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Effect of Intestinal Bacteria on Formation of Azoxymethane-Induced Aberrant Crypt Foci in the Rat Colon

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The effect of intestinal bacteria on formation of azoxymethane (AOM)-induced aberrant crypt foci (ACF) and DNA adducts in the rat colon was investigated. Male Sprague-Dawley rats were administered cultures of *Lactobacillus acidophilus, Bifidobacterium adolescentis, Bacteroides fragilis, Escherichia coli* and *Clostridium perfringens* for five weeks and given injections of AOM at 15 mg/kg body weight at the first and second weeks. The number of ACF five weeks after the start of the experiment was decreased in the rats treated with the cultures or culture supernatants of *L. acidophilus* and *C. perfringens.* The half-life of O^6-methylguanine (O^6-meG) in the *L. acidophilus* group was shorter than that in the GAM broth group. The half-life of 7-methylguanine did not differ among the groups. These results suggest that the metabolite(s) of *L. acidophilus* and *C. perfringens* inhibit(s) the ACF formation in rats treated with AOM and that the inhibitory effect of *L. acidophilus* is due to the enhanced removal of O^6-meG from the colon mucosal DNA.

Epidemiological and experimental studies suggest that besides dietary factors the gut microflora plays an important role in colon carcinogenesis (1, 2). People eating Western-style food have a higher risk of colon cancer and a higher incidence of genus *Bacteroides* in feces than those eating Japanese-style food (3). *Bacteroides fragilis* also has been implicated in production of human fecal mutagens (4). Some intestinal bacteria isolated from human feces can produce secondary bile acids, which are known to be tumor promoters in the colon, by hydroxylation of primary bile acids (5). *Escherichia coli* (6) and *Clostridium perfringens* (2) possess high activity of β-glucuronidase which reactivates glucuronide conjugates of xenobiotics such as carcinogens and mutagens by deconjugating them in the intestine (7). Mitsuoka *et al.* (8) found that the number of *C. perfringens* cells in human feces increases with aging and suggested an association of this bacterium with cancer. Kubota also reported that the number of bacteria of genus *Clostridium* was higher in patients with colon cancer than in healthy adults (9). On the other hand, it is reported that consumption of large quantities of dairy products such as yogurt and fermented milk containing genus *Lactobacillus* or *Bifidobacterium* may be related to a lower incidence of colon cancer (10, 11). Freeze-dried cells were used in most experiments to determine the inhibitory effects of lactic acid bacteria on colon cancer formation, and a few papers mentioned the effects of metabolites by the bacteria on carcinogenesis (12). Also, there is little knowledge about the effects of other intestinal bacteria on carcinogenesis *in vivo.* Therefore, we gave pure cultures of *L. acidophilus, B. adolescentis, B fragilis, E. coli* and *C. perfringens* to rats to determine their effects on formation of azoxymethane (AOM)-induced colon aberrant crypt foci (ACF), which are thought to be precursor lesions of colon cancer (13). Rodents treated with a colon carcinogen (14) and patients with colon tumor (15) have a higher number of ACF than non-treated animals and patients without colon cancer, respectively. Pretlow *et al.* demonstrated that ACF with four or more aberrant crypts per focus correlated with tumor incidence in rats (16). Therefore, these lesions have been used as biomarkers to evaluate chemopreventive effects of many chemicals on the development of colon cancer (17, 18). The cultures of *L. acidophilus* and *C. perfringens* inhibited the ACF formation. Then those cultures were separated into bacterial cells and culture supernatants to determine which component was related to the inhibitory effect. Since formation of DNA adducts is thought to be the initial step of carcinogenesis, we also examined time-dependent changes of the

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colon mucosal methylguanine levels in the treated groups and compared the half-lives of these methylated bases among the experimental groups.

