Elemental Diet Regulates Intestinal Permeability and Antibody Production in Indomethacin-Induced Intestinal Injury Rats

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Summary

Crohn’s disease is a type of inflammatory bowel disease of unknown etiology. Administration of indomethacin (Indo) to rats induces acute mucosal lesions similar to those observed in Crohn’s disease patients, but the damage can be prevented by feeding the animals an elemental diet (ED). In this study, we examined changes in intestinal macroscopic appearance, permeability, and immunoglobulin production after administration of Indo to male Sprague-Dawley rats fed normal lab chow or an ED. Intestinal damage was induced by subcutaneous injection of Indo on two successive days. Mucosal permeability, as measured by urinary excretion of phenolsulfonphthalein, peaked on day 2 after Indo injection, whereas the most severe intestinal damage, as scored by macroscopic inflammatory changes, was observed on day 3. Flow cytometric analysis of mesenteric lymph node cells revealed that the proportion of CD45RA+ cells was increased after Indo treatment. Furthermore, in vitro-cultured mesenteric lymph node and spleen lymphocytes from Indo-treated rats produced higher levels of IgA and IgG than did cells from vehicle-treated rats. In contrast, IgG and albumin concentrations in plasma were significantly decreased by Indo administration. Notably, none of the Indo-induced changes was observed in ED-fed rats. These findings suggest that an ED may prevent the appearance of Indo-induced mucosal lesions, at least in part, by modulating intestinal permeability and antibody production.

Key Words

Crohn’s disease, indomethacin, elemental diet, immunoglobulin, intestinal permeability

Crohn’s disease (CD) is an inflammatory bowel disease of unknown etiology. A number of factors have been suggested to be involved in disease development, including genetic predisposition, environmental factors, immune dysfunction, food-derived antigens, and bacterial flora (1–4). The intestinal epithelium is constantly exposed to bacteria, pathogens, and other foreign antigens such as food components (5, 6). As a result, the intestinal mucosa has a complex immune system that protects against various pathogens and functions as a barrier to prevent the invasion of toxic substances from the lumen (6–9). Dysfunction of the epithelial barrier increases intestinal permeability, which may promote uptake of luminal antigens and thus induce massive activation of immune cells and secretion of proinflammatory cytokines such as tumor necrosis factor-alpha (10–12). Immune system dysfunction is believed to be the primary pathogenic driver of CD. In particular, aberrant activation of T cells is an important component of immune dysfunction, and CD patients are reported to display dominant T helper type 1 immune responses (11, 13–15).

Administration of indomethacin (Indo), a nonsteroidal anti-inflammatory drug, to experimental animals induces intestinal ulcers similar to those seen in CD patients (16, 17). Previous work has demonstrated that an elemental diet (ED) can significantly reduce such Indo-induced intestinal damage (18, 19). An ED contains free amino acids as a nitrogen source and is low in fat; thus, it can be readily absorbed without the need for digestion (20). Indeed, EDs are commonly used as a nutritional therapy for CD in Japan, and they have proven efficacious in achieving and maintaining remission (21, 22). A number of hypotheses have been proposed to explain the beneficial effects of an ED in CD patients, including improvement of nutritional status, reduction in immune activity, and modulation of intestinal microflora (20, 23, 24).

In this study, we sought to clarify the effectiveness of an ED in CD by evaluating changes in intestinal permeability and immunoglobulin production in rats administered Indo and fed either normal chow or an ED.

