INTRODUCTION

Cigarette smoking is known to increase the development of inflammatory bowel disease (IBD) (Persson et al., 1990). Additionally, recent epidemiological studies demonstrated that increased dietary fiber intake reduced respiratory symptoms among both smokers and those subject to second-hand smoke (David et al., 2005). However these mechanisms have not been fully elucidated. Dietary fiber is delivered to the large intestine without being digested and metabolized to organic acids by micro-flora in the large intestine (Roediger, 1980). Short chain fatty acids (SCFAs) among the organic acids, in particular, are absorbed via the colon mucosa and are utilized not only as a mucosal energy substrate but also as systemic energy sources (Roediger, 1980), while SCFAs have many good effects on the host (Sugawara et al., 2006). In these contexts we hypothesized that cigarette smoke may change the levels of organic acids in the gut. In this study, we evaluated not only organic acid levels but also the population of micro-flora and pH in caecal contents after exposing rats (n = 5) to cigarette smoke for a 4-week in order to investigate whether the gut environment is altered by cigarette smoke or not. After the exposure of cigarette smoke, caecal levels of organic acids such as acetic acid, propionic acid, butyric acid and valeric acid significantly decreased. Additionally the population of Bifidobacterium significantly decreased and the pH significantly elevated. In conclusion cigarette smoke changes caecal levels of certain organic acids, the population of Bifidobacterium and the pH in caecal contents of rats. These results suggest that cigarette smoke may alter the gut environment of rats.

ABSTRACT — Cigarette smoke has been known to affect the development of bowel disease. However it has not been fully elucidated how cigarette smoke has effects on the gut. In this context we evaluated not only caecal levels of organic acids but also populations of micro-flora and pH in caecal contents after exposing rats (n = 5) to cigarette smoke for a 4-week in order to investigate whether the gut environment is altered by cigarette smoke or not. After the exposure of cigarette smoke, caecal levels of organic acids such as acetic acid, propionic acid, butyric acid and valeric acid significantly decreased. Additionally the population of Bifidobacterium significantly decreased and the pH significantly elevated. In conclusion cigarette smoke changes caecal levels of certain organic acids, the population of Bifidobacterium and the pH in caecal contents of rats. These results suggest that cigarette smoke may alter the gut environment of rats.

Key words: Bifidobacterium, Cigarette smoke, Gut environment, Organic acid, pH
ture of 22 ± 1°C, with the humidity level at 55 ± 10%, and a 12-hr light/dark cycle, the illumination extending from 08:00 to 20:00. All procedures performed during these animal experiments were carried out under the control of our committee in accordance with The Guidelines for Animal Experiments in Nara Medical University and Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society.

Method of cigarette smoke exposure

The animals underwent enforced exposure to cigarette smoke using a Hamburg II smoking apparatus (Borgwaldt, Germany) based on the method of Suemaru et al. (Suemaru et al., 1992). All smoke exposure experiments were carried out using Hi-lite® filter cigarettes (Japan Tobacco Industry Co., Ltd., Tokyo, Japan), which have nicotine and tar contents of 1.4 mg and 17 mg per cigarette, respectively.

The animals were exposed to smoke from 30 cigarettes for 20 min for five days a week, (Monday to Friday) each week. The Hamburg II apparatus was used to expose five animals to cigarette smoke simultaneously, and all the smoking exposure experiments were carried out using 5 animals. The cigarette was smoked at a rate of 15 puffs per minute with an inhalation of 2 sec of smoke mixed with 7 volumes of air, followed by 2 sec of air in the chamber. The mixture of air and smoke moved from the chamber to the connected holders, in which the animals were kept separately. The animals in the non-smoking group were kept for 20 min in the chambers without cigarette smoke.

The animals were decapitated under anesthesia by an intraperitoneal injection with sodium pentobarbital (50 mg/kg) within 24 hr after the last cigarette smoke exposure, and then pH in the caecum was measured by IQ 150 pH/Thermometer (Toho Co., Ltd., Tokyo, Japan), which have nicotine and tar contents of 1.4 mg and 17 mg per cigarette, respectively.

