Experimental enteropathy in athymic and euthymic rats: synergistic role of lipopolysaccharide and indomethacin

HIDEKI KOGA,1 KUNIHICO AYOYAGI,1 TAKAYUKI MATSUMOTO,1 MITSUO IIDA,2 AND MASATOSHI FUJISHIMA1
1Second Department of Internal Medicine, Faculty of Medicine, Kyushu University, Fukuoka 812-8582; and 2Division of Gastroenterology, Department of Medicine, Kawasaki Medical School, Kurashiki, Okayama 701-0192, Japan

Koga, Hideki, Kunihiko Aoyagi, Takayuki Matsumoto, Mitsuo Iida, and Masatoshi Fujishima. Experimental enteropathy in athymic and euthymic rats: synergistic role of lipopolysaccharide and indomethacin. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G576–G582, 1999.—The aim of this study was to investigate the immunologic and microbiological bases of indomethacin enteropathy. Athymic nude and euthymic specific pathogen-free (SPF) rats were reared under conventional or SPF conditions. In each group, indomethacin was given intrarectally for 2 days. Indomethacin enteropathy was evaluated using a previously described ulcer index and tissue myeloperoxidase activity. Both euthymic and athymic nude rats developed intestinal ulcers to the same degree under conventional conditions but not under SPF conditions. Pretreatment of conventional rats with intragastric kanamycin sulfate, an aminoglycoside antibiotic, attenuated indomethacin enteropathy in a dose-dependent fashion. Interestingly, when lipopolysaccharide was injected intraperitoneally in kanamycin-pretreated rats, it fully restored enteropathy in these rats in a dose-dependent manner. We confirmed that kanamycin decreased the number of gram-negative bacteria and endotoxin concentration of the small intestine in a dose-dependent fashion. These results indicate that indomethacin enteropathy is bacteria dependent and does not require a T cell function. Synergy between indomethacin and bacterial lipopolysaccharide may play a major role in this enteropathy.

indomethacin-induced enteropathy; T cell function; intestinal flora; gram-negative bacteria; endotoxin

INDOMETHACIN (Indo), a nonsteroidal anti-inflammatory drug (NSAID), is notorious for causing gastrointestinal injury (6). In experimental animals, Indo has been administered orally or subcutaneously to examine the pathogenesis of gastric lesions (8, 20, 48, 51, 54). We have previously investigated the effects of intrarectal administration of Indo and reported that mucosal injury was detected predominantly in the small intestine (37). This Indo enteropathy mimics the inflammatory enteritis seen in Crohn’s disease (4, 5, 16, 18, 35). Increased mucosal permeability, favorable effects of steroids and salicylatesulfapyridine, and preventive effects of polymeric or elemental diets are observed both in Indo enteropathy and in Crohn’s enteritis. Indo enteropathy seems to be a useful model for Crohn’s disease and can be expected to give clues to the pathogenesis of inflammatory bowel disease (3).

Various immunologic abnormalities have been reported in Crohn’s disease. Lymphocytes, particularly T cells, have been described as being activated in inflamed tissues or serum in Crohn’s disease (10, 12, 43). An interesting case of Crohn’s disease with remission of bowel disease after human immunodeficiency virus infection has been reported (26). The authors suggested that a decreased CD4 lymphocyte count may have a favorable effect on Crohn’s disease. Although Indo enteropathy has become an animal model for Crohn’s disease, it is not known whether T lymphocytes contribute to the pathogenesis of Indo enteropathy. We have previously reported that FK-506 inhibits Indo enteropathy more than cyclosporin A or prednisolone (36). FK-506 is a strong immunosuppressive agent that inhibits T lymphocyte function (42, 59). It is thus necessary to clarify whether Indo enteropathy is mediated by T cell-dependent immunity.

