In vivo changes in the intestinal reflexes and the response to CCK in the inflamed small intestine of the rat

D. TORRENTS AND P. VERGARA

Department of Cell Biology, Immunology, and Physiology, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain

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Torrents, D., and P. Vergara. In vivo changes in the intestinal reflexes and the response to CCK in the inflamed small intestine of the rat. Am J Physiol Gastrointest Liver Physiol 279: G543–G551, 2000.—Functional motor changes and morphological alterations have been associated with intestinal inflammation. The aim of our study was to evaluate functional alterations of intestinal reflexes and of the responses to CCK in the Trichinella spiralis model of intestinal inflammation. Rats were prepared with strain gauges and electrodes in the small intestine to evaluate spontaneous motor activity, the ascending contraction of the peristaltic reflex, and the motor responses to CCK-8 infusion. Infected animals showed increased motor activity at the duodenum and jejunum but not at the ileum. Ascending contraction was increased in both duodenum and ileum. Ascending excitation after N^o-nitro-l-arginine was still increased as well as the residual response after atropine. Response to CCK-8 during intestinal inflammation was changed in the jejunum, in which it turned from the inhibition shown in healthy animals to excitation. NADPH-diaphorase staining did not show any changes between distribution and density of positive neurons in either healthy or infected animals. In conclusion, intestinal inflammation induces functional changes in the motor activity that could explain the abnormal motor responses observed in inflammatory disorders.

intestine inflammation; cholecystokinin; ascending contraction; nitric oxide

Intraintestinal inflammation is commonly associated with changes in gut physiology, including increased secretion (7) and motor abnormalities (11, 12, 24, 31). These motor disturbances are frequently related to digestive symptoms such as diarrhea, abdominal pain, and constipation (5). They could be the result of a large number of reciprocal interactions between the immune system, the neuroendocrine system, and intestinal muscular and epithelial structures (8). These complex interactions make it necessary to conduct studies that differentiate the involvement of each component in the inflammatory response.

The peristaltic reflex is a well-studied pattern of motility that consists of an ascending excitatory response and a descending inhibitory response of the gut wall. This reflex can be induced by gut muscle stretch, distention receptors of the gut wall are activated, whereas in the second case intestinal mucosa mechanoreceptors are stimulated (19). Peristaltic reflex plays a vital role in coordinating the contractile activity that produces the aboral propulsion of intraluminal contents and requires the coordination of the enteric nervous system. The ascending excitatory response of this motor reflex is due to the activation of motoneurons containing acetylcholine and substance P (10). This ascending contraction elicited by electrical stimulation is a very good tool to study both excitatory and inhibitory innervation (1).

Furthermore, CCK is one of the major regulators of postprandial gut motility. Released by intraluminal stimuli, its effects on intestinal motility are mostly mediated via vagal afferent fibers (26, 27). Moreover, intestinal response to CCK disappeared after lidocaine or capsaicin application to the duodenal mucosa (17). As a consequence, CCK effects on the intestinal musculature depend on the predominant kind of nerves in each intestinal segment. Moreover, we previously demonstrated that, in the rat, CCK produces a motor activation of duodenum via cholinergic/substance P innervation, but in the jejunum it produces an inhibitory motor response blocked by nitric oxide (NO) synthase inhibitors (17).

The parasite nematode Trichinella spiralis is a pathogenic agent in several mammalian species, including the rat. During the enteric phase of this infection, the presence of parasites causes a predictable inflammatory response that is also associated with functional alterations on the motility of the small intestine (5, 24, 35) and its regulatory mechanisms in the inflamed zone. This infection can also affect remote segments free of parasites and of inflammation, such as the ileum (15, 22). Although the intestinal inflammatory process disappears after the intestinal phase that lasts 3 weeks, some neural and muscular alterations can persist for longer periods (3, 4).