MATERIALS AND METHODS

Experimental drink. The cultures of *B. adolescentis* strain ATCC15703, *L. acidophilus* strain ATCC4356, *E. coli* strain W3110, *B. fragilis* strain YCH46 and *C. perfringens* strain GAI0668 were prepared by inoculating overnight cultures of these bacteria into degassed fresh GAM broth (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) in dilutions of 1:25 and anaerobically incubating them at 37 °C for 23 hr. The number of viable cells in each overnight culture was as follows: *B. adolescentis,* 3.1×10^8 colony forming unit (CFU)/ml; *L. acidophilus,* 1.5×10^8 CFU/ml; *E. coli,* 4.0×10^9 CFU/ml; *B. fragilis,* 8.3×10^8 CFU/ml; *C. perfringens,* 1.2×10^9 CFU/ml. The supernatants of *B. adolescentis, L. acidophilus* and *C. perfringens* were prepared by centrifugation of their cultures at 6,000 rpm for 20 min at 4 °C and then sterilized by filtration through a filter with a pore size of 0.45 μm (Nihon Millipore Ltd., Yonezawa, Japan). The cells of *L. acidophilus* and *C. perfringens* were washed twice with an anaerobic solution (19) after centrifugation of these cultures and resuspended in the same volume of the solution. All experimental drinks were freshly prepared and changed every day throughout the experimental period.

Treatments of animals. Three-week-old Sprague-Dawley male rats were purchased from Japan SLC Inc. (Hamamatsu, Japan). The animals were housed in polycarbonate cages with wood chip bedding in a room environmentally controlled for a temperature of 23 ± 2 °C, humidity of 55 ± 10% and a 13-hr light/11-hr dark cycle, and were given the pellet diet MF (Oriental Yeast Co., Ltd., Tokyo, Japan). Experimental drinks were kept in plastic bottles and given to rats *ad libitum* until the termination of the experiment. After one week from the start of the experiments, all animals received subcutaneous injections of AOM (Sigma Chemical Co., St Louis, USA) in normal saline at a dose of 15 mg/kg body weight once a week for two weeks. The animals were sacrificed at designated times after the second AOM injection as described below.

ACF analysis. To determine the effects of the experimental drinks on ACF formation, the animals were sacrificed and the colons (including the recta) were removed three weeks after the last AOM injection. Then the colons were fixed in 10% formalin-PBS solution for 24 hr. The specimens were examined for ACF with a microscope after being stained with 0.2% methylene blue. The numbers of foci and crypts in each focus were recorded (20).

Detection of O^6-methylguanine and 7-methylguanine levels in colon mucosal cells. Rats were given tap water, sterilized GAM broth or the culture supernatant of *L. acidophilus* or *C. perfringens* as an experimental drink. They were sacrificed at 6, 12, 18, 24 and 36 hr after the second AOM injection and the colons were removed. In addition, the colons of rats treated with tap water, GAM broth and *L. acidophilus* supernatant were removed at 48 and 72 hr after the second AOM injection. DNA was extracted from the colon mucosa by the method of Gupta (21). The extracted DNA was subjected to neutral thermal hydrolysis and acid hydrolysis by the method of Netto *et al.* (22), and O^6-methylguanine (O^6-meG) and guanine in the acid hydrolysis fraction and 7-methylguanine (7-meG) in the neutral thermal hydrolysis fraction were separated by high-performance liquid chromatography (HPLC, LC-5A, Shimadzu Co., Kyoto, Japan) with Chemcosorb 7-SCX column (Chemco Co., Osaka, Japan). The methylated bases were eluted with 6 mM ammonium formate (pH 3.0) at a flow rate of 1 ml/min. These methylated and normal guanines were monitored with a spectrophotometric detector SPD-2A (Shimadzu) at 254 nm and a fluorescence HPLC monitor RF-530 (emission 365 nm, excitation 285 nm, Shimadzu). 7-MeG was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The authentic O^6-meG was kindly given to us by Drs. K. Ishizaki and M. Ikenaga (Radiation Biology Center, Kyoto University).

Statistical analysis. The data were analyzed statistically by analysis of variance.

