosal area and localized in closer proximity to nerve fibers, and there is an increased spontaneous release of mast cell-specific mediators such as histamine and tryptase in IBS patients compared to healthy subjects (Barbara et al., 2007). Thus, it has been suggested that colonic mast cell infiltration and mediator release proximal to mucosal innervation correlate with abdominal pain in IBS patients.

Probiotics are defined as “live microorganism which, when administered in adequate amounts, confer a health benefit on the host” (FAO/WHO Working Group., 2002) and are used to treat bowel movement disturbances in IBS patients in the clinical practice (Hosseini et al., 2012). However, the action mechanism of probiotic treatment of IBS remains unclear.

A probiotic bacterium, Streptococcus faecalis 129 BIO 3B (SF3B: strain classified currently as Enterococcus faecium), contained in BIOFERMIN® POWDER, has been used as an intestinal remedy for a long time to ameliorate abnormal bowel movement by controlling the balance of intestinal flora in Japanese clinical practice (Katsumata., 1954a; Katsumata., 1954b). However, it is not clear if SF3B has a beneficial effect on the abdominal pain associated with visceral hypersensitivity in IBS patients.
Recently, various animal models have been used to evaluate the efficacy of probiotics in IBS. In particular, a rat model in which 2,4,6-trinitrobenzene sulfonic acid (TNBS) is injected into the proximal colon has been used as a valuable tool for evaluating visceral hypersensitivity associated with IBS, and a decrease in the sensory threshold of the distal colon has been reported (Diop et al., 2002). Therefore, we investigated the effect and mechanism of action of SF3B on colonic pain threshold using a TNBS-induced rat visceral hypersensitivity model.

MATERIALS AND METHODS

Preparation of bacterial cells

SF3B was obtained from the Culture Collection of Biofermin Pharmaceutical Co., Ltd and cultured at 37˚C for 18 hr in GAM Broth (Nissui Pharmaceutical. Co., Ltd., Tokyo, Japan) supplemented with 0.7% glucose and 0.1% Tween 80. The bacterial cells were recovered by centrifugation for 15 min at 2,600 ×g.

Animals

Six-week-old male Sprague-Dawley (SD) rats and 7-week-old male Wistar rats (Charles River Laboratories Japan, Inc., Yokohama, Japan) were maintained at a room temperature of 22 ± 3˚C, a humidity of 55 ± 10%, and a 12-hr light/dark cycle (7:00-19:00) with ad libitum access to normal laboratory diet and water. All rats acclimated for 1 week before they were used for studies. All animal experiments were approved by the Experimental Animal Care and Use Committee of Biofermin Pharmaceutical Co., Ltd.

TNBS-induced hypersensitivity

After 16–18 hr of fasting, SD rats were anesthetized by intraperitoneal administration of somnopentyl and an abdominal laparotomy was made for injection of TNBS (50 mg/0.5 mL/kg) dissolved in 30% ethanol into the proximal colon (1 cm distal from the cecum). The sham-operated rats were received the same surgical procedure. Measurement of visceral pain threshold was carried out 7 days after the surgery as previously described (Ohashi et al., 2008a). Briefly, a latex balloon (5 cm in length, Okamoto Industries, Inc., Tokyo, Japan) was inserted through the anus and placed in the distal colon 5 cm from the anus. After 30 min of acclimation, the balloon was progressively inflated from 0 to 70 mmHg, in 5 mmHg increments every 30 sec, using an electronic barostat (Distender Series IIR, G&J Electronics, Ontario, Canada). The pain threshold was defined as the pressure inducing characteristic painful behaviors as previously described (Wesselmann et al., 1998).

Experimental protocol

SD rats were divided into four groups of eight animals each. A viable bacterial count of ten million or a billion SF3B suspended in 600 μL of PBS was orally administered to rats once a day from day −14 to day 7. TNBS (50 mg/kg) was injected into the proximal colon at day 0. The visceral pain threshold was measured using an electronic barostat at day 7.

Histological analysis

Seven days after TNBS treated, a segment was taken from the distal colon (5 to 10 cm from the anus), sectioned transversely in its entirety, and fixed overnight in 4% paraformaldehyde phosphate buffer solution. The fixed tissues were processed in
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paraffin, cut into 5-mm sections, stained with hematoxylin-eosin, and examined by light microscopy.

Mast cell degranulation analysis

The peritoneal mast cells were prepared from non-TNBS and non-SF3B treated naive rats as previously described (Sullivan et al., 1975). Briefly, Wistar rats were anesthetized with isoflurane, exsanguinated by decapitation, and 20 mL of mast cell medium (MCM; 150 mM NaCl, 3.7 mM KCl, 5.6 mM glucose, 3 mM Na₂HPO₄, 3.5 mM KH₂PO₄, 0.1% BSA, 0.1% gelatin adjusted pH 6.0 with NaOH) with 10 U/mL heparin was intraperitoneally administered. After rubbing the abdominal area for 2 min, the abdominal cavity was exposed. The harvested mast cell suspension was centrifuged for 7 min at 50 ×g and the supernatant was decanted. The cell pellet was suspended in MCM and washed by centrifugation for 7 min at 50 ×g. The cell pellet was resuspended in MCM at 10⁵ cells/mL.

