ORIGINAL

Role of unbalanced growth of Gram-negative bacteria in ileal ulcer formation in rats treated with a nonsteroidal anti-inflammatory drug

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Abstract : Nonsteroidal anti-inflammatory drugs (NSAIDs) induced formation of intestinal ulcers as side effects, in which an unbalanced increase in the number of Gram-negative bacteria in the small intestine plays an important role. To clarify how intestinal microflora are influenced by NSAIDs, we examined the effects of 5-bromo-2-(4-fluorophenyl)-3-(4-methylsulfonylphenyl) thiophene (BFMeT), an NSAID, on intestinal motility and on the growth of *Escherichia coli* and *Lactobacillus acidophilus*. Transit index, a marker of peristalsis, was not different in BFMeT-treated and solvent-treated rats, indicating that BFMeT increased the number of Gram-negative bacteria without suppression of peristalsis. The factors that affect the growth of intestinal bacteria were not found in intestinal contents of BFMeT-treated rats, because the growth of *E. coli* and that of *L. acidophilus* in the supernatants of small intestinal contents of BFMeT-treated rats and solvent-treated rats were not different. The mechanism of the increase in the number of Gram-negative bacteria is still unclear, but heat-killed *E. coli* cells and their purified lipopolysaccharide (LPS) caused deterioration of BFMeT-induced ileal ulcers, while they could not cause the ulcers by themselves without the NSAID. Concentration of LPS and myeloperoxidase activity level were elevated correlatively in the intestinal mucosa of rats treated with LPS and BFMeT. These results suggest that an increase in the number of Gram-negative bacteria and their LPS in the mucosa induces activation of neutrophils together with the help of NSAID action and causes ulcer formation. J. Med. Invest. 51:43-51, February, 2004

Keywords **:** *nonsteroidal anti-inflammatory drugs (NSAIDs), ulcer formation, lipopolysaccharide (LPS), Escherichia coli, myeloperoxidase (MPO)*

INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used as both anti-inflammatory and analgesic agents, and new NSAIDs have recent been developed. However, NSAIDs induce gastro-duodenal ulcers in humans (1-5), and Allison *et al.* (6) reported that small intestinal ulceration occurred in 8.4% of users of NSAIDs but in only 0.6% of nonusers. Furthermore, a few patients who were long-term users of NSAIDs died of ulcer perforation in the small intestine (7, 8). These studies suggest that patients who take NSAIDs have an increased risk of ulceration in the small intestine and that the development of small intestinal ulcers leads to life-threatening complications. Mechanisms of NSAID-induced ulcer formation have been studied using rat models (4, 9, 10) and have been reviewed (1, 11-13). However, there is little information on

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the role of intestinal bacteria in ulcer formation in the small intestine. Using a 5-bromo-2-(4-fluorophenyl)-3-(4-methylsulfonylphenyl) thiophene (BFMeT, a non-acidic NSAID)(14)-induced ileal ulcer model, we previously found that Gram-negative bacteria such as *Escherichia coli* play important roles in ulcer formation, although the causes of the unbalanced increase in the number of Gram-negative bacteria are unclear (10).

Intestinal microflora are affected by many kinds of factors, including diet, high doses of drugs, stress, radiation, gastric acids, intestinal motility, bile, and immunologic factors (15). Most NSAIDs have been reported to inhibit prostaglandin synthesis and to affect intestinal peristalsis (16, 17). Gastrointestinal motility disorders often cause bacterial overgrowth and enteric colonization with Gram-negative bacilli (18-20). Secretory IgA (21), antimicrobial peptides and proteins (22) produced by intestinal tissue, lysozyme, bile, and pancreatic enzymes (15) are contained in intestinal contents, and they could also affect the composition of microflora. Therefore, in this study, we examined the effect of BFMeT on intestinal peristalsis *in vivo* and the effect of intestinal contents from BFMeT-treated rats on the growth of *E. coli* and *Lactobacillus acidophilus*.