MATERIALS AND METHODS

Animals and Indo treatment. Male Sprague-Dawley rats were purchased from Kyudo (Saga, Japan) and were housed in individual cages. All rats were maintained on a 12 h light/dark cycle and were allowed access to

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food and water ad libitum. Control animals were fed normal laboratory chow (LC; CE-2, CLEA Japan, Inc., Tokyo) and the ED group were fed Elental® chow (EA Pharma Co. Ltd., Tokyo). Total numbers of animals used in this study were 106. To evoke intestinal damage, Indo was dissolved in 5% NaHCO₃ and administered to rats (n = 5/group unless specified in the legends) by subcutaneous injection (9 mg/kg body weight) once daily for two consecutive days. Control rats were injected with the same volume of 5% NaHCO₃. Intestinal damage and permeability were evaluated on LC-fed vehicle-treated (LC-Cont) rats or LC-fed Indo-treated (LC-Indo) rats on days 0, 1, 2, 3, and 7 after Indo or vehicle administration. We also examined groups of ED-fed rats at day 3 (ED-Cont and ED-Indo groups, respectively). For evaluation of lymphocyte IgA/IgG production, lymphocyte subpopulations, and plasma IgA/IgG concentrations, 4–8 rats/group were analyzed on day 3 after Indo or vehicle administration on LC and ED fed rats. Moreover, for evaluation of plasma IgA/IgG concentrations, 5 rats/group were analyzed on day 3 after Indo or vehicle administration on LC and ED fed rats. Next, plasma IgA/ IgG and albumin concentrations were also assessed on days 0, 1, 2, 3, and 7 after vehicle or Indo administration to evaluate daily changes of those. For determination of intestinal IgA/IgG and albumin concentrations, the small intestine contents were collected by washing with 50 mL phosphate-buffered saline (PBS). After centrifugation at 2,100 × g for 15 min, the supernatants were mixed with 5% soybean trypsin inhibitor (Sigma-Aldrich, St. Louis, MO) and stored at −30°C until analysis. The intestinal IgA, IgG, and albumin contents are expressed as total amount per animal (50 mL PBS). This study was approved by the Institutional Animal Care and Use Committee of Prefectural University of Kumamoto (No. 24-05, 25-03, 26-04).

Evaluation of intestinal damage and permeability. Macroscopic intestinal damage was assessed by scoring four items (Table 1) as described by Chen et al. (25). Intestinal permeability was assessed by measuring urinary excretion of phenolsulfonphthalein (PSP) after oral administration of 20 mg PSP (19, 26) via a gastric tube. Urine was collected over the next 24 h, brought to 50 mL with distilled water, and centrifuged at 2,100 × g for 10 min. The supernatants were then alkalinized with 1 M NaOH, and the PSP concentration was measured as the absorbance at 560 nm using a spectrophotometer. Permeability is presented as the percentage of the oral dose excreted in urine.

Preparation and analysis of lymphocytes. Lymphocyte culture and analysis were performed as described by Yamamoto et al. (27). In brief, mesenteric lymph nodes (MLN) and spleens (SPL) were removed and minced in RPMI 1640 medium (Life Technologies, Gaithersburg, MD). The tissue suspensions were then centrifuged at 1,300 × g for 30 min over Lymphocyte-Rat Separation Medium (Cedarlane, Hornby, Canada), and the lymphocyte layer was removed and washed with RPMI 1640 medium. The lymphocytes were resuspended at 2 × 10⁶ cells/mL in RPMI 1640 medium containing 10% fetal bovine serum and cultured for 24 h at 37°C in a 5% CO₂ atmosphere. The culture supernatants were collected and analyzed for IgA/IgG content by enzyme-linked immunosorbent assay (ELISA).

MLN and SPL lymphocyte subpopulations were analyzed by flow cytometry using a FACScalibur HG (Becton Dickinson) (27). For staining, the lymphocytes were isolated as described above, resuspended at 1.2 × 10⁷ cells/mL, and incubated on ice for 30 min with phycoerythrin- or fluorescein isothiocyanate-conjugated monoclonal antibodies against CD3, CD4, CD8, and CD45RA. The cells were then washed three times with PBS, resuspended in 0.5 mL PBS containing 0.5% paraformaldehyde, and stored at 4°C until analysis.

