Full Paper

Milk Whey Culture With *Propionibacterium freudenreichii* ET-3 Is Effective on the Colitis Induced by 2,4,6-Trinitrobenzene Sulfonic Acid in RatsMasayuki Uchida^{1,*} and Orie Mogami¹¹Food Science Institute, Division of Research and Development, Meiji Dairies Corporation,
540 Naruda, Odawara, Kanagawa 250-0862, Japan

Received July 22, 2005; Accepted October 5, 2005

Abstract. This study aimed to evaluate whether milk whey culture with *Propionibacterium freudenreichii* ET-3 (milk whey culture), which has been reported to have Bifidogenic activity, is effective on the colitis induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS) in rats. For the induction of colitis, the colon was clamped and 0.1 M TNBS in 35% ethanol was injected into the luminal side of the clamped portion under pentobarbital anesthesia. From the next day of colitis induction, milk whey culture was administered orally at doses of 1 and 3 g/kg, twice a day for 9 days. On the 10th day, rats were sacrificed and ulcer size was measured. Milk whey culture significantly accelerated the healing of the colitis in a dose-dependent manner, but culture medium did not. To clarify the active substance, the effects of propionic acid and acetic acid contained in milk whey culture was tested. Sodium propionate significantly accelerated the healing of TNBS-induced colitis, but sodium acetate did not. The above results show that milk whey culture may become a useful prebiotic for the therapy of inflammatory bowel disease and that propionic acid may be one of the active substances contained in milk whey culture.

Keywords: inflammatory bowel disease, *Propionibacterium freudenreichii* ET-3, 2,4,6-trinitrobenzene sulfonic acid, short chain fatty acid, colitis in rat

Introduction

There is increasing evidence that the intestinal microflora plays an important role in the pathogenesis of inflammatory bowel disease, although the pathogenesis of inflammatory bowel disease has not been clarified as yet. Indeed, mice with acute dextran sulfate sodium colitis treated with antibiotics have shown a significant reduction in histological parameters and myeloperoxidase activity (1).

In 1974, Parker (2) defined probiotics as “organism and substances, which contribute to intestinal microbial balance”. However, in 1989, Fuller (3) refined probiotic as “a live microbial food supplement which beneficially affects the host animal by improving microbial balance” (4). Osman et al. reported that administration of certain strains of *Bifidobacterium* significantly improves the

disease activity index and reduces bacterial translocation in the dextran sulfate sodium-induced acute colitis model of rats, and *Bifidobacterium sp.* 3B1 and *Bifidobacterium infantis* DSM 15158 seem to have the best effect (5). Tursi et al. (6) also reported that low-dose balsalazide plus VSL#3, which is a probiotic cocktail, is more effective than balsalazide alone or mesalazine in the treatment of active mild-to-moderate ulcerative colitis in clinical study.

On the other hand, prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon (4). Germinated barley foodstuff (GBF) composed mainly of dietary fiber and glutamine-rich protein, which is a prebiotic, has been reported to be effective on the colitis in human and animals (7–10). Cherbut et al. (11) also reported that fructooligosaccharides reduced intestinal inflammatory activity mainly by increasing *Lactobacillus* counts in the intes-

*Corresponding author. FAX: +81-465-36-2776
E-mail: MASAYUKI_UCHIDA@meiji-milk.com

tine in the rat 2,4,6-trinitrobenzene sulfonic acid (TNBS) colitis model. These findings mentioned above show that the improvement of bacterial flora in the colon is one of the therapeutic strategies of inflammatory bowel disease.

Milk whey culture with *Propionibacterium freudenreichii* ET-3 (milk whey culture) is a prebiotic reported to enhance the growth of *Bifidobacterium* (12, 13) and to be effective on constipation by improving the intestinal flora. Moreover, Ouwehand et al. (14) also reported the efficacy of juice supplemented with *Propionibacterium freudenreichii* on constipation, although *Lactobacillus rhamnosus* was also used as a combined supplement with *Propionibacterium freudenreichii*.

Thus, this study aimed to evaluate whether milk whey culture is effective on the colitis induced by TNBS in rats. In addition, the effects of propionic acid and acetic acid was investigated on TNBS-induced colitis from the finding that milk whey culture contained propionic acid and acetic acid.

Materials and Methods

All animal experiments were performed according to the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society.