Because FK-506 was described originally as an antibiotic, we decided to investigate whether intestinal bacteria affect Indo enteropathy. In 1969, Kent et al. (29) first reported the role of intestinal bacteria in Indo enteropathy. They found that a combination of three antibiotics inhibits the overgrowth of microorganisms in the small intestine and reduces the severity of Indo-induced ulcers. Several studies have shown that germ-free rats develop fewer intestinal lesions than conventional or specific pathogen-free (SPF) rats, suggesting a bacterial role in this enteropathy (39, 65). However, although Satoh et al. (50) confirmed the favorable effects of these same antibiotics, they concluded that antibiotics reduced gastrointestinal ulcers by their cytoprotective effect, not by their antibacterial actions. Thus the role of bacteria in Indo enteropathy remains controversial.

We report here evidence for a bacteria-mediated role in Indo enteropathy independent of T cell immunity.

MATERIALS AND METHODS

Animals. Male Wistar rats, weighing 150–250 g, aged 6–8 wk, and male F344/n rnu/rnu rats (athymic nude rats), weighing 150–200 g, aged 6–7 wk, purchased from Kyushu Animal (Tosu, Saga, Japan), were used in this study. The animals were housed in wire cages with a maximum of six animals per cage.

Care environments and administration of Indo. Athymic nude rats and Wistar rats were reared under SPF conditions; the animals were placed in sterile cages in laminar flow racks and were given an autoclaved diet and autoclaved tap water.
ad libitum. After 1–2 wk of care and maintenance, 24 mg/kg of Indo (Sigma Chemical) dissolved in carboxymethylcellulose were injected in the rectum for 2 days according to a previously described method (37). Other nude and Wistar rats were cared for under conventional conditions; they were placed in ordinary, nonsterile cages in nonsterile rooms and were given a nonsterile diet and water. The same dose of Indo was administered intraperitoneally to these rats after 1–2 wk of care.

Effects of antibiotics on Indo enteropathy. Wistar rats maintained under conventional conditions were treated with an aminoglycoside antibiotic, kanamycin sulfate (KM; 1, 10, and 100 mg/day; Meiji Seika Kaisha), which was dissolved in distilled water and administered daily via a metallic orogastric tube. After 7 days administration of KM, Indo was administered as described above.

Effects of lipopolysaccharide on Indo enteropathy in antibiotic-treated rats. Wistar rats cared for under conventional conditions were given 100 mg of KM daily via a metallic orogastric tube. After KM pretreatment for 7 days, 100, 300, or 1,000 μg of lipopolysaccharide (LPS) extracted from Escherichia coli K-235 (LPS; Sigma Chemical) per 100 g body weight were injected intraperitoneally. Twenty-four hours after LPS administration, Indo was administered as described above.

Evaluation of gastrointestinal lesions. Twenty-four hours after the second dose of Indo, all animals were killed by intraperitoneal injection of an overdose of sodium amobarbital. The stomach, small intestine, and cecum were removed, opened by a longitudinal incision, and pinned out on a wax block. The specimen was washed with saline, fixed in 10% Formalin for 2 days, and checked for any macroscopic change.

The stomach and the cecum were evaluated according to whether ulcers were present or not. Gastric or cecal ulcers were defined as ulcers of >3 mm in the greatest dimension. In the small intestine, longitudinal ulcers, which were defined as ulcers >10 mm long located on the mesenteric side of the lumen, were numbered, and their length was measured. Scattered ulcers also were assessed. The longitudinal ulcer index (UI) was calculated as the total length of longitudinal ulcers divided by the whole length of the small intestine.

Measurement of tissue myeloperoxidase activity. Tissue myeloperoxidase (MPO) activity was assayed in homogenates of the small intestinal mucosa with a spectrophotometric assay using 3,3',5,5'-tetramethylbenzidine (TMB; Sigma Chemical) as substrate (55). In brief, 100–200 mg of small intestinal tissue were homogenized in a ground-glass homogenizer in 10 vol of ice-cold PBS (20 mmol/l KH2PO4; pH 7.4). The homogenate was centrifuged at 10,000 rpm for 15 min at 4°C, and the supernatant, which contained <5% total MPO activity, was discharged. The pellet was then rehomogenized in a total volume of 50 mmol/l PBS (pH 6.0) containing 0.5% hexadecyltrimethylammonium (HETAB; Sigma Chemical) and 10 mmol/l EDTA. The pellet-HETAB suspension was freeze-thawed two times. Aliquots of the suspension were added to a solution containing 1.6 mmol/l TMB, 0.3 mmol/l H2O2, 80 mmol/l sodium phosphate buffer (pH 5.4), 8% N,N-dimethylformamide (Sigma Chemical), and 40% PBS in a total volume of 500 μl. The mixture was incubated for 3 min at 37°C and then was immersed in an ice bath. The reaction was terminated by the addition of 1.75 ml of 200 mM sodium acetate buffer (pH 3.0). One unit of enzyme activity was defined as the amount of MPO present that caused a change in absorbance of 1.0/min at 655 nm and 37°C. The data were corrected with tissue weight.