Measurement of immunoglobulin concentrations. IgA and IgG concentrations in the intestinal contents (PBS extracts), culture supernatants, and plasma samples were measured by ELISA (27). In brief, 96-well plates were coated with goat anti-rat IgA (Bethyl Laboratories, Montgomery, TX) and goat anti-rat IgG (Protos Immunoresearch, Hameenlinna, Finland), captured antibodies for 1 h at 37°C, and were washed, and blocked with PBS containing 2% cold water fish gelatin (Sigma-Aldrich) and 0.05% Tween 20 (PBST) at 4°C overnight. Aliquots of the test samples or IgA/IgG standards were added to the wells and incubated for 1 h at 37°C. After washing with PBST, horseradish peroxidase-conjugated goat anti-rat IgA (Bethyl Laboratories) or goat anti-rat IgG (MP Biological, Santa Ana, CA) diluted in blocking solution were added to the wells and the plates were incubated for 1 h at 37°C. The plates were then washed again, and a chromogenic substrate was added (0.006% H₂O₂ in 0.2 M citrate buffer, pH 4.0, distilled water, and 3-ethylbenzthiazoline-6-sulfonic acid at a ratio of 10 : 9 : 1 by vol). The plates were incubated at 37°C until the color had developed, and 1.5% oxalic acid was added to stop the reaction. Finally, the absorbance at 415 nm was measured using a spectrophotometer.

Measurement of albumin concentrations. Albumin concentrations in plasma were measured using the bromocresol green (BCG) method. In brief, 20 μL of plasma or a standard bovine serum albumin solution (4 g/dL) was mixed with 5% soybean trypsin inhibitor (Sigma-Aldrich) and 0.05% Tween 20 (PBST) at 4°C overnight. The mixture was then centrifuged at 2,100 × g for 15 min, the supernatants were alkalinized with 1 M NaOH, and the PSP concentration was measured as the absorbance at 490 nm using a spectrophotometer.

Table 1. Macroscopic damage scores.

<table>
<thead>
<tr>
<th>Score</th>
<th>Score</th>
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<tbody>
<tr>
<td>Adhesive lesions</td>
<td>None</td>
</tr>
<tr>
<td>Minimal</td>
<td>1</td>
</tr>
<tr>
<td>Involving several bowel loops</td>
<td>2</td>
</tr>
<tr>
<td>Intestinal wall thickness</td>
<td></td>
</tr>
<tr>
<td>0 &lt;1 mm</td>
<td>1</td>
</tr>
<tr>
<td>1–3 mm</td>
<td>2</td>
</tr>
<tr>
<td>&gt;3 mm</td>
<td>3</td>
</tr>
<tr>
<td>Hyperemia</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1</td>
</tr>
<tr>
<td>Recognized</td>
<td>2</td>
</tr>
<tr>
<td>Single ulcer</td>
<td>3</td>
</tr>
<tr>
<td>Ulcers</td>
<td></td>
</tr>
<tr>
<td>0 None</td>
<td>1</td>
</tr>
<tr>
<td>1 Single ulcer</td>
<td>2</td>
</tr>
<tr>
<td>2 Multiple (&lt;10 cm)</td>
<td>3</td>
</tr>
<tr>
<td>3 Multiple (&gt;10 cm)</td>
<td>4</td>
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were mixed with 6 mL of BCG color reagent (citrate buffer, BCG stock solution, and Brij-35) and incubated for 30 min at room temperature. Absorbance at 630 nm was measured using a spectrophotometer. Albumin concentrations in the PBS intestinal content extracts were measured using a Rat Albumin Quantitation kit (Bethyl Laboratories).

**Statistical analysis.** Data are presented as the mean±standard deviation (SD). Group differences were compared using one-way analysis of variance followed by Bonferroni’s or Dunnett’s multiple comparisons test. All analyses were performed using SPSS software (version 22) (IBM Corporation, Armonk, NY).

**RESULTS**

**Macroscopic intestinal damage and intestinal permeability**

In rats fed LC, intestinal lesions increased significantly between day 2 and 4 after Indo administration, with the
most severe damage observed on day 3. In contrast, intestinal permeability, as measured by urinary excretion of PSP, peaked on day 2 after Indo administration (Fig. 1). In the ED-fed group, however, these changes were suppressed. The results of intestinal damage score is 0 (ED-Cont group) and 0.260.45 (ED-Indo group), permeability of PSP is 2.060.44 (ED-Cont group) and 2.640.93 (ED-Indo group).