An unbalanced increase in the number of Gram-negative bacteria in the small intestine is likely to increase the exposure of epithelial cells to lipopolysaccharide (LPS) and enhance the production of some kinds of cytokines that induce activation of the immune system, especially neutrophils, in the mucosa of intestines (23-25). Therefore, we studied the effects of heated *E. coli* and LPS on BFMeT-induced ileal ulcer formation in rats and then measured myeloperoxidase (MPO) activity as a marker of activation of neutrophils in tissue of the small intestine.

MATERIALS AND METHODS

Chemicals

BFMeT (Lot No. S-6907-18M)(14) was obtained from Otsuka Pharmaceutical Factory, Inc., Naruto, Tokushima, Japan. GAM broth and MRS broth (Difco) were purchased from Nissui Pharmaceutical Co., Ltd., Tokyo and Nippon Becton Dickinson Co., Ltd., Tokyo, respectively. Hexadecyltrimethylammonium bromide (HTAB) and hydrogen peroxide were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. *o*-Dianisidine dihydrochloride was purchased from Sigma-Aldrich, St. Louis, MO. Other reagents were obtained from Wako.

Bacterial strains

E. coli strain MHE1 was isolated from fresh ileal contents of a specific pathogen-free male Wistar rat (5 weeks old) and was identified by Enterotube^{R}II (BBL, Becton Dickinson). *L. acidophilus* strain MHL1 was also isolated from ileal contents of a rat and was identified by the API 20 A system (BioMerieux, France).

Animals and housing

Five-week-old male Wistar rats (weighing 100-120 g) were purchased from Clea Japan, Inc., Tokyo and Charles River Japan, Inc., Hino, Japan. The rats were adapted to laboratory conditions with free access to rat pelleted diet (MF, Oriental Yeast Co., Ltd., Tokyo) and tap water. Rats were housed in plastic cages in a room environmentally controlled at a temperature of 23±2°C, and humidity of 55±10% and with a 13-hr light/11-hr dark cycle.

Peristalsis of the small intestine

To examine the effect of BFMeT on peristalsis of the small intestines of rats *in vivo*, we measured the transit index (TI), which characterizes the gastrointestinal transit *in vivo* (26-28). Rats were given 1,000 mg/kg BFMeT or gum arabic solution, and after 0-22 hr (short term) or 6-72 hr (long term) they were administered 2 ml of a charcoal solution (10% charcoal suspension in 10% gum arabic solution) by intragastric gavage. Fifteen minutes after the charcoal administration, the rats were sacrificed by cervical dislocation under anesthesia with diethyl ether. The entire small intestine of each rat was removed, and the distance from the pylorus to the front of the charcoal was measured. The transit index (TI) was defined as the ratio of the distance between the pylorus and the front of the charcoal to the total length of the small intestine.

Growth of E. coli *and* L. acidophilus *in supernatants of contents of small intestines*

As reported previously (29), intestinal ulcers first appeared 18 hr after 1000 mg/kg BFMeT treatment, following the dramatic increase of Gram-negative rods in the ileal contents. The ileal contents of the rats were removed aseptically 18 hr after administration of BFMeT or 5% gum arabic solution and suspended in 2 volumes of sterilized saline. The suspension was centrifuged and filtered through a disposable filter (DISMIC-25CS, cellulose acetate membrane filter, 0.45 μm, ADVANTEC MFS, Inc., Tokyo). Then 1.35 ml of the sterilized supernatant was inoculated with 0.15 ml of *E. coli* strain MHE1 (9.75×10^4 CFU) or *L. acidophilus* strain MHL1 (8.25×10^3 CFU) and in-

cubated at 37°C under an aerobic condition. At 0, 2, 4, 6, 8 hr after inoculation, serial 10-fold dilutions of 30 μl of each mixture were spread onto GAM agar plates for *E. coli* strain MHE1 or MRS agar plates for *L. acidophilus* strain MHL1, and the colonies on the plates were counted.