Animals and colitis induction

Male Sprague-Dawley rats weighing from 200 to 250 g were purchased from SLC (Shizuoka) and housed for 1 week prior to the commencement of the experiments under a constant temperature of $21 \pm 2^\circ\text{C}$, humidity of $55 \pm 15\%$, and a 12-h light/dark cycle.

Colitis was induced by the method reported recently by the present authors (15). In brief, the rats were anesthetized with an intraperitoneal administration of sodium pentobarbital (Nembutal; Dinabot, Tokyo) at a dose of 35 mg/kg. Following lower abdominal laparotomy, the colon was exposed. The middle part of the colon was pinched with ring forceps (inside diameter: 8 mm), and 0.2 ml of 35% ethanol solution containing a final concentration of 0.1 M TNBS (Fluka, Tokyo) was injected into the luminal site of the clamped portion of the colon. After 2 min, the colon was returned to the abdominal cavity and the incision was sutured.

Effect of milk whey culture on the colitis

Milk whey culture was administered orally at doses of 1 and 3 g/kg (10 ml/kg) twice a day (10:00 AM and 4:00 PM) from the next day of colitis induction for 9 days. On the 10th day, following cervical dislocation, the colon was excised from all animals and cut along the

mesentery to allow for ulcer evaluation. The size of the ulcer was measured, and the ulcer index was calculated from the length and width measurements (mm^2).

In the control rats, distilled water (10 ml/kg) was administered instead of milk whey culture.

Histological observation of the colitis

Following macroscopic observations, the colonic specimens were fixed in 10% neutral buffered formalin, and histological examinations were performed. Tissue sections were prepared and stained with hematoxylin and eosin, and histological observations were performed under a light microscope.

Effect of culture medium on the colitis

To clarify the effect of milk whey culture on the colitis from that of the culture medium, the effect of culture medium was investigated in comparison with milk whey culture. Milk whey culture was administered orally at a dose of 1 g/kg (10 ml/kg) as a reference compound. Culture medium was administered orally in the same manner instead of milk whey culture at a dose of 10 ml/kg. Milk whey culture and culture medium were administered orally twice a day from the next day of colitis induction for 9 days. The size of the ulcer was measured on the 10th day.

In the control rats, distilled water (10 ml/kg) was administered instead of milk whey culture or culture medium.

Effects of propionic acid and acetic acid on the colitis

From the chemical analysis data that milk whey culture contains 0.1 M propionic acid and 0.1 M acetic acid at a concentration of 1 g/10 ml, the effects of these short chain fatty acids were investigated in the same manner instead of milk whey culture. Sodium propionate (0.1 M) or sodium acetate (0.1 M) was administered orally at a dose of 10 ml/kg twice a day from the day after colitis induction for 9 days. The size of the ulcer was measured on the 10th day.

In the control rats, distilled water (10 ml/kg) was administered instead of 0.1 M sodium propionate or 0.1 M sodium acetate.

Agents

TNBS was purchased from Fluka. Milk whey culture was produced by Meiji Dairies Corporation (Tokyo). Sodium propionic acid and sodium acetic acid were purchased from Sigma (St. Louis, MO, USA).

Statistics

The results were expressed as the mean $\pm$ S.E.M. Statistical analysis was performed by Dunnett's multiple

comparison test. $P < 0.05$ was treated as statistical significance.

Results

Effect of milk whey culture on the colitis

In the control rats, the ulcer index was $64.3 \pm 11.5 \text{ mm}^2$. Milk whey culture dose-dependently and significantly accelerated the healing of the colitis as shown in

Fig. 1. Effect of milk whey culture on the colitis induced by the injection of 0.2 ml of 35% ethanol solution containing a final concentration of 0.1 M TNBS at the middle position of the colon in rats. Values represent the mean $\pm$ S.E.M. (N = 5 or 6). *, **Significant difference from the control group ($P < 0.05$, 0.01).

Fig. 2. Macroscopic findings of colitis in rats 10 days after ulcer induction with 0.2 ml of 35% ethanol solution containing a final concentration of 0.1 M TNBS at the middle position of the colon. Control rat (A): Colitis with a marked margin different from the normal mucosa and with round lesions of the colon was observed. Milk whey culture-treated rat (1 g/kg) (B): The ulcer area was smaller than that in a control rat. Milk whey culture-treated rat (3 g/kg) (C): The ulcer area was smaller than that in a milk whey culture-treated rat (1 g/kg). The blood vessels were clearly observed through the mucosa in the milk whey culture-treated rats (B and C), but in a control rat, blood vessels were not clearly observed, suggesting edematous swelling (A).