The examination of caecal bacterial flora was performed according to the method of previously reported by two of the present authors and their colleagues (Asahara et al., 2001). Briefly, One gram of caecal contents were placed in grinding tubes containing 1 ml of sterilized anaerobic transfer medium, and then homogenized with a Teflon grinder. After serial dilution of the caecal suspensions with an anaerobic buffer solution, 50 μl or 500 μl portions of the diluents were spread onto the following culture media (agar plate: 50 μl, roll tube agar: 500 μl). Heart infusion agar, supplemented with 0.2 mg/ml neomycin (Sigma, St Louis, MO, USA), 0.01% (w/v) brilliant green, 0.1% (w/v) sodium taurocholate, 0.03% (w/v) L-cysteine hydrochloride, and 5% (w/v) defibrinated horse blood (modified NBGT agar), was used for selective isolation of the Bacteroidaceae. CPLX agar was used for selective isolation of Bifidobacterium. LBS agar (Becton Dickinson and Company, Cockeysville, MD, USA), supplemented with 0.8% (w/v) Lab Lecmo powder (Oxoid Ltd., Basingstoke, UK), 0.1% (w/v) sodium acetatetrihydrate and 0.37% (w/v) acetate, was used for selective isolation for Lactobacillus. COBA agar was used for selective isolation for Enterococcus. DHL agar (Nissui Pharmaceuticals Co., Ltd., Tokyo, Japan) was used for selective isolation for Enterobacteriaceae. Statsphylococcus medium No. 110 agar (Nissui Pharmaceutical Co., Ltd.) was used for selective isolation for Staphylococcus and Bacillus. VL-G roll tube agar supplemented with 0.2% (w/v) cellobiose and 0.2% (w/v) maltose (modified VL-G roll tube agar) was used for determination of total anaerobic counts. Modified NBGT agar, CPLX agar, LBS agar were cultured under anaerobic conditions in an atmosphere of 7% H₂, 5% CO₂ in N₂ at 37°C for 72 hr. After incubation, the colonies on the plates were counted and Gram stained. Species and biotypes of the bacteria were identified with API systems (bioMerieux S.A., Montalieu-Vercieu, France): rapid ID 32 A for the Bacteroidaceae and Lactobacillaceae, API 20 STREP for the Enterococcaceae, API 20 E for the Enterobacteriaceae and API 20 STAPH for the Staphylococcaceae. The lower limit of bacterial detection with this procedure was 100 cfu/g caecal contents.

Scanning for anaerobic fujiform bacteria was carried out by microscopic bacterial counts. For quantification, 10 μl portions of the diluents were put into a 10-well immunofluorescent slide (Flow Laboratories, Inc., McLean, VA, USA), fixed and Gram-stained. Fujiform bacteria were counted with the aid of an ocular grid containing 100 squares (calibrated with a stage micrometer), a 100 × objective, and a 10 × ocular lens. Acceptable slides considered for analysis had to meet the following two criteria. (i) The bacteria appeared to be evenly distributed, and (ii) the number of bacteria per 100 grid squares was between 20 and 300. Counts were made in 10 fields chosen randomly.

The number of viable bacteria per gram wet weight of caecal contents was calculated. The lower limit of bacterial detection with this procedure was 100 cfu/g caecal contents. Results were expressed as the means ± standard deviation (S.D.) numbers of cfu per one g of caecal contents.
Detection of organic acids in caecal contents
The caecal contents were homogenized in 1 ml of distilled water, and homogenate was centrifuged at 13,000 × g at 4°C for 10 min. A mixture of 0.9 ml of the resulting supernatant and 0.1 ml of 1.5 mol/l perchloric acid in a glass tube was mixed well and allowed to stand at 4°C for 12 hr. The suspension was then passed through a filter with a pore size of 0.45 μm (Millipore Japan Ltd., Tokyo, Japan). The sample was analyzed for organic acids by high-performance liquid chromatography, as described in a previous report (Kikuchi and Yajima, 1992). The high-performance liquid chromatography was performed with a Waters system (Waters 432 Conductivity Detector; Waters, Milford, MA, USA) equipped with two columns (Shodex Rspack KC-811; Showa Denko Co., Ltd., Tokyo, Japan). The concentrations of organic acids were calculated using external standards, and the reproducibility and stability of these measurements have been reported (Kikuchi and Yajima, 1992).

Statistical analysis
Data were expressed as the means ± S.D. Comparisons of parameters between the two groups were made by the Mann-Whitney U test. A value less than 0.05 was considered to indicate a statistically significant difference.

RESULTS
Effect of exposure of cigarette smoke on body weight, body weight gain and food intake
The body weight of the smoking group was significantly less than that of the non-smoking group after 4 weeks of cigarette smoke exposure (346.0 ± 10.0 vs. 374.0 ± 16.2 g, respectively, p = 0.016) (Table 1), and this was related to the difference in body weight gain between the smoke-exposed group and the control group (34.0 ± 14.1 vs. 59.4 ± 2.2 g, respectively, p = 0.021). The food intake of the smoking group decreased significantly from the first week of the experimental period, and also was significantly reduced by about 10% compared with non-smoking group at the last experimental week (p = 0.020) (Table 1).