Preparation of LPS from **E. coli** *strain MHE1*

LPS was extracted from *E. coli* strain MHE1 by the method of Westphal (30). A suspension of 4 grams of freeze-dried *E. coli* in 74 ml of endotoxin-free pure water (Otsuka Distilled Water ; Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) was incubated with 90% aqueous phenol at 70°C for 15 min. After cooling to 10°C, the mixture was centrifuged (3,000 rpm, 30 min), and the water layer was removed. The phenol layer was extracted with another 74 ml of pure water as described above. The combined water layers were dialyzed for 3 days against distilled water to remove phenol and small amounts of low-molecular-weight bacterial substances and then lyophilized. The lyophilized crude extract was dissolved in 3 ml of pure water and centrifuged (7,000 rpm, 30 min), and the supernatant was lyophilized. The lyophilized extract was dissolved in 33 volumes of pure water and centrifuged (30,000 rpm, 8 hr). The pellet was dissolved in 5-8 ml of pure water and centrifuged (35,000 rpm, 3 hr) two times and then lyophilized. The lyophilized extract (50 mg) was resuspended in 7.5 ml of pure water, and 0.75 ml of 2% cetavlon solution was added to the suspension, and the suspension was incubated for15 min and centrifuged (3,000 rpm, 20 min). The supernatant was lyophilized and dissolved in 3 ml of 0.5 M NaCl solution. Then 30 ml of ethanol was added to the solution, and the solution was incubated at 4°C for 2 hr and centrifuged (3,000 rpm, 30 min). The pellet was dissolved in 3-5 ml of pure water, and the solution was dialyzed for 2 days to remove NaCl and then lyophilized. The purity was then tested by using an endotoxin assay kit (Endospecy ES-6 set, ToxiColor DIA-MP set ; Seikagaku Co., Ltd., Tokyo) and the purity of extracted LPS was confirmed to be 99%.

Effects of heated **E. coli** *strain MHE1 and LPS on ileal ulcer formation induced by a low dose of BFMeT*

E. coli strain MHE1 was cultured in GAM medium at 37°C for 16 hr under an aerobic condition, killed at 121°C for 20 min in an autoclave, washed twice with sterilized saline (Otsuka normal saline ; Otsuka Pharmaceutical Factory, Inc., Naruto, Tokushima, Japan), and condensed to 1.8×10^{10} or 9.0×10^{10} CFU/ml. LPS extracted from heated *E. coli* strain MHE1 was dissolved in endotoxin-free water at the concentration of 0.5 mg/ml.

BFMeT suspended in 5% gum arabic solution was administered at a dose of 250 mg/kg of body weight only one shot by intragastric gavage at 13 : 00 after an 18 hr fast as described previously (10). As the control of BFMeT, 5% gum arabic solution was administered in the same manner as that of BFMeT at a dose of 10 ml/kg of body weight. Heated *E. coli* strain MHE1 at a dose of 1.8×10^{10} or 9.0×10^{10} cells/rat or LPS at a dose of 0.5 mg/rat was administered once a day for 3 days by intragastric gavage. This administration was started 4 hr after administration of BFMeT or 5% gum arabic solution. As the control of heated *E. coli* strain MHE1 or LPS, sterilized saline or endotoxin-free water was administered in the same manner at a dose of 1 ml/rat. The rats were sacrificed 72 hr after the administration of BFMeT or gum arabic solution by cervical dislocation under anesthesia with diethyl ether, and their gastrointestinal tracts were removed. The small intestine was divided into 6 pieces of equal lengths, which were named segments-1, 2, 3, 4, 5 and 6 from the stomach to cecal side. The segments were incised and carefully examined for ulcer formation macroscopically.

Endotoxin concentration in the mucosa of the small intestine

The small intestine was washed with sterilized saline 3 times, and the mucosa of segments 5 and 6 was scraped off with sterilized glass slides on ice and homogenized in 10 volumes of ice-cold endotoxin-free pure water. After serial 10-fold dilution of the homogenate with endotoxin-free pure water, endotoxin concentration was assayed by a chromogenic substrate method (31) using an endotoxin assay kit (Endospecy ES-50M, ToxiColor DIA-MP set, Seikagaku Co., Ltd., Tokyo). A calibration curve was made by using *E. coli* O111: B4 endotoxin (ToxiColor Et-1 set, Seikagaku Co., Ltd., Tokyo). Protein concentration of tissue was assayed by the method of Bradford (32).