In the present study, we used an in vivo model that allows the investigation of both nervous and endocrine responses during inflammation. We studied 1) the

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spontaneous motor activity, 2) the motor responses to CCK, and 3) the components of ascending excitation. The study was done in areas affected by the parasite (duodenum and jejunum) as well as in the ileum (free of the parasite inflammation). The study lasted from 2–30 days and allowed investigation of the whole inflammatory process as well as the recovery. Our objective was to evaluate the functional changes in these basic reflexes and neuroendocrine responses during inflammation, which could lead to a better understanding of the motor disturbances caused by inflammatory processes.

MATERIALS AND METHODS

**Animals.** Male Sprague-Dawley rats (from Iffa-Credo), 8- to 10-wk old and weighing 300–350 g, were used in this study. They were kept under conventional conditions in a room with controlled temperature and photoperiod (12:12 h). Animals were specific pathogen free when purchased, and during the experimental period they were periodically checked for absence of intestinal parasites. Animal weight as well as food and water consumption were monitored daily.

**Trichinella infection.** Rats were infected by administering 1.0 ml of 0.9% saline solution containing 7,500 *T. spiralis* larvae by gavage. The larvae were obtained from CD1 mice infected 30–90 days before by a modification of the method described by Castro and Fairbairn (6).

**Histopathological study.** Before the functional study we performed a histopathological study to monitor the infection and the intestinal inflammation. Samples of duodenum, jejunum, and ileum were taken at 2, 6, 14, 23, and 72 days postinfection (PI) (n = 6) and processed for histopathology. Tissue sections were stained by hematoxylin and eosin. Severity of inflammation was classified according to cell infiltration of the mucosal, submucosal, and muscular levels.

**Animal preparation.** Experiments were performed in healthy rats (n = 14) and in 2- (n = 6), 6- (n = 12), 14- (n = 10), 30- (n = 18), and 72- (n = 2) days PI rats. Animals were fasted for 12–16 h before experiments. Anesthesia was induced by inhalation of halothane to allow cannulation with a polyethylene tubing of the right jugular vein. Level III of anesthesia was maintained with thiopental sodium bolus infusion in the jugular as required. Body temperature was maintained at 37°C by placing the rat on a heating pad. Rats were tracheotomized to facilitate spontaneous breathing. A polyethylene tube was inserted through the carotid artery to the aorta and placed at the bifurcation of the celiac artery for close infusion of drugs. The abdomen was opened through a small incision of the intestinal wall to induce ascending excitation of the peristaltic reflex by stimulation of intestinal mucosa, as previously described (18). Electrode holders were placed 1 cm distally to the strain gauge of the duodenum and the ileum. All experimental procedures were approved by the Ethical Committee of the Universitat Autònoma de Barcelona.

**Experimental procedures.** After an equilibration period of 30 min, spontaneous motor activity during 60 min was recorded. Afterwards, CCK-8 (3 × 10⁻⁹ mol·kg⁻¹·10⁻³ min⁻¹) was intra-arterially infused. After at least 1 h to allow the return to basal conditions, mucosal electrical stimulation of the duodenum and ileum to elicit ascending excitation was applied at 30 V, 0.6 ms, and 2, 4, and 6 Hz. Each stimulus was applied for 30 s, and the polarity of the stimulating electrodes was reversed at 15 s. An interval of 2 min was left between stimuli. Immediately afterwards, intra-arterial administration of N⁶-nitro-L-arginine (L-NNA) (10⁻⁵ mol/kg) was performed, and the pattern of electrical stimulation was repeated 15 min later. Afterwards, atropine (0.3 mg/kg) was also intra-arterially infused as a bolus, and 5 min later the electrical stimuli were repeated. Preliminary experiments were performed to ensure that ascending excitation was not modified by subsequent repetitions during the experimental protocol (data not shown).

**Drugs and solutions.** CCK-8, sulfated form (Peptide Institute, Osaka, Japan), was diluted in 1% sodium bicarbonate to 10⁻⁴ M and buffered saline solution to work concentration. Atropine (Merek, Darmstadt, Germany) and L-NNA (Sigma, St. Louis, MO), a nonspecific NO synthase inhibitor, were diluted in buffered saline solution. WIN 51.708, a nonpeptide NK-1 tachykinin receptor antagonist (RBI, Natick, MA) was diluted in DMSO to 10⁻² M and in buffered saline to work concentration.