Effect of exposure of cigarette smoke on organic acid levels in caecal contents
Table 2 shows that in the smoking group, the total organic acid concentration in caecal contents was significantly less than in the non-smoking group (72.2 ± 9.4 vs. 107.6 ± 10.8 μmol, respectively, p = 0.009); specifically in acetic acid levels (44.4 ± 4.8 vs. 62.9 ± 6.3 μmol, respectively, p = 0.009), in propionic acid levels (7.8 ± 2.0 vs. 17.3 ± 3.3 μmol, respectively, p = 0.009), in butyric acid levels (6.7 ± 2.6 vs. 11.9 ± 2.2 μmol, respectively, p = 0.021), and in valeric acid levels (1.0 ± 1.1 vs. 2.4 ± 0.4 μmol, respectively, p = 0.036).

Effect of exposure of cigarette smoke on population of micro-flora and pH in caecal contents
Table 3 shows that in the smoking group, the population levels of Bifidobacterium in cecal contents was significantly less than in the non-smoking group (5.4 ± 1.4 vs. 7.2 ± 0.7 log_{10} CFU/g caecal contents, respectively, p = 0.036). However, for other kinds of micro-flora, there were no significant differences between the smoking group and the non-smoking group. The pH in caecal contents of the smoking group was significantly greater than that of the non-smoking group (7.1 ± 0.3 vs. 6.7 ± 0.1 μmol, respectively, p = 0.047).

DISCUSSION
This study demonstrated that not only certain organic acid levels but also the population of Bifidobacterium and the pH in caecal contents changed after exposure to cigarette smoke.

Table 1. Effect of cigarette smoke on body weight and food intake
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body weight (g)</th>
<th>Food intake (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-smoke</td>
<td>Smoke</td>
</tr>
<tr>
<td>Before exposure</td>
<td>314.6 ± 8.4</td>
<td>312.0 ± 10.8</td>
</tr>
<tr>
<td>After exposure</td>
<td>374.0 ± 16.2</td>
<td>346.0 ± 10.0*</td>
</tr>
</tbody>
</table>

The animals underwent enforced exposure to cigarette smoke for 4-week. The body weight was measured just before cigarette smoke exposure and after the last cigarette smoke exposure. The food intake from Monday to Friday was measured at the last week of smoke-exposure period. Each point indicates the mean ± S.D. of 5 animals. *p < 0.05: significant relative to the control, the non-smoking group, by Mann-Whitney U test.
Results are expressed as log 10 cfu, the mean ± S.D. per one g of mean ± S.D. of 5 animals. *p after the last cigarette smoke exposure. Each point indicates the Caecal contents were obtained from the animals within 24 hr relative to the control, the non-smoking group, by Mann-Whitney U test.

Caecal contents were obtained from the animals within 24 hr after the last cigarette smoke exposure. Each point indicates the mean ± S.D. of 5 animals. *p < 0.05, **p < 0.01: significant relative to the control, the non-smoking group, by Mann-Whitney U test.

Table 2. Changes in concentrations of organic acids in caecal contents after 4 weeks of cigarette smoke exposure

<table>
<thead>
<tr>
<th>Organic acids</th>
<th>Organic acid concentration (μmol/g caecal contents)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-smoking group</td>
</tr>
<tr>
<td>Total organic acids</td>
<td>107.6 ± 10.8</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>62.6 ± 6.3</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>17.2 ± 3.3</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>11.9 ± 2.2</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>8.4 ± 3.8</td>
</tr>
<tr>
<td>Valeric acid</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>1.5 ± 0.9</td>
</tr>
</tbody>
</table>

Table 3. Influence of 4 weeks of cigarette smoke exposure on the population of bacteria in caecal contents

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Number of bacteria (log_{10} cfu/g caecal contents)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-smoke</td>
</tr>
<tr>
<td>Total</td>
<td>10.2 ± 0.1</td>
</tr>
<tr>
<td>Fusiform bacteria</td>
<td>10.4 ± 0.1</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>9.3 ± 0.4</td>
</tr>
<tr>
<td>Bacteroidaceae</td>
<td>8.0 ± 0.5</td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td>7.2 ± 0.7</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>6.1 ± 0.6</td>
</tr>
<tr>
<td>Enterococcus</td>
<td>5.5 ± 0.3</td>
</tr>
<tr>
<td>Staphylococcus</td>
<td>5.4 ± 0.4</td>
</tr>
<tr>
<td>Bacillus</td>
<td>3.4 ± 0.3</td>
</tr>
</tbody>
</table>

Results are expressed as log_{10} cfu, the mean ± S.D. per one g of caecal contents for five animals. *p < 0.05: significant relative to the control, the non-smoking group, by Mann-Whitney U test.

