Intestinal motor disorders associated with cyclical bacterial overgrowth in a rat model of enteritis

Monica Porras, María Teresa Martín, Mercè Soler, and Patri Vergara


The aims of this study were: 1) to obtain an experimental model reproducing the characteristics of chronicity and spontaneous relapses found in inflammatory bowel disease (IBD) and 2) to correlate these changes with intestinal motility and bacterial translocation. For this purpose, two groups of Sprague-Dawley rats were used: a treated group that received two subcutaneous injections of indomethacin (7.5 mg/kg) 48 h apart and a control group that received saline. Blood leukocytes, TNF, and fecal parameters were monitored for 90 days after treatment. In treated rats, a cyclic oscillation of blood leukocytes and TNF concomitant with an inverse relation of fecal output was observed. Treated rats were then selected either during their highest or lowest blood leukocyte values for motor activity and microbiological evaluation. Controls were obtained in age-matched rats. Rats with high leukocyte levels showed a decrease of motor activity. In contrast, animals with low leukocyte levels presented hypermotility. Bacterial overgrowth accompanied by bacterial translocation was found in the group with high leukocytes, whereas no differences were observed between the control and indomethacin groups during the lowest leukocyte phase. We obtained a model of IBD characterized by a chronic cyclic oscillation of intestinal motility, flora, and inflammatory blood parameters. During the high-leukocyte stage, motor activity decrease is related to bacterial translocation. This phase is followed by a reactive one characterized by hypermotility associated with a decrease in both bacterial growth and leukocytes. However, as in IBD, this reaction seems unable to prevent a return to relapse.

INFLAMMATORY BOWEL DISEASE (IBD) in its more common forms, Crohn’s disease (CD) and ulcerative colitis, is characterized by presenting alternative phases of activity and quiescence and by its chronicity (11). Several studies (12, 22, 29) indicate an abnormal immune reactivity to intestinal flora as the cause for the inflammation, probably caused by impairment of the mucosal barrier function.

Systemic administration of indomethacin in the rat has been used to cause inflammation of the small intestine, which presents some histopathological similarities to lesions found in CD (5, 37). It has been suggested that inhibition of cyclooxygenase activity induced by this drug alters mucosal permeability (7), facilitating the entry of bacteria and other harmful substances into the lamina propria and producing inflammation (24). Luminal bacteria seems to be critical to the induction of intestinal damage, because both germ-free rats (24) and animals treated with antibiotics (8) develop minimal lesions after the administration of indomethacin.

Although IBD patients present symptoms such as abdominal pain and diarrhea that suggest motor alterations, few motility studies have been performed (2). Moreover, it has been demonstrated that changes in motility are concomitant with inflammation, constituting a self-defense mechanism against the aggression (10, 34). Morphological and functional changes at the neuroendocrine structures controlling intestinal motility have also been observed using other models of inflammation such as those induced by nematode infection (30, 34).

Thus the objectives of our study were: 1) to obtain a chronic model of enteritis using indomethacin, 2) to look for indicators of both active and reactive phases of the disease that can be easily determined, 3) to study both intestinal motility during both phases of the disease, 4) to evaluate the physiological mechanisms involved in the cyclical motor changes, and finally 5) to correlate motor changes with markers of inflammation, bacterial growth, and changes in bacteria location in the intestinal area.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Charles River, Lyon, France), 8–10 wk old and weighing 300–350 g, were kept under conventional conditions in an environmentally controlled room (20–21°C, 12:12 h light-dark cycle) with water and standard laboratory rat chow available ad libitum. All experimental protocols were approved by the Ethical Committee of the Universitat Autònoma de Barcelona.