**NADPH-diaphorase histochemistry.** Segments from the duodenum, jejunum, and ileum were taken from healthy rats, and at 2, 6, 14, and 30 days PI (n = 6). The intestinal segments were opened longitudinally and fixed in 4% paraformaldehyde. Whole mount and longitudinal muscle layer with the myenteric plexus attached (LMMP) preparations were incubated in PBS containing 0.3% Triton X-100, 1 mM L-NADPH, and 0.1 mM nitroblue tetrazolium (Sigma) at 37°C for 45 min. After several washes, preparations were mounted on glass slides and examined under the microscope.

**Data analysis.** Presence or absence of spontaneous activity and of clustered contractions was estimated and compared by means of a χ²-test. The area under the curve (in mm²) during electrical stimulation and CCK-8 infusion was measured. These data were expressed as means ± SE, and statistical analysis of the results was performed using one-way ANOVA and Bonferroni’s post hoc test. Ascending excitation after L-NNA or atropine were compared with their control response by a t-test. In all cases, differences were considered significant when P < 0.05.

**RESULTS**

A significant decrease of food consumption and body weight was observed for the first 2 wk after *T. spiralis* infection. Average loss in body weight was 1.2% per day from 2–12 days PI. Afterward, both parameters were back to normal. Digestive pathophysiological symptoms such as vomiting, diarrhea, or constipation were absent in all animals.

**Histopathological study.** As extensively described (30), *T. spiralis* infection induced a mixed inflammatory infiltrate with neutrophil and eosinophil cells that affected the mucosa and submucosa of the duodenum and jejunum. No signs of inflammation were observed at the muscular layers. Inflammation was already present at 2 days PI, accompanied by a hypertrophy of both circular and longitudinal smooth muscle in all the intestinal areas including the ileum, where no signs of
inflammation were observed. Inflammation was more severe at the jejunum, in which from 2 to 14 days PI an intense (level 4) inflammation was observed. Duodenal inflammation was less severe at 2 and 6 days PI (level 3) and reached level 4 at 14 days PI. By 23 days PI intestinal mucosa had mostly recovered its normal appearance, although a slight inflammation was still present in jejunum (level 1). Although muscular hypertrophy was visible from 2 days PI and lasted over 72 days PI, it was most noticeable at 6, 14, and 23 days PI. Duodenum circular muscle thickness during the period of study is shown in Table 1. Our results in jejunum and ileum were similar to those already reported (4, 33).

Spontaneous activity. Spontaneous activity recorded during the first 60 min of the experiment was altered in the enteric phase of the *T. spiralis* infection. Healthy rats showed spontaneous activity consisting of isolated contractions with a frequency of 3–6 contractions/10 min in the duodenum (70% of the experiments), jejunum (42%), and ileum (50%) (Table 2). During intestinal inflammation, these contractions were substituted by intense bursts of clustered contractions followed by periods of inactivity (Fig. 1). However, these changes were different according to the time after infection. At 2 days PI there was a decrease of the cases in which spontaneous activity was present (33%). At 6 and 14 days PI there was an increase of spontaneous activity in both duodenum and jejunum, whereas the ileum remained quiescent in most of the animals (Table 2). Clustered contractions were never observed in healthy animals or at 2 days PI. In contrast, they were present in ~50% of the 6- and 14-days PI rats in the duodenum and jejunum and in the duodenum of 30-days PI rats. The ileum never showed clustered activity (Fig. 1, Table 3).

CCK motor response. In healthy rats, as previously described (17), intra-arterial infusion of CCK-8 (3 × 10⁻⁹ mol·kg⁻¹·10 min⁻¹) induced an increase of both tonic and phasic contractions in the duodenum. In contrast, an inhibition of spontaneous mechanical activity was observed in the jejunum during CCK-8 infusion (Fig. 2). In infected rats, response to CCK was modified both in duodenum and jejunum. In the duodenum a quantitative increase of the response was observed after 14 days PI. This increase still remained at 30 days PI (Fig. 3). But the most dramatic change was observed in the jejunum. Response to CCK in this organ turned from the inhibition observed in healthy and 2-days PI rats into a strong excitatory response observed at 6 and 14 days PI. Jejunal CCK response was back to normal in 30-days PI rats (Figs. 2 and 3). Finally, in the ileum no effect of CCK-8 infusion on motor activity was observed in either healthy or infected rats at any time of study.