RESULTS

The body weights of the rats were not significantly different among the groups in the experiments (data not shown). The effects of experimental drinks on the number of AOM-induced ACF and the crypt multiplicity, that is, the number of aberrant crypts per focus (AC/focus), are summarized in Tables 1 and 2. As shown in Table 1, the number of ACF in the GAM broth treated group was 214.4 ± 29.4 per colon. The number of ACF in the *L. acidophilus* treated group was significantly lower (73.6%) than that of the GAM broth treated group ($P < 0.05$). The culture of *C. perfringens* also decreased the number of ACF to 68.6% of that in the GAM broth treated group ($P < 0.01$). The overnight cultures of *B. adolescentis* and *B. fragilis,* and the culture supernatant of *B. adolescentis* had no effect on the number of ACF. The number of ACF of the *E. coli* culture group was significantly higher (126%) than that of the GAM broth treated group ($P < 0.05$) (Table 1). The crypt multiplicity was not influenced by the experimental drinks. A supplementary experiment was performed with culture supernatants and bacterial cells of *L. acidophilus* and *C. perfringens* to determine which component was associated with the inhibitory effect (Table 2). The number of ACF in the GAM broth group was 245.8 ± 40.9 per colon and in the *L. acidophilus* culture supernatant group it was 77.2% of that in the GAM broth group ($P < 0.05$). However, this inhibitory effect was lost after the supernatant was kept at 4 °C for 2 weeks. The culture supernatant of *C. perfringens* also reduced the number of ACF to 68.8% of that in the GAM broth group ($P < 0.05$). The number of ACF in the GAM broth group was 87.0% of that of the water treated group (282.6 ± 64.8 per colon), and the difference between these groups was not significant. *L. acidophilus* or *C. perfringens* cells in an anaerobic solution did not influence the ACF formation. These results indicate that the inhibitory effects of *L. acidophilus* and *C. perfringens* overnight cultures come from these bacterial culture supernatants.

The effect of culture supernatants of *L. acidophilus* and *C. perfringens* on methylated guanine levels in the colon mucosa was determined at various times after the second AOM injection. Figure 1 shows time-dependent changes in O^6-meG levels in the colons of rats taking water, GAM broth and culture supernatants of *L. acidophilus* and *C. perfringens.* The O^6-meG levels in all groups reached a maximum at 12 hr after the second AOM injection, and the levels at that time were not significantly different among the groups (data not shown). This figure also shows that the decrease in O^6-

TABLE 1

Effect of Experimental Drinks on the Number and Crypt Multiplicity of Aberrant Crypt Foci in the Colons of Sprague–Dawley Rats Treated with Azoxymethane

Treatment	*n*	ACF/colon	AC/focus
GAM broth	6	214 ± 29[a]	2.05 ± 0.14
Bifidobacterium adolescentis culture	6	237 ± 71	2.10 ± 0.17
Lactobacillus acidophilus culture	6	158 ± 31[b1]	2.10 ± 0.16
Escherichia coli culture	7	269 ± 42[b1]	2.03 ± 0.12
Bacteroides fragilis culture	7	210 ± 36	2.06 ± 0.11
Clostridium perfringens culture	8	147 ± 36[b2]	1.99 ± 0.20
Bifidobacterium adolescentis supernatant	6	199 ± 41	2.06 ± 0.16

[a] Mean ± SD.
[b1, b2] Significantly different from the GAM broth group at $P < 0.05$ and < 0.01, respectively.

meG levels was more rapid in the GAM broth group and the culture supernatant groups than in the water group. The half-lives in the water and GAM broth groups were 25.1 and 17.7 hr, respectively (Table 3). The half-life in the *L. acidophilus* supernatant group was 52.2% and 74.4% of that in the water and GAM broth groups, respectively. The half-life in the *C. perfringens* supernatant group calculated from O^6-meG levels at 12, 18, 24 and 36 hr was almost the same as that in the GAM broth group. The half-life of 7-meG was the same among all the experimental groups (Table 3). These results suggest that removal of O^6-meG from DNA of the colon mucosa is accelerated by drinking the culture supernatants of *L. acidophilus.*

DISCUSSION

The correlation between large bowel cancer and the gut microflora has been clarified by many epidemiological and experimental studies (1, 2). These reports show that intestinal bacteria are associated with production of mutagens (4), inactivation of mutagens by adsorption to bacterial cells (23), production of tumor promoters (5), activation of the immune system resulting in anti-tumor effects (24), and metabolical activation of detoxified conjugates (7). Therefore, these findings suggest that intestinal bacteria play a role at all stages of carcinogenesis. To determine the effect of intestinal bacteria on colon carcinogenesis in the animal model, *Bifidobacterium* and/or *Lactobacillus* were used in most experiments (25, 26). However, there is little knowledge about the effects of other kinds of intestinal bacteria on carcinogenesis *in vivo.* Therefore, we administered overnight cultures of various kinds of intestinal bacteria to rats treated with AOM and investigated the effect of these bacteria on formation of ACF and DNA adducts.