IgA and IgG production by MLN and SPL lymphocytes
MLN and SPL lymphocytes were isolated from treated rats and cultured in vitro for 24 h. IgA and IgG concentrations were higher in the culture supernatants of cells from the LC-Indo rats compared with LC-Cont rats. In contrast, there were no differences in IgA or IgG production by cells isolated from the ED-Cont and ED-Indo groups (Fig. 2).

Lymphocyte subpopulations
Flow cytometric analysis of lymphocytes showed decreased populations of CD3+ and CD4+ cells and an increased population of CD45RA+ cells in the MLNs isolated from LC-Indo compared with LC-Cont rats. Notably, these Indo-induced changes were not seen in the ED-fed rat groups. Moreover, Indo had no effects on the SPL lymphocyte populations in either the LC or ED groups (Fig. 3).

IgA, IgG, and albumin concentrations
Plasma IgA, IgG, and albumin levels were analyzed 3 d after the rats were treated with vehicle or Indo (Fig. 4). We found that plasma IgG but not IgA concentrations were significantly lower in the LC-Indo group compared with the LC-Cont group (Fig. 4A and B). To evaluate whether this change was specific for IgG, we measured plasma albumin concentrations and observed that the levels were also significantly lower in the LC-Indo group than in the LC-Cont group. Interestingly, these Indo-induced reductions in IgG and albumin were not observed in the ED-fed groups. Plasma IgA concentrations were lower in the ED-Cont group than the LC-Cont group, but no effect of Indo administration was detected in either group. We also analyzed daily changes in IgG and albumin concentrations in plasma and the small intestine contents. Consistent with the results described above, Indo administration caused a significant decrease in plasma IgG concentrations, with nadir levels detected on day 3 (Fig. 5B), and in plasma albumin concentrations (Fig. 5C). In contrast, IgG and albumin levels in the small intestine contents were increased by Indo administration (Fig. 5E, F).

DISCUSSION
In this study, we investigated the effects of Indo treatment and ED feeding on various features of CD in rats. Specifically, we evaluated Indo-induced intestinal inflammation and damage in LC- and ED-fed rats, as indicated by analysis of macroscopic damage, permeability, lymphocyte subsets in the MLN and SPL, and levels of IgA, IgG, and albumin in the small intestine and plasma. Previous work reported that Indo administration induces mucosal ulceration and increases intestinal permeability in rats (28). Here, we demonstrated an increase in mucosal PSP permeability and macroscopic damage after Indo administration in rats, which peaked on days 2 and 3, respectively. These results suggest that the effect on mucosal permeability preceded the intestinal damage. In contrast, animals fed an ED were completely protected from the destructive effects of Indo on the intestine.

CD is thought to be caused, at least in part, by an aberrant immune response to intestinal microbiota and dietary antigens (29). To assess this, we analyzed Indo-induced changes in plasma IgA/IgG levels, the abundance of MLN and SPL lymphocytes, and their production of IgA/IgG ex vivo. We found that Indo decreased the proportion of CD3+ and CD4+ cells but increased the abundance of CD45RA+ cells in the MLNs. In addition, MLN lymphocytes from Indo-treated animals produced more IgA/IgG in vitro than did their counterparts from vehicle-treated rats. Since B cells also express CD45RA, it is possible that the elevated proportion of
CD45RA^+^ cells may include B cells, which could explain the observed increase in IgA/IgG production after Indo administration. However, we did not examine this directly in the present study, and a more detailed analysis will be needed to confirm our hypothesis. Similarly, although CD4^+^ T cells are known to play an important role in the pathogenesis of CD (30, 31), we observed a decrease in the proportion of CD4^+^ cells among MLN lymphocytes in the LC-Indo rats compared with LC-Cont rats. Further studies are also needed to confirm and investigate this unexpected observation.