Fig. 1. The values of ulcer index at 1 and 3 g/kg were 24.2 ± 7.8 and 10.4 ± 4.3 , respectively.

Macroscopic findings of the colitis

Macroscopic findings of colitis 10 days after colitis induction are shown in Fig. 2. In a control rat, colitis was observed with a marked margin different from the normal mucosa and with round lesions of the colon. The normal mucosa showed edematous swelling, and the blood vessels were not observed clearly through the mucosa. In the milk whey culture-treated rats, the ulcer area became smaller than that in a control rat in a dose-dependent manner, and the blood vessels were observed through the mucosa, suggesting the decrease in edematous swelling.

Fig. 3. Microscopic findings of colitis in rats 10 days after ulcer induction with 0.2 ml of 35% ethanol solution containing a final concentration of 0.1 M TNBS at the middle position of the colon. Hematoxylin and eosin staining, magnification $\times 100$. Control rat (A): The regenerated epithelial cells were seen on the ulcer base, but the regenerated epithelium is scar tissue and did not cover the ulcer base firmly (arrowhead). Milk whey culture-treated rat (3 g/kg) (B): Many regenerated epitheliums were seen around the ulcer base (arrowhead), and the regenerated epithelium firmly covered the ulcer base, suggesting the healing of the colitis (arrowhead).

Histological observation of the colitis

Histological observations characterized the lesions by the presence of infiltration of inflammatory cells (Fig. 3). In a control rat, epithelial regeneration was scarcely observed around the ulcer. On the contrary, in a rat treated with milk whey culture at a dose of 3 g/kg, many regenerated epitheliums are observed around the ulcerated area. The regenerated epithelium firmly covered the ulcer base as shown in Fig. 3.

Fig. 4. Effect of milk whey culture and culture medium on the colitis induced by the injection of 0.2 ml of 35% ethanol solution containing a final concentration of 0.1 M TNBS at the middle position of the colon in rats. Milk whey culture at a dose of 1 g/kg (10 ml/kg) and culture medium at a dose of 10 ml/kg were administered orally twice a day from the day after colitis induction for 9 days. The size of the ulcer was measured on the 10th day. Values represent the mean $\pm$ S.E.M. (N = 5 or 6). *Significant difference from the control group ($P < 0.05$).

Fig. 5. Effect of sodium propionate and sodium acetate on the colitis induced by the injection of 0.2 ml of 35% ethanol solution containing a final concentration of 0.1 M TNBS at the middle position of the colon in rats. Sodium propionate at a dose of 10 ml/kg (0.1 M solution) and sodium acetate at a dose of 10 ml/kg (0.1 M solution) were administered orally twice a day from the day after colitis induction for 9 days. The size of the ulcer was measured on the 10th day. Values represent the mean $\pm$ S.E.M. (N = 5 or 6). **Significant difference from the control group ($P < 0.01$).

Effect of culture medium on the colitis

In the control rats, the ulcer index was 49.7 ± 9.7 mm² (Fig. 4). Culture medium did not accelerate the healing of the colitis as shown in Fig. 4. On the contrary, milk whey culture significantly accelerated the healing of the colitis and its value of ulcer index was 20.9 ± 3.3 mm² (Fig. 4).

Effects of sodium propionate and sodium acetate on the colitis

In the control rats, the ulcer index was 53.8 ± 6.1 mm² (Fig. 5). Sodium acetate did not significantly accelerate the healing of the colitis at a dose used in this study, and the ulcer index was 41.8 ± 5.9 mm² (Fig. 5). On the contrary, sodium propionate significantly accelerated the healing of the colitis ($P < 0.01$), and the ulcer index was 27.8 ± 3.0 mm² (Fig. 5).

Discussion

Inflammatory bowel disease is divided into two diseases, ulcerative colitis and Chron's disease. However, the pathogenesis of inflammatory bowel disease has not been clarified as yet. In the clinic, 5-aminosalicylic acid, azathioprine, methotrexate, and steroid have been used for the therapy.

Recently, there is increasing evidence that the intestinal microflora plays an important role in the pathogenesis of inflammatory bowel disease. Some probiotics and prebiotics have been reported to be effective on inflammatory bowel disease. Milk whey culture is a prebiotic reported to enhance the growth of *Bifidobacterium* (12, 13) and to be effective on constipation by improving the intestinal flora. Thus in this study, we aimed to evaluate if milk whey culture is effective on TNBS-induced colitis in rats.