Experimental Model

Rats were randomized in two groups: 1) indomethacin group (n = 25) receiving two subcutaneous injections of indomethacin (7.5 mg/kg) 48 h apart and 2) control group (n = 12) receiving saline. Dosage of indomethacin was decided according to a preliminary validation study designed to indicate a dosage that would produce a moderate inflammatory reaction with absence of ascitis and a average body weight loss of not more than 10%. Body weight, food consumption, and number of fecal pellets were monitored daily. Blood samples were taken every 3 days between 10:00 and 11:00 AM to monitor both blood leukocytes (BL) by using a Neubauer chamber and TNF by a specific ELISA (rat TNF-α; Biosource International, Camarillo, California) with a minimum detectable concentration of 4 pg/ml. The BL cyclic oscillation observed in indomethacin-treated rats enabled the selection of animals according to their BL level: 50% at high blood level (Hbl) an 50% at low blood level (Lbl). Studies were performed in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Address for reprint requests and other correspondence: M. T. Martin, Edificio V, Unidad de Fisiologia, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain (E-mail: maite.martin@ub.es).
at different times, starting 15 days after and ≤90 days after treatment. An additional group of rats \((n = 8)\) was studied just 2 days after indomethacin treatment to evaluate the lesions induced and the acute motility changes.

**Motility Studies**

Motor activity was evaluated in indomethacin-treated rats at acute phase \((n = 4)\), Hbl \((n = 8)\), and Lbl \((n = 9)\) as well as in the control group \((n = 8)\).

**Animal preparation.** At 12:00 AM, after a fasting period of 6 h, animals were anaesthetized by inhalation of halothane to allow cannulation of the right jugular vein as previously described (34). Level III of anesthesia was maintained with intravenous thiopental sodium as required. A laparatomy was performed to suture three strain gauges in each animal. Follow-up studies. Rats were killed by CO2 inhalation, and pieces of distal duodenum, jejunum, ileum, and colon were obtained under sterile conditions.

**Bacterial growth.** Intestinal contents from 2-cm pieces of each segment from control animals was considered to be the value of reference for that segment. Samples from indomethacin rats showing a result 10-fold higher than the control group were considered positive.

**Histopathological Study**

Samples of duodenum, jejunum, ileum, and colon were taken from the same animals used in microbiological studies. Tissues were fixed in 10% neutral buffered formalin and processed for histopathology according to standard procedures. Signs of inflammation were evaluated by hematoxylin and eosin staining.

**Drugs and Solutions**

Indomethacin (Sigma, St. Louis, MO) was dissolved in absolute ethanol (50 mg/ml) and diluted in a 0.1 M NaHCO3 solution to obtain a 7.5 mg/kg dose in 0.3 ml final volume. CCK-8 (Peptide Institute, Osaka, Japan), was diluted in 1% NaHCO3 solution to a concentration of 10−4 M and in buffered saline solution to work concentration. Atropine (Merck, Darmstadt, Germany) and L-NNA (Sigma) were diluted in saline solution.

**Data Analysis**

Data were expressed as means ± SE. Motor response was quantified measuring the area under the curve delimited by the tracing, and the results were expressed in square millimeters. Results of animal monitoring and of motility obtained in acute studies were analyzed using an unpaired t-test. Data obtained from motility studies at chronic stages were analyzed using ANOVA and Bonferroni’s post hoc test. In all cases, differences were considered significant when \(P < 0.05\).

**RESULTS**

**Animal Monitoring**

Two days after treatment, the indomethacin group showed a decrease of food consumption \((9.0 ± 1.9 \text{ vs. } 29.4 ± 0.6 \text{ g in control group, } P < 0.05)\) related to a reduced body weight gain (expressed as percentage of the initial weight) \((-8.3 ± 1.2 \text{ vs. } 5.9 ± 0.5%, P < 0.01)\). After the first week, these parameters returned to normal values. Moreover, a high increase of blood leukocyte levels was also observed, from 10,872 ± 140 to 25,695 ± 707 cells/mm3, followed by a cyclic oscillation of this parameter (Fig. 1A). This oscillation determined a high leukocyte \((19,614 ± 222 \text{ cells/mm}^3; \text{Hbl})\) and a low leukocyte \((15,369 ± 259.7 \text{ cells/mm}^3; \text{Lbl})\) phase. Despite observation of a steady decrease over the monitored 90 days, no animal was free of this cyclical oscillation. In contrast, BL concentration was quite constant in the control group \((10,872 ± 140 \text{ cells/mm}^3; \text{Fig. 1B})\), except for the normal decrease observed with aging \((28)\). In a similar way, TNF in the same blood samples showed a parallel cyclical oscillation of its value in indomethacin rats, whereas TNF concentration was undetectable in control animals (Fig. 1, A and B).