Among the bacteria tested, overnight cultures of *L. acidophilus* and *C. perfringens* inhibited the formation of ACF induced by AOM (Table 1). It was confirmed that these inhibitory effects came from culture supernatants, not from bacterial cells, of both bacterial cultures (Table 2). The effect of *L. acidophilus* culture supernatant disappeared when it was kept at 4 °C for 2 weeks (Table 2). These results suggest that *L. acidophilus* and *C. perfringens* culture supernatants contain substance(s) which inhibit ACF formation.

DNA adduct formation is the first step in the process

TABLE 2

Effect of Culture Supernatants and Bacterial Cells on the Formation of Aberrant Crypt Foci in the Colons of Sprague–Dawley Rats Treated with Azoxymethane

Treatment	*n*	ACF/colon	AC/focus
GAM broth	4	246 ± 41[a]	2.15 ± 0.17
Lactobacillus acidophilus supernatant, new	6	190 ± 22[b]	1.96 ± 0.13
Lactobacillus acidophilus supernatant, old (4 °C, 2 weeks)	6	206 ± 60	1.99 ± 0.15
Clostridium perfringens supernatant	6	169 ± 33[b]	2.09 ± 0.16
Anaerobic solution (AS)	5	201 ± 35	1.84 ± 0.06
Lactobacillus acidophilus cells in AS	6	198 ± 37	1.86 ± 0.08
Clostridium perfringens cells in AS	6	207 ± 63	2.06 ± 0.23
Water	5	283 ± 65	1.93 ± 0.14

[a] Mean ± SD.
[b] Significantly different from the GAM broth group at $P < 0.05$.

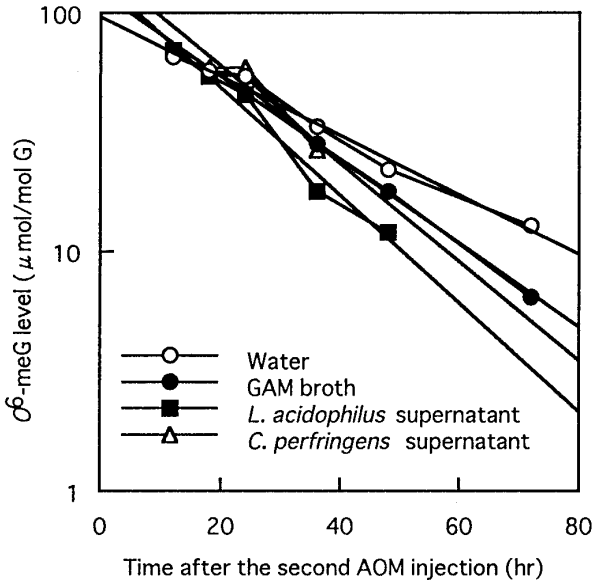

FIG. 1. Removal of O^6-methylguanine from the colon mucosal DNA of Sprague-Dawley rats treated with culture supernatants of *L. acidophilus* and *C. perfringens* following the second AOM injection. Data were plotted semilogarithmically to determine the half-life of the methylated bases.

of chemical carcinogenesis. Especially O^6-meG induced by AOM plays an important role in the initiation stage of carcinogenesis because this methylguanine causes point mutation of GC pair to AT pair and activation of the k-*ras* oncogene (27). Also a positive correlation between the formation of ACF and the formation of O^6-meG is shown by an experiment with transgenic mice which possess high activity of the O^6-methylguanine-repair enzyme, methylguanine-methyltransferase (MGMT) (27). Therefore we investigated time-dependent changes in the colon mucosal O^6-meG levels in the *L. acidophilus* culture supernatant treated group and calculated the half-lives of the methylated bases. Since the half-life of O^6-meG in the *L. acidophilus* supernatant group was reduced to 52.2% and 74.4% of that in the water and GAM broth groups, respectively (Table 3), it was thought that feeding *L. acidophilus* supernatant enhanced removal of O^6-meG from the colon mucosal DNA.