Significant decrease was seen in acetic acid, propionic acid, butyric acid and valeric acid in the caecal contents of rats exposed to cigarette smoke. Organic acids are produced by micro-flora in the gut. In particular, acetic acid, propionic acid and butyric acid are SCFAs, which are primarily utilized by intestinal epithelial cells as energy substrates (Goodlad et al., 1989). In particular, butyric acid is not only the main energy source of the luminal muco-

sa but also has anti-inflammatory effects (Säemann et al., 2000). The present study did not show whether the decreased SCFAs had a deleterious effect on colon mucosa or not, because histological changes in colon mucosa in the smoking group were not recognized (results not shown). The effect of cigarette smoke on colon mucosa in patients with IBD remains unclear, because while cigarette smoke contributes to the development of Crohn’s disease (Persson et al., 1990), it improves mucosal lesions in cases of ulcerative colitis (Green et al., 1998). This study, which was performed only in normal animals, therefore did not attempt to show whether cigarette smoke had good or bad effects on colon mucosa with IBD. The effects of cigarette smoke on colon mucosa may vary according to the mucosal condition. Further investigations on interaction between cigarette smoke and colon mucosa are needed.

The pH in the caecal contents of the rats exposed to cigarette smoke was higher than that of non-smoking rats. This result has been linked to a decrease in organic acid levels (Asahara et al., 2004; Shimizu et al., 2006). The pH in the colon of patients with colonic cancer is more alkaline, indicating a reduction in colonic carbohydrate fermentation by organic acids (Fallingborg, 1999). Cigarette smoke promotes growth of colon cancer in mice model (Wong et al., 2009). The elevation in pH in the colon and the decreases in certain organic acids levels by cigarette smoke may be related to the development and growth of colon cancer.

Interestingly, this study demonstrated that the population of Bifidobacterium as well as certain organic acid levels was decreased. This is apparently the first study to show that the population of Bifidobacterium is decreased after exposure to cigarette smoke. Bifidobacterium not only protects against the growth of pathogenic bacteria (Asahara et al., 2004) but also helps counteract inflammatory bowel disease (Dotan and Rachmilewitz, 2005). Moreover it up-regulates the systemic immune response by activation of natural killer (NK) cytotoxicity (Chiang et al., 2000). It was previously demonstrated that in smokers, the activity of NK cells in peripheral blood is decreased (Kusaka et al., 1992). The decrease in population of Bifidobacterium in the gut may contribute to impairment in both local and systemic defenses among smokers. This study has not clarified the mechanism of how the Bifidobacterium population was decreased. Change in pH in the gut during exposure to cigarette smoke may contribute to the decrease in the population of Bifidobacterium. Further investigations about the effects of cigarette smoke on population of the micro-flora in the gut are needed.

The most important change in the gut environment revealed by this study seems to be a decrease in the con-
Cigarette smoke changes the gut environment in the caecum of rats

centration of organic acids. Organic acid formation by gut micro-flora varies according to host conditions and microbiologic factors. In this study, the decrease of organic acid levels was caused mainly by a decrease in food intake during exposure to cigarette smoke. In patients with systemic inflammatory syndrome (SIRS) (Shimizu et al., 2006), it was reported that the concentration of organic acids in caecal contents decreased accompanied with a decrease in micro-flora population. This study demonstrated that the population of Bifidobacterium, which produces mainly acetic acid (Macfarlane et al., 1995), was decreased after exposure to cigarette smoke. The decreased population of Bifidobacterium may partially contribute to the decreased level of organic acid levels of the rats exposed to cigarette smoke. Food intake in the smoking group decreased to only about 90% of the non-smoking group. It has been demonstrated that after even 40% food restriction does not change intestinal micro-flora in rats (Henderson et al., 1998). Besides a decrease in food intake and population of Bifidobacterium, changes in influx of dietary fiber or production of organic acids from dietary fiber by micro-flora may contribute to the decreased levels of certain organic acids by cigarette smoke. Further investigations about how cigarette smoke effects on production of organic acids by the micro-flora in the gut are needed.

One of the limitations of the present study is the small size of the animal groups. However despite that, both the population of Bifidobacterium and total organic acid levels remarkably decreased with statistical significance (p = 0.036, p = 0.009). A particular large difference (10^{7.2} vs. 10^{5.4} CFU/g caecal contents) was observed in the population of Bifidobacterium because the population was expressed as log scales. We therefore feel that the sample size of this study, although limited, is enough to provide some insight into changes in the gut environment caused by cigarette smoke.

Another limitation is the lack of extended chronological follow up of the population of the gut micro-flora and concentration of organic acid in caecal contents. The present study did not clarify precisely when cigarette smoke changed the gut environment. However the results of the present study do demonstrate that cigarette smoke changes the gut environment, underlining the need for further study on how cigarette smoke may change the gut environment in humans.

In conclusion, the caecal levels of acetic acid, propionic acid, butyric acid and valeric acid which are the organic acids metabolized by micro-flora significantly decreased in Wistar rats exposed to cigarette smoke for 4 weeks. Not only was there a decrease in certain organic acid levels but also in the population of Bifidobacterium while the pH was elevated. These results suggest that cigarette smoke may alter the gut environment.

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