Tissue endotoxin assay. Small intestinal tissue (100–200 mg) was removed under sterile conditions and was homogenized in 10 vol of ice-cold PBS. Endotoxin concentration was assayed in the homogenates with a chromogenic endotoxin-specific test (Endospec SP; Seikagaku Kogyo). Briefly, this test consists of factor G-free Limulus-amebocyte lysate and a chromogenic substrate, t-butyloxycarbonyl-Leu-Gly-Arg-p-nitroanilide. The sample was added to a portion of Endospec SP test solution and incubated. The sample was cleared by centrifugation and 0.2 ml of test solution was added and read at 405 nm at 37°C. The data were corrected with tissue weight.

Bacteriological examination of the small intestine. Small intestinal tissue (100–200 mg) was removed under sterile conditions and washed out rapidly and fully with PBS. The tissue was homogenized in 10 vol of PBS. The homogenate was diluted with Ringer solution. The aerobic plates were inoculated and incubated at 37°C for 24 h, and the anaerobic plates were incubated for 48–72 h of incubation. All isolates were identified by standard methods, and surface counts at the various dilutions were performed. The colony counts were expressed as colony-forming units per gram of tissue. Statistical analyses. Overall significance was determined by a one-way ANOVA for the length and number of small intestinal ulcers, MPO activity, endotoxin concentration, and bacteriological status and by χ2 test for the incidence of cecal ulcers. P values <0.05 were considered significant.

RESULTS

Gastrointestinal damage under different care environments. Table 1 summarizes the intestinal lesions resulting under various conditions. Wistar rats cared for under conventional conditions developed a mean longitudinal ulcer length of 213.1 ± 16.8 mm in the small intestine, whereas under SPF conditions the mean ulcer length was significantly decreased to 7.0 ± 21.0 (SD) mm. The length of the small intestine of SPF-maintained Wistar rats was significantly longer than that of conventionally maintained Wistar rats. The number of small intestinal scattered ulcers was significantly less in SPF-maintained Wistar rats than in conventionally maintained Wistar rats. The mean UI for the small intestine, as shown in Fig. 1, was 26.7 ± 3.0 in conventionally maintained Wistar rats vs. 0.6 ± 1.8 in SPF-maintained Wistar rats. In athymic nude

Table 1. Indomethacin induced small intestinal ulcers in rats maintained under various conditions

<table>
<thead>
<tr>
<th>Rat Type</th>
<th>Animal Condition</th>
<th>No. of Rats</th>
<th>Length of Small Intestine, mm</th>
<th>Length of Longitudinal Ulcers, mm</th>
<th>No. of Scattered Ulcers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wistar</td>
<td>Conventional</td>
<td>10</td>
<td>802.5 ± 55.4</td>
<td>213.1 ± 16.8</td>
<td>26.3 ± 3.4</td>
</tr>
<tr>
<td></td>
<td>SPF</td>
<td>9</td>
<td>1,049.3 ± 98.5‡</td>
<td>7.0 ± 21*</td>
<td>20.9 ± 5.1†</td>
</tr>
<tr>
<td>Athymic nude</td>
<td>Conventional</td>
<td>6</td>
<td>772.8 ± 49.8</td>
<td>210.8 ± 16.2</td>
<td>24.2 ± 8.2</td>
</tr>
<tr>
<td></td>
<td>SPF</td>
<td>6</td>
<td>921.2 ± 23.5‡</td>
<td>0‡</td>
<td>7.8 ± 4.1‡</td>
</tr>
</tbody>
</table>