Table 2. Incidence of spontaneous motor activity in healthy and *T. spiralis*-infected rats

<table>
<thead>
<tr>
<th></th>
<th>Healthy</th>
<th>2 PI</th>
<th>6 PI</th>
<th>14 PI</th>
<th>30 PI</th>
</tr>
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<tbody>
<tr>
<td>Duodenum</td>
<td>71</td>
<td>33</td>
<td>91</td>
<td>90</td>
<td>66</td>
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<tr>
<td>Jejunum</td>
<td>42</td>
<td>33</td>
<td>75</td>
<td>80</td>
<td>15</td>
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<tr>
<td>Ileum</td>
<td>50</td>
<td>33</td>
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<td>n</td>
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Values are percentages; *n* = no. of rats.
Effect of inflammation on the ascending contraction. Electrical stimulation on the intestinal mucosa at all frequencies of stimuli always induced an ascending contraction recorded at the orad strain gauge (Fig. 4). This response was significantly increased by L-NNA and reduced by atropine (Figs. 5 and 6). Table 4 shows the results observed during inflammation. Responses did not show any change when the same protocol was repeated in 2-days PI rats. However, after 6 days PI the duodenum showed an increase of the response to electrical stimulation (Fig. 4). This effect was more prominent at higher frequencies of stimulus. The response was back to normal at 30 days PI. The ileum of infected rats also presented bigger responses, but the values did not reach statistical significance (Fig. 4).

L-NNA intra-arterial infusion (10^{-5} mol/kg) also significantly increased the response to electrical stimulation in infected rats (Fig. 5). However, the response after 6 days PI was of larger amplitude than the one observed in healthy rats. The response was back to normal after 30 days PI. In the ileum, a progressive increase of the response after L-NNA was observed, with the maximum effect at 30 days PI, although the results did not reach statistical significance.

Ascending contraction elicited after atropine intra-arterial infusion (0.3 mg/kg) was reduced in infected animals as in healthy rats (Fig. 6). However, the magnitude of the remaining response was dependent on the phase of inflammation. In the duodenum, the remaining response after atropine was reduced at 2 days PI.
but significantly increased at 6 days PI and back to normal at 30 days PI. In the ileum after 6 days PI, atropine did not modify the response to electrical stimulation at low frequencies (2 and 4 Hz). In fact, at 14 days the response was the same irrespective of the frequency of stimulus.

In some experiments with 14-days PI rats, WIN 51,708 (10^{-6} mol/kg), a tachykinin antagonist, was infused at the end of the experiment and the electrical stimulation was repeated (n = 4). In these cases, the remaining response to electrical stimulation was blocked (data not shown). The responses in the 72-days PI rats were no different from healthy rats.

NADPH-diaphorase staining. Both whole mount and LMMP preparations showed an organized network of NADPH-diaphorase-positive neurons. Whole mount preparations from inflamed specimens did not show a consistent staining. However, in the LMMP preparations in which the hypertrophied circular muscle had been removed a well-organized network of NADPH-diaphorase-positive neurons was visible in both healthy and inflamed specimens. Figure 7 shows NADPH-diaphorase staining at 14 days PI. No difference was observed in the distribution or the number of neurons in the tissue of healthy and infected rats at any stage of the parasite infection.

DISCUSSION

The results of this study show that responses to inflammation are complex and vary in accordance with the parameter of study, the experimental model, and the phase of inflammation. Our experimental model made it possible to differentiate several components of the neuroendocrine control of the small intestine motor activity and their alteration by intestinal inflammation. This study demonstrates that motor disturbances observed during inflammation could be due to changes
in sensitivity of intestinal reflexes and in the response to postprandial hormones.