MPO activity in mucosa of the small intestine

MPO activity in the same mucosa as that used in the endotoxin assay was measured according to a modified version of the method of Bradley *et al.* (33, 34). The mucosa of the small intestine was homogenized in 50 mM phosphate buffer containing 0.5% HTAB, pH 6.0, and centrifuged at 2,000 rpm for 10 min at 4°C. The supernatant (0.1ml) was mixed with 2.9 ml of 50 mM phosphate buffer, pH 6.0, containing 0.167 mg/ml *o*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. Time-dependent increase in absorbance at

460 nm was monitored to calculate MPO activity. The protein concentration of the tissue was determined by the method of Bradford (32). One unit of MPO activity was defined as that degrading one micromole of hydrogen peroxide per minute at 25°C (35).

Statistical analysis

The significance of difference in the mean values of two groups was tested by one-way analysis of variance.

RESULTS

Transit index as a marker of peristalsis of the small intestine

There was no difference in the transit index at any point of the time course between the rats treated with BFMeT(1,000 mg/kg) and those treated with 5% gum arabic (10 ml/kg) (Figure 1a and b). These data show that BFMeT had no influence on peristalsis of the small intestine, indicating that the unbalanced growth of Gram-negative bacteria was not caused by retarded peristalsis.

Growth of **E. coli** *and* **L. acidophilus** *in the supernatants of contents of the small intestines of rats treated with BFMeT or gum arabic*

E. coli strain MHE1 grew in the supernatants of small intestinal contents with no difference in the contents collected from BFMeT-treated rats and those from gum arabic-treated rats (Figure 2a). Growth of *L. acidophilus* strain MHL1 in the small intestinal contents of rats treated with BFMeT and that in the small intestinal contents of rats treated with gum arabic were also similar (Figure 2b). Therefore, there were no

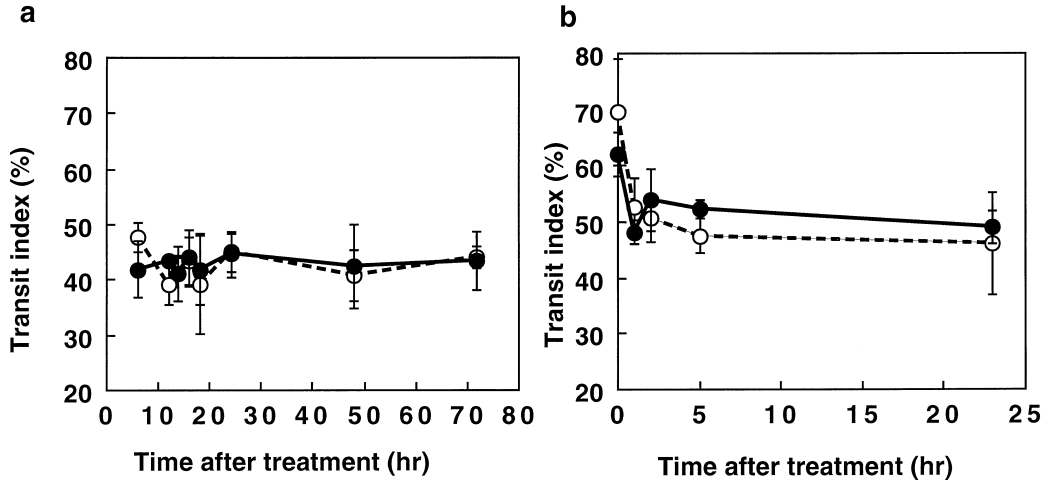

Fig. 1. Time course of transit index in the small intestine. Rats were treated with BFMeT (1,000 mg/kg)(●) or 5% gum arabic solution(10 ml/kg)(○),and transit index was determined as described in MATERIALS AND METHODS at 5-72 hr (a) or 0-22 hr (b) after treatment with BFMeT or gum arabic. Values are means±SDs.