Fecal pellets increased 24 h after the first dose of indomethacin \((51.0 ± 0.8 \text{ pellets vs. } 45.0 ± 1.2 \text{ pellets in control group, } P < 0.05)\). Afterward, a cyclical oscillation in this parameter
was also observed, showing an inverse correlation with leukocyte and TNF values (Fig. 1C).

Motility Studies

Chronic-phase rats were studied from 15 to 90 days after treatment, and animals were selected at Hbl or Lbl phases. Acute-phase rats were studied at 2 days after indomethacin treatment.

Chronic phase. SMA. In the control group, SMA was characterized by isolated phasic contractions occurring at regular frequency. Treated rats at the Lbl phase presented an increase of SMA, with higher frequency of contractions and the appearance of clustered phasic contractions, whereas SMA during the Hbl phase was practically nonexistent (Fig. 2).

RESPONSE TO CCK AND L-NNA. In control rats, CCK caused an increase in motor activity in the duodenum (from 18.6 ± 7.3 to 602.8 ± 120.2 mm², \( P < 0.001 \)). In the indomethacin group, CCK response was higher at the Lbl phase and lower during the Hbl phase, compared with the response observed in control animals (Fig. 3). CCK has an inhibitory effect in the jejunum similar to that observed in previous studies (13, 14, 34). This inhibitory response was not modified in indomethacin rats.

The increase of motor activity induced by L-NNA in control rats showed a tendency to be greatest at the Lbl phase of indomethacin-treated animals. However, the differences only reached statistical significance in the jejunum (242.4 ± 41.7 vs. 117.0 ± 23.4 mm² in control group, \( P < 0.05 \)). By contrast, no differences were observed in the response to L-NNA between the Hbl and the control group.

RESPONSE TO EMS. In control rats, EMS at 2 and 6 Hz always induced an ascending contraction recorded at the oral strain gauge, both in the duodenum and ileum. This response was

Fig. 1. A: representative graph showing blood leukocytes (BL) and TNF evolution after indomethacin treatment in an individual animal. B: representative graph showing BL evolution in a control animal. TNF was not detectable in this and other control animals. C: representative graph showing the oscillatory inverse correlation between BL and fecal pellets (FP) in an individual rat after indomethacin treatment.

Fig. 2. A: representative recordings of mechanical activity showing the spontaneous motor events in the duodenum of one animal for each experimental group. Similar recordings were found in all the animals of the same group. B: average of frequency of contraction observed in the duodenal spontaneous motor activity in each group. **Significant difference (\( P < 0.01 \)) vs. control group; ++significant difference (\( P < 0.01 \)) between high blood level (Hbl) and low blood level (Lbl) groups.
increased by L-NNA and reduced by atropine. Differences in the response to EMS were observed according to the blood leukocyte stage of the animals.

In the Hbl group, a decrease in the magnitude of the response to EMS was observed in both studied segments at both frequencies of stimuli (Fig. 4). However, after L-NNA administration, the response to EMS was similar to the response after L-NNA in the duodenum of the control group. The ileal response after L-NNA was still lower than in the control group. In all cases, ascending contraction elicited after atropine administration was similar to the control group.

In the Lbl group, the response induced by stimulation at 2 Hz was not modified, whereas the contraction obtained after stimulation at 6 Hz was higher compared with the control group, especially in the ileum (Fig. 4). The response to EMS after L-NNA administration significantly increased in this group compared with the control group, and the magnitude of the remaining response after atropine in the ileum was also significantly higher.

Acute phase. The intestine of the indomethacin group did not show any SMA 2 days after the treatment. In contrast, no changes were observed in the CCK response in relation to the control group (490.9 ± 79.2 mm²), whereas a significant increase of activity was found after L-NNA infusion, both in the jejunum (343.4 ± 47.2 vs. 131.3 ± 35.4 mm² in control, P < 0.01) and ileum (323.0 ± 33.4 vs. 65.0 ± 31.3 mm² in control, P < 0.01). Response to EMS after L-NNA administration also significantly increased both in duodenum (2 Hz: 24.7 ± 5.1 vs. 7.8 ± 2.5 mm² in control, P < 0.01; 6 Hz: 56.0 ± 11.1 vs. 30.3 ± 10.0 mm² in control, P < 0.05) and ileum (2 Hz: 22.7 ± 6.7 vs. 3.0 ± 1.7 mm² in control, P < 0.01; 6 Hz: 48.8 ± 7.1 vs. 30.1 ± 6.8 mm² in control, P < 0.05), whereas no differences were observed after atropine administration in relation to the control group.

