Muscularis inflammation and the loss of interstitial cells of Cajal in the endothelin ETB receptor null rat

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congenital aganglionosis rats; megaileum; Hirschsprung’s disease; macrophages; microflora

ENDOTHELIN RECEPTOR NULL RATS [ETB(−/−)] possess an autosomal recessive gene (sl) that leads to traits such as white coat color, a small pigmented spot on the head, and defects in enteric ganglia (4, 19). ETB(−/−) rats display severely distended intestines proximal to a constricted region caused by long-segment aganglionosis (14–16). Heterozygote rats (sl/+ ) appear anatomically and histologically identical to wild-type animals (25). However, ETB(−/−) rats display histological and pathophysiological features similar to human patients with long-segment Hirschspring’s disease (1, 16), making these animals a model for intestinal obstructive diseases, such as Hirschspring’s disease. Although several morphological and functional abnormalities have been identified in the gastrointestinal (GI) tracts of these animals, the pathophysiology and underlying mechanisms leading to these intestinal changes are not fully understood.

Immunologically activated cells, including macrophages in the intestinal mucosa, are thought to be involved in inflammatory bowel diseases. Recently, macrophages within the tunica muscularis of the GI tract were reported as a population distinct from mucosal macrophages (17, 21, 22, 29). These macrophages are regularly distributed in the subserosa, at the level of myenteric plexus (MY), and within the tunica muscularis. Although their unique distribution and great numbers imply an important role, the function of resident macrophages in the tunica muscularis remains to be clarified, and their role in pathological responses of the GI tract is not fully understood. Recently, Schroeder et al. (32) and Eskandari et al. (8) reported that intraperitoneal injection of LPS into rats upregulated inducible nitric oxide (NO) synthase (iNOS) expression in resident macrophages. The resulting increase in NO production in these animals suppressed smooth muscle contractility. Zheng et al. (38) reported that incubation with LPS for 4–5 h in vitro also increased iNOS mRNA levels in the small intestine and decreased smooth muscle contractions. Hori et al. (13) showed that LPS-induced iNOS expression was mediated by autocrine regulation by prostaglandins through induction of cyclooxygenase-2 (COX-2) gene expression. These findings suggest that the resident macrophages in the tunica muscularis might contribute to endotoxin-induced gut dysmotility. At present, however, studies of the immunological properties of resident macrophages and how these cells contribute to abnormal GI motility under pathophysiological conditions are incomplete.

In the present study, we investigated the inflammatory state of the tunica muscularis in the distended region of small intestine in ETB(−/−) rats to determine whether the intestinal distension was 1) purely a consequence of the constricted region distal to this site or 2) because of changes within the distended region itself, such as intestinal dysmotility resulting...
from inflammation of the tunica muscularis. It was found that resident macrophages affect the network of pacemaker interstitial cells of Cajal (ICC), which lie in close spatial association with these immune cells in the myenteric region of the intestine, and that these changes are associated with contractile abnormalities and distension in the region of intestine in which ganglia are intact.

**MATERIALS AND METHODS**

**Experimental animals.** Animal care and treatment were conducted in conformity with the institutional guidelines of the University of Tokyo. ETB(−/−) rats were generated in our animal facility by mating heterozygous ETB(+/−) males and ETB(+/+) females. The genotypes were determined by coat color and pigment scattering (15, 16). ETB(−/−) rats ~12–15 days old were used for the studies. Age-matched ETB(+/+) or ETB(+/+) rats were used as controls (see RESULTS). Rats were anesthetized with ether and killed by a sharp blow to the neck followed by exsanguination. The intestines were dissected out and immediately rinsed with physiological salt solution (PSS).

**Measurement of contractile activity.** The entire small intestine was dissected in PSS containing (in mM): 136.5 NaCl, 5.4 KCl, 1.5 CaCl2·H2O, 2.0 MgCl2·6H2O, 23.8 NaHCO3, 0.01 EDTA, and 5.5 glucose. This solution was saturated with a 95% O2–5% CO2 mixture at 37°C and pH 7.4. For the measurement of contractile activity, the mucosa was removed from the intestinal segments. The segments were then cut into rectangular strips oriented toward the longitudinal axis of the circular muscle cell layer. The length of the muscle strips were equal to the circumference of the intestine, and the width was 2–3 mm. Muscle contractions were measured isometrically under a resting tension of 5–10 mN.