Subcutaneously injected AOM is metabolized to methylazoxymethanol in the liver and delivered to the colon mucosa via the blood stream or bile after conjugation with glucuronic acid (28). The glucuronide conjugate is deconjugated by β-glucuronidase of the microflora, and the released methylazoxymethanol attacks the colon mucosal DNA (7). Kinouchi *et al.* reported that ACF formation induced by dimethylhydrazine in the rat colon was inhibited by antibiotics which were administered orally to reduce the number of intestinal microorganisms (29). β-Glucuronidase inhibitors also reduce the ACF formation (20). These results indicate that intestinal bacteria play an important role in ACF formation. In this experiment, fecal β-glucuronidase activity and the maximum level of O^6-meG in the colon mucosa were not affected by *L. acidophilus* treatment (data not shown), suggesting that the inhibitory effect against the ACF formation can not be ascribed to β-glucuronidase.

The reduction of O^6-meG level may be caused by MGMT (30) or dilution of O^6-meG by cell proliferation. Since the half-life of 7-meG was not affected by the *L. acidophilus* treatment, O^6-meG in the *L. acidophilus* group was not diluted by cell proliferation. The MGMT activity is induced by exposure to chemicals including carcinogens, by physical stimuli such as UV irradiation or by hormones (31, 32). *L. acidophilus* metabolite(s) may induce MGMT in the colon mucosa of rats.

Although the major metabolite in sugar fermentation by *L. acidophilus* is lactic acid (33), this short chain fatty acid does not inhibit the ACF formation induced by AOM (34). Therefore, other metabolite(s), such as peptides and other kinds of fatty acids (12) might be associated with the inhibitory effect of the *L. acidophilus* culture supernatant. *Lactobacillus* is also known to activate the immune system, to have an antitumor effect (24) and to suppress mutagenicity by adsorption of the mutagens to its cell surface (23). These anti-tumor and anti-mutagenic effects come mainly from cells of this bacterium. The mechanism of inhibition of ACF formation by the *L. acidophilus* culture supernatant may not be related to adsorption of AOM and its activated metabolite to the cell surface and enhancement of the immune system.

Since *C. perfringens* has strong β-glucuronidase activity, this bacterium is thought to be involved in the process of colon carcinogenesis (2). Unexpectedly, the culture and the culture supernatant of *C. perfringens* reduced the number of ACF induced by AOM in the rat colon (Tables 1 and 2). The mechanism of this inhibitory effect is unclear because β-glucuronidase activity of feces from *C. perfringens* -treated rats (data not shown) and half-lives of O^6-meG and 7-meG in the *C. perfringens* supernatant group were not significantly different from those of the GAM broth group. The major

TABLE 3

Effect of Culture Supernatants on the Half-Life of 7-Methylguanine and O^6-Methylguanine in the Colon Mucosa of Sprague–Dawley Rats Treated with Azoxymethane

Treatment	Half-life (hr)	
	7-meG	O^6-meG
Water	10.6	25.1
GAM broth	10.1	17.7
Lactobacillus acidophilus supernatant	9.2	13.1
Clostridium perfringens supernatant	10.3	20.1

metabolites in sugar fermentation by *C. perfringens* are acetic acid and butyric acid (35). Butyrate has been found to slow cell proliferation and promote expression of phenotypic markers of differentiation in the large bowel cancer cell line LIM1215 (36). McIntyre *et al.* (37) reported that butyrate produced by fiber fermentation in the distal large bowel is protective against the large bowel cancer induced by dimethylhydrazine. Therefore, butyrate produced in the *C. perfringens* culture might inhibit cell proliferation of the initiated cells.

In our experiment the *B. adolescentis* culture and its supernatant did not show an inhibitory effect on the ACF formation (Table 1). However, Kulkarni and Reddy (38) and Challa *et al.* (39) reported the inhibitory effect of *Bifidobacterium longum* on the ACF formation induced by AOM. This contradiction may be due to the difference in bacterial species used in the two experiments.

In this study we demonstrated that the culture supernatants of *L. acidophilus* and *C. perfringens* decreased the number of ACF induced by AOM. The mechanism of the inhibitory effect of the *L. acidophilus* supernatant may be enhancement of O^6-meG removal from colon mucosal DNA by metabolite(s) produced in this culture. We are planning to examine whether this culture supernatant can actually induce the activity of MGMT or not.

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