HISTOLOGICAL STUDY. Indomethacin induced an important acute inflammatory reaction in the small intestine, especially in the distal jejunum and proximal ileum. No changes were observed in the colon.

Macroscopically, alterations were observed until 15 days after treatment, characterized by a severe distention of the small bowel loops with an edematous appearance. White nodules were located on the serosal side of the mid-small intestine and multiple adherences were also found. Microscopically, damage was maximal at 4 days after treatment, showing an inflammatory infiltrate with neutrophil and macrophage cells, accompanied by an extensive edema that affected both the mucosa and serosa. Multifocal subepithelial vacuolization and multiple mucosal ulcers on the mesenteric side of the small intestine were also found.

In contrast, 15 days after treatment no evident microscopic differences were observed between control and treated rats at Hbl or Lbl phases using the conventional staining procedure previously described.

MICROBIOLOGICAL STUDIES. Despite the normal appearance of the intestine from 15 days after treatment, marked changes in both bacterial luminal load (Table 1) and enteric bacterial wall colonization (Table 2) were observed in the small intestine as well as in the colon.

Species isolated under anaerobic conditions were mainly facultative anaerobes (gram-positive flora) both in small intestine and colon. In addition, a few strict anaerobes were also identified in the colon (Clostridium). Isolated gram-positive flora identified include the following: Enterococcus faecalis, Staphylococcus, and Lactobacillus. The main enterobacteria cultured was Escherichia coli. Similarity of results from anaerobic and aerobic cultures allowed us to discard the anaerobic cultures and focus only on aerobic cultures.

In the acute phase, luminal enterobacteria increased in duodenum, jejunum, ileum, and colon, whereas a significant increase of gram-positive flora was only observed in colon. Moreover, bacterial translocation of both enterobacteria and gram-positive flora was found in all intestinal segments studied.

In the chronic phase, results obtained during the Lbl phase were similar to those observed in the control group. During the Hbl phase, an increase in the luminal enterobacteria was observed 30 days after treatment. Moreover, intestinal wall colonization of both enterobacteria and gram-positive flora was also detected in all segments studied.
DISCUSSION

This study demonstrates that there is a high correlation between intestinal motor activity, bacterial translocation, and indicators of inflammation. We reproduced in an experimental model the chronic relapses reported in IBD patients. In this model, alternative phases of motor activity and quiescence occurred. These phases could be predicted by blood leukocyte monitoring, as well as by the cyclic oscillation of TNF in serum. Hypomotility was related to high blood leukocyte and TNF concentration, whereas hypermotility was observed when leukocyte counting decreased to normal levels. Our results also show that high leukocyte values and hypomotility coincided with bacterial invasion of the intestinal wall.

Histological similarities between the rat indomethacin model and CD are well known (3, 5). However, because the described protocols using indomethacin cause extensive intestinal damage (19, 37), studies could only be performed a few days after treatment. Table 1. Results from cultures of luminal specimens 2 days (acute) and 30 days (Hbl and Lbl) after indomethacin treatment

<table>
<thead>
<tr>
<th>Intestinal Area</th>
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<th>Acute</th>
<th>Hbl</th>
<th>Lbl</th>
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<tbody>
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<tr>
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Results from luminal cultures in both acute and chronic studies are expressed as positive cases/total number of rats. Positive cases are samples showing bacterial concentrations 10-fold higher than the control group; MC, mean concentration of control group; Hbl, high blood level; Lbl, low blood level. *Includes facultative anaerobes.

Table 2. Results from cultures of intestinal wall specimens 2 days (acute) and 30 days (Hbl and Lbl) after indomethacin treatment

<table>
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<td><strong>Group 2: Enterobacteria</strong></td>
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Results from luminal cultures both in acute and chronic studies are expressed as positive cases/total number of rats. *Includes facultative anaerobes.
days after treatment. By modifying a previously reported protocol (27), we obtained rats with less-severe lesions, which allowed us to perform chronic studies. As previously reported (37), an initial inflammatory reaction was observed especially in the mid-small intestine, which was concomitant with enterobacteria overgrowth and wall invasion by both enterobacteria and gram-positive bacteria. Interestingly, a marked increase in wall invasion by bacteria was also observed in the colon despite the absence of any sign of inflammation, suggesting that functional alteration might also happen in noninflamed areas. As previously described (31), indomethacin markedly enhanced motility within 4 h (data not shown), an effect related to the increased number of fecal pellets observed 24 h after treatment. This hypermotility was transitory, as shown by a decrease in motor activity observed 2 days after treatment. L-NNA reverted this hypomotility, indicating an overexpression of nitric oxide synthase induced by the inflammatory process, similar to other models of inflammation (32).