Values are means ± SD. SPF, specific pathogen free. *P < 0.001 and †P < 0.05 vs. Wistar rats maintained under conventional conditions. ‡P < 0.001 vs. athymic nude rats maintained under conventional conditions.
rats, conventionally maintained rats had a mean ulcer length of 210.8 ± 16.2 mm, whereas SPF-maintained rats developed no longitudinal ulcers. The degree of small intestinal longitudinal ulceration seen in conventionally maintained nude rats was as severe as in conventionally maintained Wistar rats (Fig. 1). In both Wistar and nude rats, the small intestinal ulcers were located in the midportion of the small intestine. In the stomach, no ulcers were induced in Wistar or nude rats under any condition. Cecal ulcers were produced in one of nine SPF-maintained Wistar rats and in none of the SPF-maintained nude rats. In contrast, cecal ulcers were seen in 5 of 10 conventionally maintained Wistar rats and 4 of 6 conventionally maintained nude rats. These results suggest that environmental factors are more important in Indo enteropathy than T cell-dependent immunity.

Effect of antibiotics on Indo enteropathy. Wistar rats cared for under conventional conditions with 1, 10, or 100 mg of KM pretreatment developed mean ulcer lengths of 155.3 ± 60.6, 89.3 ± 42.8, or 50.9 ± 41.8 mm in the small intestine, respectively. The length of the entire small intestine in rats pretreated with 1, 10, or 100 mg of KM was 902.3 ± 52.4, 930.4 ± 37.4, or 934.8 ± 78.8 mm, respectively (Table 2 and Fig. 2). Scattered ulcers in the small intestine were not affected by KM pretreatment. The incidence of gastric or cecal lesions was unchanged by KM pretreatment. As another marker of the intestinal inflammation, we evaluated MPO activity of the small intestine. MPO activity in rats without KM pretreatment measured 91.3 ± 20.9 (SE) U/g, whereas in rats with 1, 10, and 100 mg of KM pretreatment, MPO decreased by 37.0, 73.6, and 83.7%, respectively (Fig. 3). The UI of the small intestine and MPO activity were well correlated. These data show that KM inhibits Indo enteropathy in a dose-dependent fashion, suggesting a bacterial etiology for this enteropathy.

Bacteriological status and endotoxin concentration of the small intestine in rats. In Wistar rats cared for under SPF conditions, tissue endotoxin concentration measured only 0.03 ± 0.01 µg/g (Fig. 4). However, in rats cared for under conventional conditions, endotoxin remarkably increased to 98.4 ± 42.4 µg/g. With 1, 10, and 100 mg of KM pretreatment, endotoxin decreased by 33.2, 90.8, and 99.9%, respectively. Table 3 shows the results of tissue cultures of the small intestine in rats under various conditions. KM pretreatment decreased the number of gram-negative bacteria, especially Bacteroides species, in a dose-dependent manner. Gram-positive bacteria was killed with the least dose of KM. These findings support that endotoxin derived from gram-negative bacteria may play a key role in Indo enteropathy.

Effects of LPS on Indo enteropathy in antibiotic-treated rats. After Indo administration subsequent to one injection of 100, 300, or 1,000 µg/100 g body wt of LPS, KM-pretreated Wistar rats had mean ulcer lengths of 97.6 ± 36.4, 150.6 ± 26.9, or 206.7 ± 26.4 mm, respectively, in the small intestine. The length of the small intestine in the LPS-treated groups was 139.3, or 888.9 ± 70.1 mm, respectively.