_T. spiralis_ infection in the rat is a long-lasting inflammatory process that, apart from the slight decrease in food intake and body weight, is clinically asymptomatic in the rat. However, the infection generates dramatic morphological changes in the small intestine that have been extensively studied (4, 33). Our results agree with previous findings and show that the mucosal inflammation is dependent on the presence of the parasite in the intestine (4). Mucosal inflammation is restricted to the duodenum and jejunum, although jejunum mucosa is affected to a greater extent. Inflammation is well correlated to the presence of parasites in the intestine and disappears as soon as the parasites are expelled. As already reported, the maximum intensity of inflammation is observed between 6 and 14 days, and around 23 days PI the intestinal mucosa is back to normal. In contrast, the induced muscle hypertrophy seems to be a more generalized response, affecting both inflamed and noninflamed areas and lasting much longer than mucosal inflammation.

In addition, cytokine release during _T. spiralis_ infection has been well monitored and correlated with immune cell activity. Furthermore, two different phases have been found, one at the early stage with a predominance of interleukin-5 secretion, and another one in which interleukin-4 was predominant and lasted over the inflammatory period. Moreover, interleukin-4 peak occurred at parasite rejection (25). Similarly, we observed that, despite the clear signs of inflammation and a slight hypertrophy already present at 2 days PI, there was no difference in the motor response of the gut except for a moderate decrease of activity. On the
contrary, after 6 days PI, with a peak at 14 days PI, we observed an increase of activity in all of the parameters studied.

The increase of motor activity, which in some cases lasted over the inflammation period, indicates that inflammation had already modified the structures controlling intestinal motor activity. However, the fact that the different parameters of study (spontaneous motility, CCK responses, and ascending contraction) involve different mechanisms makes it necessary to discuss each parameter individually. Concerning spontaneous motility, functional changes differ from the inflamed areas to the noninflamed. Whereas the duodenum and the jejunum (inflamed areas) showed an increase of spontaneous motility, mainly due to the presence of strong clustered contractions, the ileum (noninflamed) showed a significant decrease in spontaneous activity. Clustered contractions propagated along the small intestine have been described during inflammation by *T. spiralis* in the dog and associated with rapid transit and diarrhea (11, 12). In the rat, an increase of the intestinal transit and of propulsion has also been described after *T. spiralis* infection (5), which could probably be a consequence of the clustered contractions. However, under our conditions, a gradient of propagation for clustered contractions seemed to occur, so that they were more frequent in the duodenum than in the jejunum. Moreover, clustered contractions seem to be restricted to the inflamed areas because they were absent in the ileum, which in fact remained quiescent in most animals.

Some authors (22) have also indicated that a difference between jejunum and ileum of *T. spiralis*-infected rats can be observed in vitro. These authors suggested that the inflammation induced changes in the muscle. However, without rejecting the possibility that these changes could be due to local effect of cytokines in the muscle, the fact that in our model spontaneous motility is hexamethonium sensitive (17) could indicate changes in the innervation of the intestine. This hypothesis is corroborated by the results of the present study in respect to CCK and electrical field stimulation.

The alteration of postprandial motility has also been reported during inflammation (12). An abnormal response to CCK has been suggested in patients with irritable bowel syndrome (20). We had previously characterized the motor effects of CCK (17). This peptide induced a complex response in the small intestine consisting of duodenal excitation and jejunal inhibition. The excitatory component of this response was mediated by stimulation of mucosal afferents, whereas the jejunal inhibition persisted after hexamethonium and was blocked by L-NNA.

In the present study, CCK response in the duodenum did not vary significantly from that observed in healthy animals, except for the increase in magnitude that lasted over the inflammatory period. In contrast, in the
jejenum the response completely changed from inhibition to excitation. The change observed in the jejunum is coincident with inflammation, recovers as soon as inflammation is over, and indicates the complete impairment of nitrergic inhibition during jejunal inflammation. However, both the NADPH-diaphorase staining and the responses to ascending excitation indicate that there is no alteration, either morphological or functional, of NO neurons during inflammation. Controversial results have been presented in relation to a possible destruction of NO synthase neurons in the enteric nervous system during inflammation (21, 23). However, our experience in this study clearly indicates that the hypertrophy of the muscle can mask the staining, because removal of the circular muscle showed the preservation of the NO synthase-containing neurons during inflammation. In consequence, the qualitative change in the response to CCK observed in the jejunum during inflammation has to be caused by functional changes in other components of the enteric reflexes that participate in the modulation of inhibitory neurons. In fact, intestinal NO neurons are modulated by several neurotransmitters such as noradrenaline (14), somatostatin (34), and opioids (16).