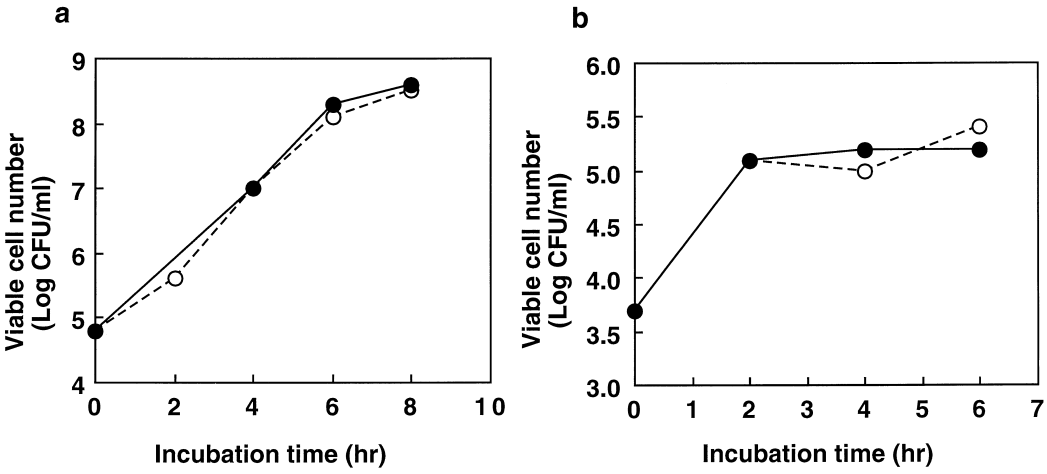

Fig. 2. Growth of *E. coli* and *L. acidophilus* in the supernatants of contents of the small intestines of rats treated with BFMeT (1,000 mg/kg) or 5% gum arabic solution (10 ml/kg). (a) *E. coli* strain MHE1 and (b) *L. acidophilus* strain MHL1 were incubated with the sterilized supernatant of ileal contents collected at 18 hr after treatment with BFMeT (1,000 mg/kg)(●)or 5% gum arabic solution (10ml/kg)(○). At indicated times, viable cell numbers were determined using GAM agar or MRS agar.

BFMeT-specific factors that promoted the growth of *E. coli* in small intestinal contents collected from BFMeT-treated rats.

Effects of heated **E. coli** *cells and LPS on ileal ulcer formation*

A small number of ileal ulcers were observed in all of the rats treated with 250 mg of BFMeT per kg, and most of the ulcers were observed in segments 4-6. The number of ulcers was significantly increased by administration of heated *E. coli* strain MHE1 (1.8×10^{10} cells/rat) compared with that in the BF+saline group (Figure 3a), especially in segments 5 and 6 (Figure 3b). No ulcer was observed in rats treated with 5% gum arabic and heated *E. coli* strain MHE1