The treatment with milk whey culture significantly accelerated the healing of the colitis in a dose-dependent manner, but culture medium had no effect on the healing. These findings suggest that the fermented material by *Propionibacterium freudenreichii* ET-3 has activity to accelerate healing of the colitis. In the macroscopical findings, the blood vessels were clearly observed though the mucosa in the milk whey culture-treated rats, but in a control rat, blood vessels were not clearly observed, suggesting edematous swelling. Moreover, by the histological observation in a milk whey culture-treated rat, many regenerated epitheliums were observed around the ulcerated area as compared with those of a control rat. These findings show that milk whey culture enhances the regeneration of the epithelium and inhibits the inflammation of the mucosa around the ulcer. However, proliferating cell nuclear antigen

staining was not different between control rats and milk whey culture-treated rats on Day 10 (data not shown). On this point, further studies would be needed.

A short chain fatty acid, such as butyric acid, propionic acid, and acetic acid, provides energy to colonocytes (16) and stimulates epithelial cell proliferation of the gut in vivo (17–19). Shimotoyodome et al. (20) reported that short chain fatty acids but not lactate or succinate stimulate mucus release in the rat colon. Mucus plays the important role of colonic protection against luminal pathogens and toxins. Indeed, short chain fatty acids enemas are expected to be a therapeutic alternative in patients with diversion colitis or distal ulcerative colitis either by providing an energy source for colonocytes or by stimulating the renewal of injured epithelium (18, 21). However, Tarrerias et al. (22) reported that the beneficial effect of short chain fatty acid (especially butyrate) enemas on hypersensitivity and inflammation in inflammatory bowel disease is questionable in the TNBS-induced colitis model.

From the chemical analysis of milk whey culture, it was found that milk whey culture contains propionic acid and acetic acid, but does not contain butyric acid. *Propionibacteria* has been known to generate short chain fatty acids, such as propionic acid and acetic acid. Although butyric acid has been known to be effective on colitis (23), the efficacy of propionic acid or acetic acid on colitis has not been evaluated as far as we know. Thus to clarify the active substance for accelerating the healing of colitis, we tested the effects of propionic acid and acetic acid which are contained in milk whey culture. In the TNBS-induced colitis model of this study, propionic acid enhanced the healing of TNBS-induced colitis, but acetic acid did not at the dose used in this study. In human colonic mucosa in vitro, Sheppach et al. (19) found that short chain fatty acids, especially butyrate and propionate, are luminal trophic factors for the cecal epithelium and that the effect of acetate on the proliferation was minor. Recently, Ichikawa et al. (24) reported that the oral instillation of short-chain fatty acids stimulated gut epithelial cell proliferation in rats fed an elemental diet. These findings support our results that propionic acid contained in milk whey culture accelerated healing in the TNBS-induced colitis model.

In conclusion, it was found that milk whey culture may be a useful prebiotic for the therapy of inflammatory bowel disease and that propionic acid may be one of the active substances contained in milk whey culture.