Table 2. Effect of kanamycin sulfate on indomethacin-induced enteropathy

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Rats</th>
<th>Incidence of Gastric Ulcers, %</th>
<th>Incidence of Small Intestinal Ulcers, %</th>
<th>Incidence of Intestinal Scattered Ulcers</th>
<th>Incidence of Cecal Ulcers, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indo only</td>
<td>10</td>
<td>10</td>
<td>100</td>
<td>26.3 ± 3.4</td>
<td>50</td>
</tr>
<tr>
<td>KM 1 + Indo</td>
<td>9</td>
<td>0</td>
<td>100</td>
<td>34.8 ± 14.5</td>
<td>0</td>
</tr>
<tr>
<td>KM 10 + Indo</td>
<td>8</td>
<td>0</td>
<td>100</td>
<td>31.0 ± 9.7</td>
<td>0</td>
</tr>
<tr>
<td>KM 100 + Indo</td>
<td>10</td>
<td>0</td>
<td>70</td>
<td>24.9 ± 13.2</td>
<td>30</td>
</tr>
<tr>
<td>Vehicle + Indo</td>
<td>8</td>
<td>0</td>
<td>100</td>
<td>27.8 ± 4.9</td>
<td>38</td>
</tr>
<tr>
<td>KM 100 + vehicle</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are means ± SD. Indomethacin (Indo; 24 mg/kg) was intraperitoneally administered two times at intervals of 24 h. Kanamycin sulfate (KM; 1, 10, or 100 mg/day) or distilled water (vehicle + Indo) was given for 7 days before Indo treatment. KM 100 + vehicle, 100 mg/day KM + carboxymethylcellulose.
The mean UI for the small intestine in the 1,000 µg LPS group returned to almost the same level seen in conventionally maintained rats without KM pretreatment, as shown in Fig. 2. The distribution of longitudinal ulcers was similar to that of conventionally maintained rats with no KM pretreatment and no LPS injection, i.e., the midportion of the small intestine. Wistar rats receiving 1,000 µg of LPS without Indo did not develop ulcers in the small intestine. The number of scattered ulcers in the small intestine was increased by LPS treatment in a dose-dependent manner. Rats treated with 1,000 µg of LPS developed many more scattered ulcers than LPS-untreated rats (41.7 ± 18.4 vs. 24.9 ± 13.2, \( P < 0.05 \)). No gastric ulcers were seen in any group. Cecal ulcers developed in 4 of 5 rats receiving 100 µg of LPS and in 6 of 7 rats receiving 300 or 1,000 µg of LPS, but in only 3 of 10 rats not receiving LPS. Rats receiving 300 or 1,000 µg of LPS tended to have a higher incidence of cecal ulcers than LPS-untreated rats, but this difference was without statistical significance (\( P = 0.07 \)). These findings suggest that LPS is involved in Indo enteropathy (Table 4).

**DISCUSSION**

Indo, an analgesic-antipyretic and anti-inflammatory agent, was introduced in 1963 for the treatment of rheumatoid arthritis and related disorders. The toxicity of this drug often provokes many adverse effects: gastrointestinal ulcerations, pancreatitis, central nervous system disorders, and hematopoietic reactions. Therefore, Indo has been used to experimentally induce ulcerations of the stomach and the intestine in animals. In most previous reports, Indo has been given orally or subcutaneously. With these methods of Indo administration, gastric ulcers are prominent and have been regarded as more important than intestinal ulcers. However, intrarectal administration of Indo, which we previously described in detail (37), chiefly induces longitudinal ulcers in the small intestine, mimicking Crohn’s disease.

Various reports have described mechanisms of Indo gastropathy. Indo inhibits cyclooxygenase activity, resulting in a decrease in endogenous cytoprotective prostaglandins and an increase in cytotoxic leukotrienes (32, 33, 46, 48, 63, 66). Prostaglandin \( \text{E}_2 \) or 5-lipoxygenase inhibitor has been reported to prevent NSAID-induced gastric ulcers (47, 61). Some studies have shown that Indo provokes gastric hypermotility (40, 56, 57). Agents blocking peristalsis, such as neomycin and urethan, also prevent Indo-induced gastric lesions. Recently, much attention has been paid to the role of oxygen-derived free radicals in many disorders. Several investigations have shown that antioxidants such as superoxide dismutase have a preventive effect on NSAID-induced gastropathy (15, 57, 61). Thus Indo gastropathy is a multifactorial disorder. Although these mechanisms of Indo gastropathy in part can be applied to Indo enteropathy, the precise mechanisms underlying the latter still remain unresolved.