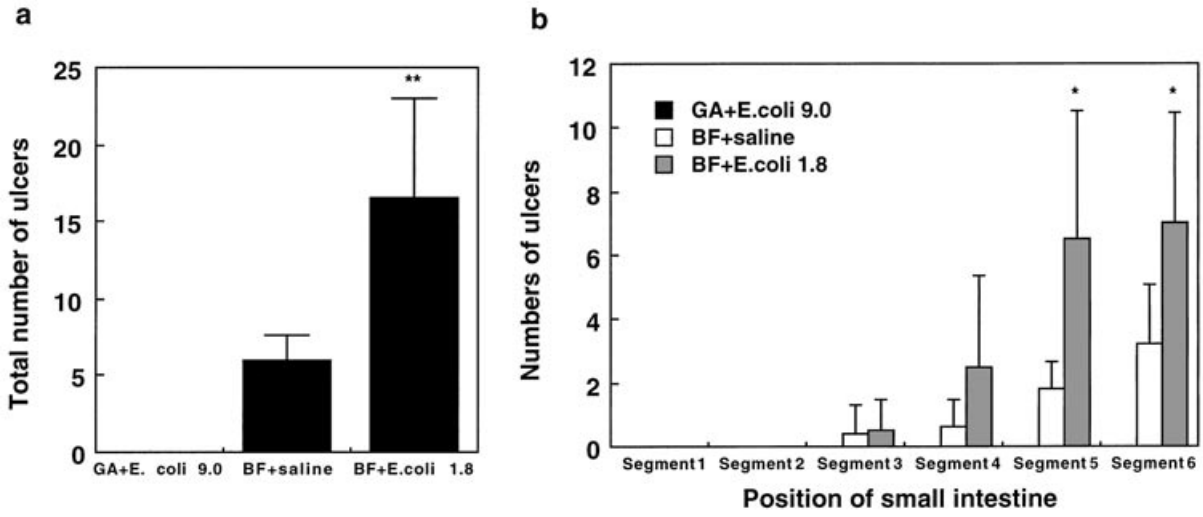

Fig. 3. Effects of heated *E. coli* strain MHE1 cells on the ulcer formation induced by BFMeT in the small intestines of rats. Rats were treated with 5% gum arabic solution (10 ml/kg) and heated *E. coli* strain MHE1 (9.0×10^{10} cells/rat/day for 3 days) (GA+*E.coli* 9.0 group), BFMeT (250 mg/kg) and saline (1 ml/rat/day for 3 days) (BF+saline group), or BFMeT (250 mg/kg) and heated *E. coli* strain MHE1 (1.8×10^{10} cells/rat/day for 3 days) (BF+*E.coli* 1.8 group). Rats were sacrificed 72 hr after administration of BFMeT or gum arabic solution. The small intestines were removed and divided into 6 equal segments, and the number of ulcers in each segment was counted macroscopically. (a) Total number of ulcers in the small intestine and (b) number of ulcers in each segment.
*Significantly different from other groups ($P<0.05$).
** Significantly different from other groups ($P<0.005$).

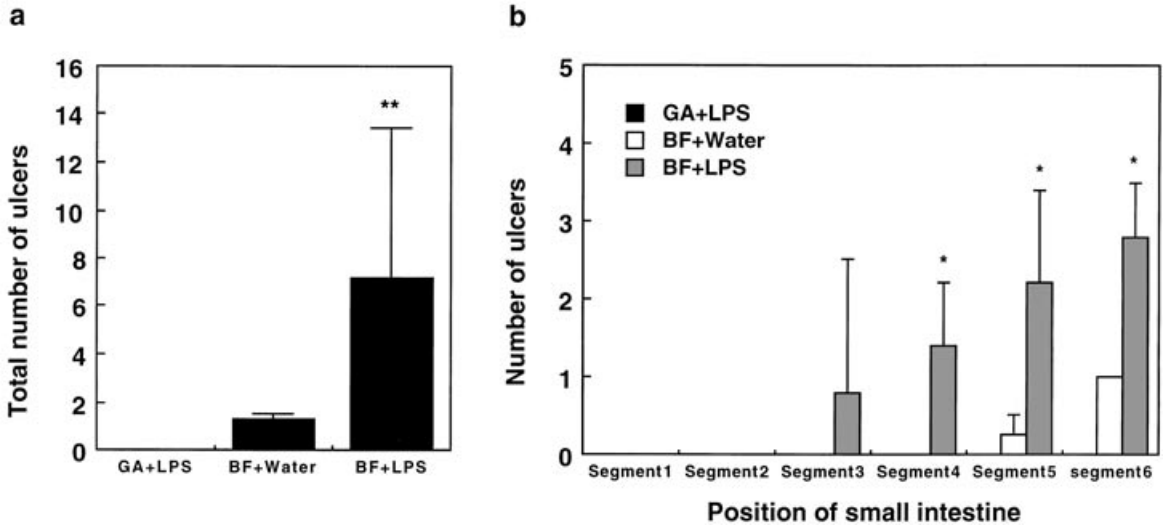

Fig. 4. Effect of LPS extracted from *E. coli* strain MHE1 on ulcer formation in the small intestines of rats treated with BFMeT. Rats were treated with 5% gum arabic (10 ml/kg) and LPS (0.5 mg/rat/day for 3 days) (GA+LPS group), BFMeT (250 mg/kg) and endotoxin-free pure water (1 ml/rat/day for 3 days) (BF+Water group), or BFMeT (250 mg/kg) and LPS (0.5 mg/rat/day for 3 days) (BF+LPS group). Rats were sacrificed 72 hr after administration of BFMeT or gum arabic solution. The small intestines were divided into 6 equal segments, and the number of ulcers in each segment was counted macroscopically. (a) Total number of ulcers in the small intestine and (b) number of ulcers in each segment.
*Significantly different from other groups ($P<0.005$).
**Significantly different from other groups ($P<0.0001$).