References

- 1 Hans W, Scholmerich J, Gross V, Falk W. The role of the resident intestinal flora in acute and chronic dextran sulfate sodium-induced colitis in mice. *Eur J Gastroenterol Hepatol*. 2000;12:267–273.
- 2 Parker RB. Probiotics, the other half of the antibiotic story. *Animal Nutr Health*. 1974;29:4–8.
- 3 Fuller R. Prebiotics in man and animals. *J Apply Bacteriol*. 1989;66:365–378.
- 4 Gibson GR, Roberfroid MB. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J Nutr*. 1995;222:1401–1412.
- 5 Osman N, Adawi D, Ahrne S, Jeppsson B, Molin G. Modulation of the effect of dextran sulfate sodium-induced acute colitis by the administration of different probiotic strains of *Lactobacillus* and *Bifidobacterium*. *Dig Dis Sci*. 2004;49:320–327.
- 6 Tursi A, Brandimarte G, Giorgetti GM, Forti G, Modeo ME, Gigliobianco A. Low-dose balsalazide plus a high-potency probiotic preparation is more effective than balsalazide alone or mesalazine in the treatment of acute mild-to-moderate ulcerative colitis. *Med Sci Monit*. 2004;10:PI126–PI131.
- 7 Kanauchi O, Iwanaga T, Andoh A, Araki Y, Nakamura T, Mitsuyama K, et al. Dietary fiber fraction of germinated barley foodstuff attenuated mucosal damage and diarrhea, and accelerated the repair of the colonic mucosa in an experimental colitis. *J Gastroenterol Hepatol*. 2001;16:160–168.
- 8 Kanauchi O, Iwanaga T, Mitsuyama K. Germinated barley foodstuff feeding. A novel nutraceutical therapeutic strategy for ulcerative colitis. *Digestion*. 2001;63 Suppl 1:60–67.
- 9 Hanai H, Kanauchi O, Mitsuyama K, Andoh A, Takeuchi K, Takayuki I, et al. Germinated barley foodstuff prolongs remission in patients with ulcerative colitis. *Int J Mol Med*. 2004;13:643–647.
- 10 Kanauchi O, Mitsuyama K, Homma T, Takahama K, Fujiyama Y, Andoh A, et al. Treatment of ulcerative colitis patients by long-term administration of germinated barley foodstuff: multicenter open trial. *Int J Mol Med*. 2003;12:701–704.
- 11 Cherbut C, Michel C, Lecannu G. The prebiotic characteristics of fructooligosaccharides are necessary for reduction of TNBS-induced colitis in rats. *J Nutr*. 2003;133:21–27.
- 12 Kaneko T, Mori H, Iwata M, Meguro S. Growth stimulator for bifidobacteria produced by *Propionibacterium freudenreichii* and several intestinal bacteria. *J Dairy Sci*. 1994;77:393–404.
- 13 Mori H, Sato Y, Taketomo N, Kamiyama T, Yoshiyama Y, Meguro S, et al. Isolation and structural identification of bifidogenic growth stimulator produced by *Propionibacterium freudenreichii*. *J Dairy Sci*. 1997;80:1959–1964.
- 14 Ouwehand AC, Lagstrom H, Suomalainen T, Salminen S. Effect of probiotics on constipation, fecal azoreductase activity and fecal mucin content in the elderly. *Ann Nutr Metab*. 2002;46:159–162.
- 15 Uchida M, Orie Mogami O. An improved and reliable method for the induction of colitis in rats using 2,4,6-trinitrobenzene sulfonic acid. *J Pharmacol Sci*. 2005;97:285–288.
- 16 Kripke SA, Fox AD, Berman JM, Settle RG, Rombeau JL. Stimulation of intestinal mucosal growth with intracolonic infusion of short-chain fatty acids. *J Parenter Enteral Nutr*. 1989;13:109–116.
- 17 Sakata T. Stimulatory effect of short-chain fatty acids on

- epithelial cell proliferation in the rat intestine: a possible explanation for trophic effects of fermentable fibre, gut microbes and luminal trophic factors. *Br J Nutr.* 1987;58:95–103.
- 18 Mortensen FV, Hesseov I, Birke H, Korsgaard N, Nielsen H. Microcirculatory and trophic effects of short chain fatty acids in the human rectum after Hartmann's procedure. *Br J Surg.* 1991;78:1208–1211.
- 19 Scheppach W, Bartram P, Richter A, Richter F, Liepold H, Dusel G, et al. Effect of short-chain fatty acids on the human colonic mucosa in vitro. *J Parenter Enteral Nutr.* 1992;16:43–48.
- 20 Shimotoyodome A, Meguro S, Hase T, Tokimitsu I, Sakata T. Short chain fatty acids but not lactate or succinate stimulate mucus release in the rat colon. *Comp Biochem Physiol A Mol Integr Physiol.* 2000;125:525–531.
- 21 Harig JM, Soergel KH, Komorowski RA, Wood CM. Treatment of diversion colitis with short-chain-fatty acid irrigation. *N Engl J Med.* 1989;320:23–28.
- 22 Tarrerias AL, Millecamps M, Alloui A, Beaughard C, Kemeny JL, Bourdu S, et al. Short-chain fatty acid enemas fail to decrease colonic hypersensitivity and inflammation in TNBS-induced colonic inflammation in rats. *Pain.* 2002;100:91–97.
- 23 Venkatraman A, Ramakrishna BS, Shaji RV, Kumar NS, Pulimood A, Patra S. Amelioration of dextran sulfate colitis by butyrate: role of heat shock protein 70 and NF-kappaB. *Am J Physiol Gastrointest Liver Physiol.* 2003;285:G177–G184.
- 24 Ichikawa H, Shineha R, Satomi S, Sakata T. Gastric or rectal instillation of short-chain fatty acids stimulates epithelial cell proliferation of small and large intestine in rats. *Dig Dis Sci.* 2002;47:1141–1146.