Athymic nude rats carry an autosomal recessive mutation, resulting in an absent thymus (21). The resulting severe T cell deficiency causes profoundly impaired T cell and T cell-dependent reactivity. Athymic nude rats characteristically contain few CD4^-CD8^- intestinal intraepithelial lymphocytes (58). Normal B cell function has been observed in nude rats (9), and natural killer cell activity is also normal or higher than that in euthymic rats (14). Proliferation of intestinal mucosal mast cells, cytokine production, and the eosinophil response to parasites are also normal (2, 11, 41). Therefore, in some experiments, athymic nude rats have been used to investigate the role of T cells in the gastrointestinal tract (13, 17, 30, 38, 53). In both athymic nude rats and Wistar rats, conventionally maintained rats developed intestinal ulcers, whereas SPF-maintained rats did not. This suggests that Indo enteropathy is not related to T cell function but to environmental factors. The most important difference
in environmental factors between conventional and SPF conditions is the presence of bacteria. We treated rats with KM before Indo administration to sterilize the intestine. KM, an aminoglycoside antibiotic, was first produced and isolated in 1957 and is used for treating serious gram-negative bacterial infections. Orally administered KM is not absorbed, and its effect is limited to killing the gut flora. As expected, KM alone prevented Indo enteropathy in a dose-dependent manner in our study. Kent et al. (29) have reported that neomycin prevents Indo enteropathy, but they did not investigate what organisms may play a pathogenetic role. Although Satoh et al. (50) also have suggested a role for gram-negative bacteria in Indo enteropathy, they did not investigate this in detail. They did report that neomycin prevents Indo enteropathy but not its antibacterial action but by a cytoprotective effect. The favorable effect of oral KM in our study suggests a close relation between intestinal gram-negative bacteria and enteropathy. Actually, we confirmed that oral KM decreased the number of gram-negative bacteria and tissue endotoxin concentration in a dose-dependent manner. However, because antibiotics have various actions, a bacterial etiology for Indo enteropathy cannot be confirmed only by the preventive actions of KM. For example, neomycin is reported to accelerate orocecal transit in patients with hepatic cirrhosis (62), suggesting that neomycin increases intestinal and gastric motility (40).

To clarify the nature of bacterial involvement in Indo enteropathy, we injected LPS intraperitoneally before Indo administration in KM-pretreated rats because LPS is the major physiologically active substance derived from bacteria, especially the gram-negative bacteria frequently seen as part of the gut flora. We observed that LPS restored Indo enteropathy in a dose-dependent fashion. With 1,000 µg of LPS, Indo enteropathy returned almost to the level seen without KM treatment. This finding strongly suggests a role for bacteria, especially those containing LPS, in this enteropathy. In several knockout mouse models of inflammatory bowel disease, the presence of gut flora is necessary for the development of mucosal injury (34). Our results would support these previous studies.

Inhibition of cyclooxygenase is widely accepted as a major pharmacological effect of Indo, resulting in changes in prostanooids (32, 33, 48, 66). However, these changes in prostanooids may not affect the intestine directly, because Indo alone produced few ulcers in this study. This result suggests the presence of other pathways in forming intestinal ulcers. Our study demonstrates that intraperitoneal LPS and intrarectal Indo affected the small intestine. This phenomenon may support the importance of bile in Indo enteropathy, because Indo undergoes enterohepatic recirculation (19, 52) and because bile is rich in LPS (45). In Indo enteropathy, histological examination shows early infiltration of eosinophils and neutrophils (1). We had questioned why leukocyte infiltration and accumulation is induced by Indo administration despite Indo being an anti-inflammatory agent that can inhibit leukocyte accumulation. Because LPS is a strong chemotactic substance that promotes leukocyte accumulation (7, 24, 25, 31, 64), our observation that LPS is involved in Indo enteropathy may resolve this question. Although LPS also acts as a leukocyte stimulator (22, 23,
LPS AND T CELLS IN INDOMETHACIN ENTEROPATHY IN RATS


15. Del Soldato, P., D. Boschi, G. Benori, and C. Scarpignato. Oxygen free radicals interact with indomethacin to cause gastrointestinal injury and thrombosis. This may reinforce our results and hypothesis. Further investigations are required to elucidate the precise mechanism(s) of this experimental enteropathy.

REFERENCES


G582

LPS AND T CELLS IN INDOMETHACIN ENTEROPATHY IN RATS