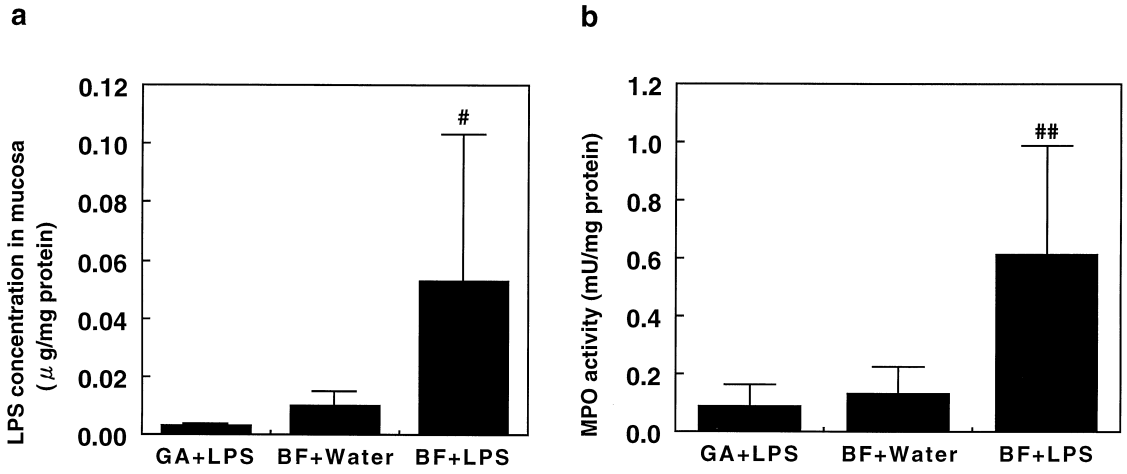

Fig. 5. Mucosal LPS concentrations and myeloperoxidase (MPO) activities in segments 5 and 6. They were determined as described in MATERIALS AND METHODS. (a) Mucosal LPS concentrations and (b) MPO activities in segments 5 and 6.
Significantly different from other groups ($P < 0.05$).
Significantly different from other groups ($P < 0.01$).

even at a higher dose up to 9.0×10^{10} cells/rat.

Administration of LPS also caused significant deterioration of ulcer formation in rats treated with 250 mg of BFMeT per kg compared with that in the BF+endotoxin-free pure water group (Figure 4a); the number of ulcers was significantly increased in segments 4-6 (Figure 4b) as in the case of heated *E. coli*.

LPS concentration and MPO activity in the mucosa

The concentration of LPS in segments 5 and 6 was significantly increased in the intestinal mucosa of rats treated with LPS (Figure 5a). MPO activity in the same segments was significantly higher in BF+LPS group than in BF+Water or GA+LPS group. Figure 5a and b show that LPS concentration and MPO activity were not elevated in response to LPS alone or BFMeT alone. The level of MPO activity was strongly correlated with LPS accumulation in the BF+LPS group. The results indicate that both treatment with NSAIDs and increased concentration of LPS in the mucosa are necessary for ulcer induction.

DISCUSSION

NSAIDs are widely used as both anti-inflammatory drugs and analgesics, but they induce gastrointestinal ulcers as side effects. Many researchers have tried to elucidate the mechanisms of gastrointestinal ulcer formation (1-5). Since NSAIDs enhance the permeability of the intestinal mucosa (36), luminal aggressive factors such as bacteria and their degradation products easily infiltrate the mucosa, and then the migration and activation of neutrophils, the start of inflammation, is observed in the intestinal mucosa (11, 12). However, NSAIDs did not induce ulcer formation in germ-free rats (10, 37), antibiotic-treated rats and gnotobiotic rat monoassociated with *L. acidophilus* or *Bifidobacterium adolescentis* (10). In addition, overgrowth of Gram-negative bacteria was observed in the intestines of NSAID-treated rats, and an NSAID induced the formation of intestinal ulcers in gnotobiotic rats monoassociated with *E. coli* (10, 29). Therefore, the unbalanced growth of Gram-negative bacteria plays an important role in NSAID-induced ulcer formation. However, the mechanism of the unbalanced growth of Gram-negative bacteria has not been elucidated yet. In this study, we tried to determine the cause of unbalanced growth of Gram-negative bacteria in the small intestine of BFMeT-treated rats.