**Morphological studies.** Studies were performed to observe resident macrophages in tunica muscularis. Segments of intestine were removed and opened along the mesenteric border, and the mucosa and submucosa were removed by sharp dissection. Tissues were fixed in 10% neutral buffered formalin solution and embedded in paraffin. Tissue sections were cut with a Reichert ultramicrotome, double-stained with uranyl acetate and lead citrate, and observed with a JEM1200 EX II electron microscope.

**Bacteriological examination.** In some experiments, the dilated region of the intestine was removed aseptically and the contents of the intestine were isolated and homogenized in 4.5 ml of anaerobic transport medium with a glass homogenizer. Bacterial cultivation was performed by the method of Mitsuoka et al. (24). Serial 10-fold dilutions of the homogenates with anaerobic diluents were performed in an anaerobic phosphate-buffered medium and plated on 3 non-selective and 11 selective agar media. Isolated bacteria were identified by gram staining, colony and cell morphology, aerobic growth, and spore formation. The numbers of bacteria were expressed as log10 counts of viable bacteria per gram wet weight of intestinal contents.

**Quantitative RT-PCR analysis.** Total RNA was extracted from the muscle strips by the acid guanidinium thiocyanate-phenol chloroform method and the concentration of RNA was adjusted to 0.4 μg/μl with RNAse-free distilled water. Quantitative RT-PCR was performed as follows. First strand cDNA was synthesized by using random 9-mer primer and avian myeloblastosis virus reverse transcriptase XL at 30°C for 10 min, 55°C for 45 min, 99°C for 5 min, and 4°C for 5 min. PCR amplification was performed by the hot starting method using Taq Gold (PerkinElmer). The oligonucleotide primers for cytokines and rat GAPDH designed from rat is in Table 1. After initial denaturation at 95°C for 10 min, 35–45 cycles (5-cycle interval) of amplification at 94°C for 40 s, 55°C for 1.0 min, and 72°C for 1.5 min were performed by using a thermal cycler (Takara PCR Thermal Cycler MP; Takara Biomedicals). PCR products in each cycle were electrophoresed on 2% agarose gel containing 0.1% ethidium bromide and 3% glutaraldehyde in 0.1 M PBS (pH 7.3) at room temperature. After being rinsed in fresh PBS, tissues were postfixed in 1% osmium tetroxide for 2 h at 4°C. The samples were then rinsed in distilled water, block-stained with uranyl acetate solution for 3 h, dehydrated in a graded series of ethyl alcohols, and embedded in Epon epoxy resin. Ultrathin sections were cut with a Reichert ultramicrotome, double-stained with uranyl acetate and lead citrate, and observed with a JEM1200 EX II electron microscope.

**RESULTS**

**Anatomical and histological observations.** As previously reported by Gariepy and Yanagisawa (10), ETB(−/−) rats had

<table>
<thead>
<tr>
<th>Table 1. Primers used for RT-PCR</th>
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<td><strong>Cytokines</strong></td>
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| IL-1β | Forward primer 5’-CTCTGGAATCTGGAGAATGA-3’  
Reverse primer 5’-AGTTGGGAACTGTGCAGAC-3’ |
| IL-6 | Forward primer 5’-GGTCTCTGAGGCTTCTGGTTC-3’  
Reverse primer 5’-GGTGGGCAAGATGACCTCA-3’ |
| GAPDH | Forward primer 5’-TACAGAGCGGGGGAACAC-3’  
Reverse primer 5’-CAGACTGAAGATGTA-3’ |

For electron microscopy, tissues were fixed with 4% paraformaldehyde and 3% glutaraldehyde in 0.1 M PBS (pH 7.3) at room temperature. After being rinsed in fresh PBS, tissues were postfixed in 1% osmium tetroxide for 2 h at 4°C. The samples were then rinsed in distilled water, block-stained with uranyl acetate solution for 3 h, dehydrated in a graded series of ethyl alcohols, and embedded in Epon epoxy resin. Ultrathin sections were cut with a Reichert ultramicrotome, double-stained with uranyl acetate and lead citrate, and observed with a JEM1200 EX II electron microscope.
dark eyes and a white coat and displayed obvious distension of the GI tract. (Fig. 1A). Gross morphological observation of the GI tracts of ETB(−/−) rats has shown a constricted region in the distal ileum and an area proximal to this site that was markedly distended (e.g., Fig. 1). In our animals, the constricted region extended from the colon, through the cecum, and into a very short segment of the terminal ileum above the ileocecal sphincter. The ileum proximal to this point was distended.