Two milliliters of the mast cell suspension with 20 μL of 90 mM CaCl₂ were incubated at 37˚C for 5 min. Subsequently, the cell suspensions were incubated with 100 μL of SF3B at concentrations of 10⁷, 10⁶, 10⁵, and 10⁴ cfu/mL for 10 min and induced by 20 μl of compound 48/80 at 37˚C for 10 min. After cooling, the supernatant solutions were obtained by centrifugation for 5 min at 1700 ×g.

A histamine assay was performed using fluorescence as previously described (May et al., 1970). Standards were prepared by diluting histamine in MCM. Total histamine was determined in mast cell suspension boiled with 1% perchloric acid for 8 min. Fluorescence was measured with excitation at 360 nm and emission at 450 nm.

Statistical analysis

Results are presented as mean ± standard error of mean (SEM). Pain threshold was statistically analyzed using the Mann-Whitney U-test. Histamine release was statistically analyzed in comparison with the control using the Dunnett’s test. Probability values of p < 0.05 were considered statistically significant.

RESULTS

Effect of SF3B on TNBS-induced hypersensitivity

We investigated the effect of oral administration of SF3B on TNBS-induced colonic pain threshold in the distal colon. In the control group (32.8 ± 2.8 mmHg), the pain threshold was significantly decreased in comparison with sham group (45.9 ± 3.2 mmHg), while rats receiving oral administrations of SF3B at doses of 10⁷ (40.6 ± 1.5 mmHg) and 10⁹ cfu/day (42.5 ± 0.9 mmHg) had significantly increased pain threshold in comparison with the control group (Fig.2).

Histological changes

We assessed the histological appearance of the distal colon where the distention procedure was applied. No tissue damage was observed in the control or SF3B groups in the distal colon by staining with hematoxylin-eosin (Fig.3).

FIGURE 2. Effect of SF3B on visceral hypersensitivity in the distal colon. SF3B was orally administered once daily at a viable bacterial count of 1×10⁷ or 1×10⁹ for 22 days. TNBS was injected into the proximal colon after administration of SF3B for 14 days. Pain threshold was measured 7 days after TNBS injection. Data are presented as mean ± SEM (n = 8). #: p < 0.01 compared with sham. †: p < 0.05, ††: p < 0.01 compared with control.
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In this experimental model, the visceral hypersensitivity results from the degranulation of mucosal mast cells (Ohashi et al., 2007). Therefore, we investigated the effect of SF3B on the compound 48/80-induced histamine release from rat peritoneal mast cells. SF3B at concentrations of $10^5$–$10^8$ cfu/mL inhibited histamine release in a concentration-dependent manner.

**FIGURE 3. Hematoxylin-eosin stain of the distal colon tissues at day 7.** SF3B was orally administered once daily at a viable bacterial count of $1 \times 10^9$ for 22 days. TNBS was injected into the proximal colon after administration of SF3B for 14 days. Rats were sacrificed 7 days after TNBS injection. A segment was taken from the distal colon (5 to 10 cm from the anus), and hematoxylin-eosin stains were performed. (A) Control group. (B) SF3B (administration at $1 \times 10^9$ cfu/day) group. Imaged at 20x (left panels) and 100x (right panels). The bar indicates 100 μm.

**FIGURE 4. Effect of SF3B on histamine release from rat peritoneal mast cells.** Mast cells were incubated with SF3B at concentrations of $10^5$, $10^6$, $10^7$, and $10^8$ cfu/mL or without SF3B (control) for 10 min. The degranulation of mast cells was induced by 0.1 μg/mL of compound 48/80 for 10 min. The degranulation was determined by measuring the release of histamine.

Histamine release (%) = \((B)-(S)/(T)-(S)\)×100 where,
(T) = total histamine content measured after mast cells were boiled with 1% perchloric acid for 8 min. (B) = histamine content in the supernatant measured after mast cells were incubated with SF3B at each dose for 10 min and subsequently induced by 0.1 μg/mL of compound 48/80 for 10 min. (S) = histamine content in the supernatant measured after mast cells were incubated without SF3B or compound 48/80. All data are presented as mean ± SEM (n=3). ###: $p < 0.001$ vs. control.
Compounds 48/80-induced histamine release was completely inhibited by SF3B at a concentration of 10^6 cfu/mL (Fig.4).

**DISCUSSION**

IBS is characterized by chronic abdominal discomfort or pain associated with bowel habit changes such as diarrhea and constipation. Currently, IBS patients are treated with symptomatic therapies such as stress relief or amelioration of abnormal bowel movements (Bijkerk et al., 2003; Talley, 2003). Chronic abdominal pain is an important factor of IBS that reduces the quality of life for IBS patients (Wilson et al., 2004). However, there is no established effective therapy for the treatment of chronic abdominal pain associated with IBS (Talley, 2003).