In our model, evident morphological signs of inflammation disappeared in the second week after indomethacin treatment, whereas cyclical oscillation of motility, bacterial translocation, and inflammatory mediators continued to be present in the chronic phase. These findings agree with previous studies demonstrating that inflammation induces chronic changes at the structures controlling intestinal motor activity (6, 16). Moreover, our studies demonstrate that structures controlling motor activity react to bacterial translocation and inflammation.

The differentiated motility patterns observed in the indomethacin group indicate cyclical changes in the mechanisms controlling gastrointestinal motility. For instance, in the Lbl group, CCK response that is mediated by vagal afferent innervation (26, 32) was higher than that observed in healthy animals, indicating an increase in sensitivity of afferent mucosal fibers. Moreover, whereas the nitricergic innervation was preserved, as indicated by the increase in EMS response after L-NNA treatment, the remaining response after atropine was greater in response to EMS. This result is in agreement with other studies indicating the existence of functional changes in the excitatory acetylcholine/substance P neurons as a reaction to intestinal inflammation (1, 34) and with elevated levels of SP and increased expression of NK-1R in the myenteric plexus described in CD (15, 21).

In contrast, the lower response induced by CCK observed in the Hbl group suggests an impairment of excitatory pathways and/or an increase of the tonic nitricergic innervation. However, the basal ascending contraction elicited by EMS is decreased and significantly increased after L-NNA administration, further indicating that the hypomotility observed in these animals is related to an increase of the tonic nitricergic innervation. Moreover, the fact that no differences were observed in the response after atropine treatment indicates the preservation of excitatory pathways.

Luminal microflora is important for physiological gut motility (17), and its effect depends on the species involved (18). In addition to the role of pathogenic bacteria to induce motility disorders (20, 23), there is increasing evidence that luminal bacteria are implicated in the pathogenesis of IBD (9, 22, 36). Our results clearly show that both leukocytes and TNF increases (Hbl group) are concomitant with an enterobacterial overgrowth and a bacterial translocation. In contrast, during the reactive phase (Lbl group), the microbiological results were similar to those observed in the control group, indicating that gut microflora was back to normal. It is well documented that bacterial overgrowth might be present in motility disorders (35). In our study, active phases of the inflammatory response (Hbl group) are concomitant with hypomotility, which contributes to the luminal enterobacteria overgrowth and allows the wall invasion by the flora. In contrast, the bacterial load decreases during the Lbl stage most probably as a result of the hypermotility observed in these animals. The lack of bacteria inside the wall reduces the inflammatory reaction as shown by the return to basal values of both leukocytes and TNF values.

Unfortunately, our results do not explain why, after a period of relative normality, the intestine returns to the active inflammatory phase. A prolonged period of intestinal permeability alteration has been demonstrated after inflammation (4). In consequence, our hypothesis is that bacterial translocation induces inflammatory reaction and thus hypermotility. Once hypermotility is established, both bacterial translocation and inflammatory reaction decrease, reducing the drive for hypermotility. However, if permeability remains altered, the decrease of motility could then favor a new bacterial translocation and the perpetuation of the disease. The high correlation between motility and active and reactive phases opens a new perspective on the study of the mechanisms implicated in IBD and its treatment.

In conclusion, we have obtained a chronic model of enteritis characterized by spontaneous cyclical alternation of active and reactive phases that are correlated with cyclical long-lasting motor changes. During the active phase, there is a decrease in motor activity that coincides with wall invasion by intestinal flora and an increase in the blood level of inflammatory indicators. This phase is followed by a reactive phase characterized by hypermotility that helps to restore both flora and BL values. However, as in IBD, this reaction seems unable to prevent a return to cyclic relapse.

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