In control rats, there were no inflammatory changes in the mucosal and muscular layers of the ileum. The presence of vacuoles in the cytoplasm of intestinal absorptive epithelial cells characteristically suggests these animals were in a developmental stage before gut closure. In contrast to wild-type rats, ETB(−/−) animals showed severe atrophy of intestinal villi, which were covered with irregularly vacuolated epithelial cells. Mild cellular infiltration with predominant monocytes was observed in the lamina propria mucosa (Fig. 2A). In contrast, neutrophilic infiltration of the tunica muscularis (Fig. 2B; higher magnification) was rarely or not observed. There was no apparent inflammation of the tunica muscularis and no significant differences in the thickness of both muscular layers in both mouse types.

**Intestinal microflora and plasma endotoxin levels.** Examination of the bacterial microflora in the lumen of the dilated region of ileum showed that the bacterial composition of the intestinal flora was abnormal in ETB(−/−) rats (Table 2). In the region of the ileum in control animals, lactobacilli were predominant bacteria (10^9/g). The total bacteria and the number of each bacterial group were abnormally high in ETB(−/−) rats compared with control rats. The number of anaerobes such as Bacteroidaceae, eubacteria, and clostridia, and aerobes such as Enterobacteriaceae and enterococci in the dilated region in the ETB(−/−) rats were significantly higher than in control animals.

Marked increases of gram-negative aerobes (Enterobacteriaceae) and anaerobes (Bacteroidaceae) in the distended region of the small intestine of ETB(−/−) rats suggest the possibility of enhanced endotoxin production and/or bacterial translocation. Thus we measured the plasma endotoxin level in ETB(−/−) rats with a limulus reagent assay kit. The plasma endotoxin levels of ETB(−/−) rats was twofold higher (P < 0.01) than in control rats (Fig. 3).

### Table 2. Composition of intestinal flora in the proximal ileums of control and ETB (−/−) rats

<table>
<thead>
<tr>
<th>Bacterial Groups</th>
<th>Control</th>
<th>ETB (−/−)</th>
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<tbody>
<tr>
<td>Enterobacteriaceae</td>
<td>4.0 ± 1.2 (6)</td>
<td>9.1 ± 1.8** (8)</td>
</tr>
<tr>
<td>Enterococci</td>
<td>6.6 ± 1.1 (7)</td>
<td>9.7 ± 0.6** (8)</td>
</tr>
<tr>
<td>Staphylococci</td>
<td>5.4 ± 0.9 (7)</td>
<td>5.2 ± 1.1 (8)</td>
</tr>
<tr>
<td>Gram (negative) aerobic rods</td>
<td>5.0 (1)</td>
<td>ND (0)</td>
</tr>
<tr>
<td>Yeasts</td>
<td>N.D. (0)</td>
<td>3.0 (1)</td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>8.8 ± 0.3 (7)</td>
<td>9.1 ± 0.7 (8)</td>
</tr>
<tr>
<td>Bacteroidaceae</td>
<td>5.8 ± 1.3 (5)</td>
<td>10.0 ± 0.6* (8)</td>
</tr>
<tr>
<td>Bifidobacteria</td>
<td>5.0 (1)</td>
<td>ND (0)</td>
</tr>
<tr>
<td>Eubacteria</td>
<td>6.8 ± 2.1 (5)</td>
<td>9.9 ± 0.6* (8)</td>
</tr>
<tr>
<td>Clostridia</td>
<td>N.D. (0)</td>
<td>8.8 ± 1.1 (3)</td>
</tr>
<tr>
<td>Total count</td>
<td>8.9 ± 0.3</td>
<td>10.6 ± 0.5</td>
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Means ± SD of log/g ileal contents of 8 rats in each group. No. in parentheses is no. of subjects harboring the intestinal flora. Significantly different from control with *P < 0.05 or **P < 0.01. ND, not detected; ETB(−/−), endothelin receptor null rats.
ileum compared with tissues from control animals. In contrast, there was no significant difference in the number of FITC-dextran positive macrophages in the region of the jejunum between ETB(−/−) and control animals, in which distension of the intestines was not observed.