Recently, many clinical trials investigating the therapeutic benefits of probiotics in IBS have been reported (Clarke et al., 2012). The putative pathophysiological mechanisms of probiotics include improvement of intestinal microbiota (Kajander et al., 2008), intestinal permeability (Francavilla et al., 2010), gastrointestinal dysmotility (Agrawal et al., 2009), and modulation of immune functions (O’Mahony et al., 2005). However, it is not clear how probiotics improve symptoms of IBS.

In this study, we investigated the effect of probiotic bacterium SF3B on colonic pain threshold using an experimental rat model of visceral hypersensitivity induced by TNBS. We found that SF3B suppresses the increase in pain threshold induced by TNBS. In the experimental model used in this study, an injection of TNBS into the proximal colon of rats resulted in a sustained decrease in the sensory threshold of the distal colon, however, no histological tissue damage, such as inflammation, was observed. Since the TNBS-induced increase in colonic sensitivity lasted for 21 days (Diop et al., 2002), it is considered a valuable experimental tool to elucidate the pathophysiology of chronic functional disorders characterized by visceral hypersensitivity, including IBS. Additionally, visceral pain threshold was measured by balloon distension of the distal colon. This tool is a method to quantitatively measure the degree of pain with high reproducibility. Therefore, it has been used to evaluate the visceral hypersensitivity in humans and animals (Mayer and Gebhart, 1994; Ness and Gebhart, 1990).

Studies suggest that oral administration of probiotics are effective against visceral hypersensitivity in various murine models. Oral administration of *Lactobacillus acidophilus* NCFM strain improved visceral perception by inducing the expression of μ-opioid and cannabinoid receptors on intestinal epithelial cells in a rat model of chronic colonic hypersensitivity elicited by butyrate enemas (Rousseaux et al., 2007). *Lactobacillus paracasei* NCC2461 strain attenuated visceral hypersensitivity and inflammatory activity in mice with disrupted gut flora due to use of antibiotics. Changes in gut flora may be a reason for the variability of IBS symptoms and could potentially be prevented by administration of probiotics (Verdu et al., 2006). The administration of multispecies probiotics prevented the epithelial disruption induced by intracolonic infusion of fecal supernatants from IBS patients in *vitro* and prevented visceral hypersensitivity in a water avoidance stress mouse model (Nebot-Vivinus et al., 2014). Early life administration of probiotics VSL#3 protected against the development of visceral hypersensitivity in a rat model of neonatal maternal separation by resetting colonic expression of subsets of genes mediating pain and inflammation (Distrutti et al., 2013). Fermented milk containing *Bifidobacterium lactis* CNCM I-2494 strain reduced visceral hypersensitivity by normalizing colonic occluding and Jam-A expressions, as well as blood endotoxin levels in a partial restraint stress rat model (Agostini et al., 2012).

As mentioned above, it has been suggested that probiotics exert an inhibitory effect on the visceral hypersensitivity associated with IBS. In this study, it was suggested that SF3B suppresses visceral hypersensitivity similarly to these reports. In this experimental model, it was demonstrated that the visceral hypersensitivity induced by TNBS is associated with an increase in the number and degranulation rate of colonic mucosal mast cells, which is suppressed by pretreatment with mast cell stabilizer doxantrazole (Ohashi et al., 2007). Additionally, TNBS-induced colonic pain threshold did not develop in mast cell-deficient (W/Ws) rats (Ohashi et al., 2008b). In another rat model, IBS symptoms developed after a subsidence of acetic acid-induced colitis, although the number of mucosal mast cells was unchanged in the colon, and the increase of degranulation of mucosal mast cells was shown to be responsible for visceral hypersensitivity (La et al., 2004). These results suggest that the degranulation of mucosal mast cells is an important factor in the pathogenesis of visceral hypersensitivity in these IBS models.

In this study, SF3B suppressed the degranulation of rat peritoneal mast cells in a dose-dependent manner. Although the difference in the response of mucosal mast cells and peritoneal mast cells to SF3B is unknown, it has possibility that SF3B suppresses the degranulation of mucosal mast cells. This hypothetical mechanism is supported because doxantrazole which suppresses the degranulation of not only colonic mucosal mast cells but also peritoneal mast cells (Scott et al., 1993) suppresses a decrease of visceral pain threshold of this model. In further research, we need to examine the effect of SF3B on the degranulation of mucosal mast cells in the distal colon using this model.

**CONCLUSION**

Our results suggest that probiotic strain SF3B suppresses the visceral hypersensitivity associated with IBS. Although further research is needed to confirm the mechanism of action, SF3B therapy may be an efficacious option for treating abdominal pain associated with visceral hypersensitivity in IBS patients.

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