Gastrointestinal motility disorders have recently been reported to induce bacterial overgrowth in the gastrointestinal tract (18, 19). In the microflora of the small intestine of non-treated rats, the percentage of Gram-negative bacteria was found to be less than 5% (29), but Gram-negative bacteria were found to be more predominant in the cecum than in the ileum (15, 38). On the other hand, NSAIDs have been reported to inhibit peristalsis in the isolated guinea-pig ileum by affecting prostaglandin synthesis(16, 17). Therefore, we hypothesized that the reverse migration of Gram-negative bacteria from the cecum to the ileum causes the unbalanced growth of Gram-negative bacteria because of reduced peristalsis of the ileum induced by BFMeT. However, transit index, an indicator of peri-

stalsis, in the group of rats administered BFMeT was not different from that in the group of rats administered gum arabic solution (Figure 1a and b).

It has recently been reported that factors defending the intestinal mucosa against bacteria were produced in the mucosa or secreted from the mucosa as a part of the mucosal immune system (21, 22). Bile or enzymes secreted into the lumen have been reported to affect microflora in the small intestine (15). We carried out an experiment to determine whether factors in the small intestinal contents affected the growth or viability of Gram-negative bacteria or Gram-positive bacteria in the small intestine. Acceleration of the growth of *E. coli* was not observed in the intestinal contents of BFMeT-treated rats compared with that in the intestinal contents of gum arabic-treated rats.

In this study, BFMeT did not affect peristalsis, and the intestinal contents of BFMeT-treated rats did not affect the viability and growth of either Gram-negative or Gram-positive bacteria. It has been reported that diclofenac, an NSAID, has antibacterial activity (39-41). Since BFMeT does not dissolve in water or any other solvent, we could not examine the direct effect of BFMeT on the growth of *E. coli* and *L. acidophilus*. Therefore, factors affecting the unbalanced increase in number of Gram-negative bacteria could not be clarified in this study. Further study is needed to elucidate the mechanism of unbalanced growth of Gram-negative bacteria in the intestine.

It has been reported that Gram-negative bacteria play an important role in small intestinal ulcer formation induced by NSAIDs (10, 29, 42, 43). LPS is a well known virulent factor of Gram-negative bacteria, and it promotes inflammation by interference with the host immune response and can activate neutrophils (44-46). Intestinal epithelial cells recognize LPS by Toll-like receptor 4 and CD 14 on their surfaces (47) and secrete cytokines to induce migration of neutrophils (25). In the present study, heated *E. coli* and its LPS caused deterioration of the BFMeT-induced ulcer formation in the small intestine when they were administered together with the NSAID. LPS concentration and MPO activity, a marker of activated neutrophils (33), in mucosa of the small intestine increased correlatively in rats treated with LPS and BFMeT (Figure 5a and b), suggesting that the increased infiltration of LPS into the mucosa causes migration of neutrophils and their activation and that this phenomenon might be necessary for ulcer formation.

Although the mechanism of unbalanced increase in the number of Gram-negative bacteria has not been elucidated yet, the increased Gram-negative bacteria might easily infiltrate into the mucosa when mucosal permeability is increased by NSAIDs (36). In the next stage, the intestinal epithelial cells recognize LPS by the receptors (47) and secrete cytokines to induce migration of neutrophils (25). Activation of neutrophils is amplified by LPS (44-46). Then the oxidative damage is triggered by MPO-derived hypochlorous acid and/or other reactive oxygen species. Therefore, the ileal ulcer formation induced by NSAIDs does not occur in germ-free rats and in gnotobiotic rats monoassociated with *L. acidophilus* or *B. adolescentis*, both Gram-positive bacteria (10). This result suggests that maintenance of normal microflora is important for preventing damage to the intestinal mucosa induced by NSAIDs.

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