Expression of CD14 (LPS receptor). We further examined the dilated region of intestinal smooth muscle of ETB(−/−) rats using CD14 antibody (ED9). The expression of CD14 was determined by using immunohistochemical techniques, and reaction product was determined by pixel number using NIH image software. CD14 was significantly (P < 0.01) increased in the dilated region of the intestine compared with control tissues (Fig. 6, A and B). Although we did not specifically identify the immune cells, high expression of CD14 appeared localized to the macrophage-like cells (compare Figs. 4A and 6A).

Expression of proinflammatory cytokines, IL-1β and IL-6 mRNA. RT-PCR analysis was performed on RNA extracted from the dilated region of intestinal smooth muscle in control and ETB(−/−) rats. As shown in Fig. 7, A and B, the expression of RT-PCR product encoding IL-1β mRNA and IL-6 mRNA was strongly expressed in the ETB(−/−) rats com-
pared with control. The expression of IL-1β mRNA ratio to GAPDH mRNA in ETB(−/−) was significantly greater than that of control tissues. The expression of IL-6 mRNA ratio to GAPDH mRNA in ETB(−/−) was also significantly greater than that of control.

**ICC networks are disrupted in ETB(−/−) intestines.** Mikkelsen et al. (22) first demonstrated that the area around the MY region of the small intestine contained a large number of resident macrophage cells. These cells are in close morphological association with the ICC-MY region (23), regarded as the pacemaker cells in the GI tract (30, 31). This morphological association would suggest a possible physiological interaction between macrophages and ICC, which may play a role in GI motility especially under conditions when macrophages become activated. With the use of Kit immunoreactivity, ICC appeared as a dense network in the ICC-MY of control rat ileal tissues (Fig. 8A). However, the ICC-MY appeared greatly disrupted or absent in the distended regions of intestine from ETB(−/−) rats (Fig. 8B). Analysis of the Kit-like immunopositive cells in ETB(−/−) rats showed that the immunopositive area was significantly (P < 0.01) decreased compared with control animals. In this study, we also observed a decrease in the density of ICC-DMP in ETB(−/−) intestines but did not analyze these data because of technical reasons.

**ICC-MY.** To examine the ultrastructural features of macrophages and ICC in the distended regions of ETB(−/−) muscles, we observed tissues by using transmission electron microscopy. In the ileums of ETB(−/−) rats, numerous macrophages were observed in the subserosal layer, MY, and deep muscular plexus regions (Fig. 9). Macrophages were ultrastruc-
turally identified by well-developed cell processes, lysosomes, vacuoles, and coated vesicles within the cytoplasm. Electron microscopy also confirmed observations using Kit-like immunohistochemistry that the number of ICC at the level of the MY was decreased in ETB(-/-) rat ileal tissues. Occasional close morphological associations were observed between macrophages and ICC-MY in ETB(-/-) ileal tissues.

**Changes in rhythmic contractile activity.** To determine whether changes had occurred to the spontaneous mechanical activity of the ileal muscles of ETB(-/-) rats, phasic contractions were recorded by using isometric force measurements. Figure 10A shows the typical spontaneous contractile activity of circular muscle strips isolated from control and ETB(-/-) tissues (from 4 different animals). Ileal muscles isolated from control animals displayed regular spontaneous contractions at a frequency of 20.8 ± 0.7 cycles/min. In contrast, spontaneous contractile activity of tissues from ETB(-/-) rats was attenuated and highly irregular (Fig. 10A). Analysis of the frequency of spontaneous contractions in ETB(-/-) intestines showed that this activity was significantly (P < 0.05) reduced compared with that of control tissues (Fig. 10B).

![Figure 7](http://ajpgi.physiology.org/)

Fig. 7. The expression of IL-1β mRNA and IL-6 mRNA in the tunica muscularis of ETB(-/-) rats as determined by RT-PCR at 40 cycles. IL-1β mRNA and IL-6 mRNA were significantly increased in the distended region of the ileaum. *Significantly different from control with P < 0.05 (n = 4 each).