The conservation of the excitatory response to CCK, sensitive to both intraluminal capsaicin application as well as to systemic hexamethonium, indicates that afferent mucosal fibers are mostly preserved during inflammation, in contrast with that observed with other, probably more abrasive, models of intestinal inflammation (29). However, the fact that we did not examine the effect of capsaicin on CCK response during inflammation does not allow us to reject the possibility that CCK during inflammation might act in other sites where it does not act under physiological conditions.

Distal electrical stimulation has been used extensively to induce ascending excitation, a well-characterized component of the peristaltic reflex. Recently, we established that ascending contraction can also be elicited by electrical stimulation of the mucosa. This response is blocked by intraluminal infusion of lidocaine (18). Although ascending contraction is an excitatory response mediated by cholinergic/tachykinin pathways, NO synthase inhibitors increase the response to electrical stimulation (1, 13, 17). Therefore, induction of ascending contraction is a good method to evaluate the functionality of excitatory neurons and the tone of inhibitory motor neurons.

During inflammation we observed significant changes in ascending contraction in the duodenum, but these changes, although similar, were of less intensity in the noninflamed ileum. This result indicates that the response to the presence of the parasite is not restricted to the inflamed areas and, in consequence, suggests a systemic response to inflammation as already reported in the same model (4). However, the results we observed in the ileum were much more variable than those in the duodenum, indicating an individual susceptibility on the extension of the consequences of inflammation.

Ascending excitation is mediated by enteric neurons, and, in consequence, our results indicate changes in the enteric nervous system during inflammation. Since ascending excitation is the result of several components, we then considered it necessary to study each component separately. The first finding was that the increase of the response was not due to an impairment of inhibitory neurons, as is clearly shown by the L-NNA results. In fact, L-NNA caused a very significant increase of ascending excitation, indicating that the tone of nitrergic innervation was not decreased during inflammation. A previous study using a model of colitis also reported a similar result (2). However, after L-NNA treatment, excitatory response during inflammation remained of significantly larger amplitude than in healthy rats, indicating that either an alteration of excitatory pathways or the hypertrophy of the muscle could be responsible for this increase.

As mentioned in this study, the time course of hypertrophy and of increased ascending contraction did not correlate well, and, in consequence, we believe that the observed changes are most likely a consequence of changes in excitatory innervation. Both impairment of acetylcholine release and increase of substance P-positive fibers have been reported in T. spiralis infection at 6 days PI (9, 32). In our study, we observed a functional decrease of the atropine-sensitive component and, more significantly, an increase of the tachykinin component of the response. This is in agreement with other studies that indicate the existence of functional changes in the excitatory acetylcholine/substance P neurons as a consequence of inflammation. However, changes in the number of functional receptors for these two neurotransmitters in the muscle or in its associated intracellular mechanisms cannot be discarded. Alterations of these functional parameters have been described in other models of inflammation (28, 36). The contradictory responses to CCK and to electrical stimulation that we observed could be caused by the different mechanisms involved. Although CCK induces a physiological reflex, electrical stimulation is probably acting in a less discriminative way. However, the fact that we used the two parameters in this study allowed us to show that nitrergic neurons are still functional, although their regulation seems to be altered.

In conclusion, inflammation induces functional changes in the enteric nervous system that increase sensitivity of the peristaltic reflex and induce abnormal responses to CCK. These two events could be responsible for the abnormal responses to physiological neuroendocrine stimuli observed during inflammation. Moreover, although some changes correlate well with inflammation and the presence of parasites in the intestine, which they help to eliminate, others are prolonged and could be representative of long-lasting abnormal symptoms observed in inflammatory bowel disease.
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