Interestingly, although Indo treatment increased IgA/IgG production by MLN lymphocytes ex vivo, plasma IgG concentrations in the rats were markedly reduced compared with vehicle-treated rats. This was also the case for albumin, another major plasma protein. We hypothesize that the reductions in plasma IgG and albumin levels may result from plasma protein leakage into the intestinal lumen of Indo-treated rats, as supported by the observed increase in intestinal permeability. CD patients are often reported to be hypoalbuminemic and hypoglobulinemic, which is thought to be caused by malnutrition and the use of immunosuppressive agents (32, 33). However, their causes are still incompletely understood. Our results suggest that increased intestinal permeability may be one of the triggers for hypoalbuminemia and hypoglobulinemia in CD patients (33, 34). Unlike IgG and albumin, plasma IgA concentrations did not decrease after Indo administration. IgA is a secretory antibody that is in complex with a secretory component (35). Although the precise relationship between Indo and IgA secretion is not clear, it has been speculated that Indo does not affect the secretory mechanism. Further investigation into this process will be necessary.

As for plasma IgA concentrations being low in the ED-fed group, it is thought that this is caused by lower IgA production capability than for the LC group.

Increased intestinal permeability may promote the invasion of food allergens and bacterial components that can induce or aggravate various diseases (36–38). Epithelial uptake of protein antigens is elevated in the non-involved ileum of CD patients, which could provoke an immune response (39, 40). In our study, an Indo-induced increase in intestinal permeability may be related to the elevated levels of IgA/IgG production by MLN lymphocytes shown here. An Indo-induced
increase in intestinal permeability may promote the invasion of antigens and intestinal bacteria and increase the production of the specific antibody against them. There are some reports of serum IgG antibodies in patients with IBD and experimental animals. Bentz et al. suggested that a nutritional intervention diet based on serum IgG antibodies against food antigens showed effects with respect to stool frequency, abdominal pain and general well-being for CD patients, and the authors have considered it is due to enhanced invasion of food antigens according to disruption of epithelial barrier (41). Kawaguchi et al. reported that in CD colitis mice, intestinal inflammation was induced by food antigens with high serum IgG levels and was ameliorated by the elimination of food antigens (42). In the present study, we did not show the pathological effects of elevated IgG production in MLN of intestinal damaged rats. More work is necessary to confirm whether the passage of food allergens across the intestinal barrier is altered and to determine antigen specification of the produced antibodies after Indo administration.

In contrast to the animals fed an LC, the ED-fed rats did not display notable intestinal damage or immunological changes following Indo administration. Sanderson et al. have shown that lactulose/rhamnose permeability is altered in CD patients receiving an ED, and the authors speculate that the diet had a direct effect on intestinal physiology (43). Furthermore, EDs have been shown to reduce acute inflammation and intestinal permeability (as measured by a $^{51}$Cr-EDTA clearance assay), likely as a result of interactions between several factors (44). Along these lines, analysis of mucosal tissue samples from CD patients has shown that an oral polymeric diet contributes to downregulation of proinflammatory cytokines (45), and another study demonstrated direct effects of an enteral diet on cytokine production and IL-1 receptor antagonist/IL-1β ratios in vitro (45, 46). However, despite many reports of the efficacy of elemental and enteral diets on CD, the mechanisms underlying the beneficial effects of an ED remain unknown. We speculate that EDs may prevent intestinal lesions by stabilizing intestinal permeability, thereby reducing the abundance of luminal antigens and suppressing immune activation. The absence of protein in EDs may be an important contributor to their protective effects. Moreover, it has been reported that EDs reduce the diversity and composition of microbiota, which might also contribute to the suppression of inflammation observed in colitic mice after transfer of IL-10 deficient cells (47). Therefore, it is possible that an ED modulates intestinal permeability via effects on the intestinal microbiome. Studies are ongoing to assess the intestinal microbiota of ED-fed compared with LC-fed rats and to determine the intestinal permeability of macromolecules, including dietary antigens.

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