![Figure 8](http://ajpgi.physiology.org/)

Fig. 8. A: immunohistochemistry of c-Kit in whole mount preparations of ileum. Scale bar: 20 μm. B: area of c-Kit positive cells was demonstrated by using NIH image by pixel number. The number of c-Kit-positive cells in ETB(-/-) rats was significantly decreased compared with controls. **Significantly different from control with P < 0.01 (n = 4 each).
Enterocolitis is the most common cause of significant morbidity and death in Hirschsprung’s disease. Although the etiology of the enterocolitis in Hirschsprung’s disease is still obscure, cytopathic toxin originating from Clostridium difficile may play a causal role in the pathogenesis of enterocolitis (34, 35). It is important to isolate specific microorganisms that could be implicated in the pathogenesis of Hirschsprung’s enterocolitis. However, systemic examination on the balance of intestinal microflora is also important, because a defense system in the intestine is believed to be related to a balance of the intestinal bacteria. Therefore, we then examined the bacterial flora in the contents of the dilated region of intestine in the ETB(−/−) rats. We found that the total amount of intestinal bacteria significantly increased, and its composition was abnormal in the distended region of the ileum of ETB(−/−) rats. In addition, there was an abnormal increase of aerobes, such as Enterobacteriaceae and enterococci that are usually present in low numbers in the small intestine. The number of anaerobes increased abnormally in ETB(−/−) rats. Summers and Kent (33) reported highly significant increases in the number of coliforms, enterococci, bacteroides, and clos-
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tridium in the ileum of rats when propulsion is drastically retarded by total obstruction at the ileocecal junction. Thus it is suggested that the abnormal pattern of ileum flora could be a secondary phenomenon after the extreme stagnation of intestinal contents. Abnormal overgrowth of intestinal bacteria forms excessive bacterial products, e.g., endotoxin, and causes failure of the mucosal barrier system that leads to the induction of bacterial translocation and inflammatory reactions (20, 36). In human Hirschsprung’s disease, chronic obstruction of the colon has been reported to induce enterocolitis (3). In the subsequent in advanced cases. Consequently, as demonstrated in Fig. 3, the plasma level of endotoxin in ETB(−/−) rats was significantly increased compared with controls. However, endotoxin levels were below those found in sepsis, indicating that ETB(−/−) rats did not develop systemic disease.

Cytokines and macrophage activation. IL-1β is known as an important proinflammatory cytokine, and LPS is known to induce IL-1β expression in macrophages (6). It is also known that IL-1β promotes the induction of other proinflammatory cytokines, such as IL-6 or TNF-α (26, 28). We found that expression of IL-1β mRNA in the dilated region of the intestinal smooth muscle in ETB(−/−) rats was significantly increased. IL-6 is also an important cytokine that promotes gut inflammation, and IL-6 mRNA expression was also increased in the dilated regions of intestinal smooth muscles in ETB(−/−) rats (Fig. 7).

CD14 and toll-like receptor 4 (TLR4), which are known as LPS receptors, are expressed at the surface of membranes in immune cells. LPS binds to LPS-binding protein (LBP) in plasma and is delivered to the cell surface receptor CD14. LPS-LBP and CD14 complex are transferred to transmembrane signaling receptor TLR4 and its accessory protein MD2 to activate NF-κB. This signaling may coordinate the induction of many genes encoding inflammatory mediators (7, 11). Hopkins and Hunninghake (12) have suggested that LPS stimulation increased the expression of CD14 in human alveolar macrophages to promote the function of macrophages. In the present study, we found expression of CD14 in the tunica muscularis, and also found a significant increase in the dilated regions of intestinal smooth muscles in ETB(−/−) rats (Fig. 6).

Recently, Schroeder et al. (32) and Eskandari et al. (8) reported that resident macrophages within the tunica muscularis can be induced to express iNOS by LPS treatment. The subsequent elaboration of NO in affected tissues suppresses smooth muscle motility. Hori et al. (13) also confirmed this finding in an in vitro study and showed that LPS-induced iNOS gene expression was mediated by autocrine regulation of prostaglandins through the induction of COX-2 gene expression. It is possible that the overgrowth of intestinal bacteria and the secondary occurring bacterial translocation are capable of altering myenteric macrophage populations and their state of activation. As demonstrated in Fig. 5, the ETB(−/−) rat showed significantly greater numbers of myenteric macrophages per unit area in the ileum (both constricted and dilated regions) compared with tissues from the same region of ileum from control animals, whereas no significant differences were observed in the region of the jejunum in which distension of the intestines was not observed. Although little is known about the effects of intestinal bacterial overgrowth and translocation on macrophages, the relationship among Kupffer cells, the resident macrophages of the liver, and intestinal flora have been discussed (2). These authors demonstrated that intestinal gram-negative bacterial overgrowth significantly increases the sensitivity of hepatic resident macrophage to respond to LPS and produce IL-1 and prostaglandin E2. At present, the mechanism(s) underlying the increases in number of the muscularis resident macrophages is not known. However, there are at least two possibilities: 1) chemokine such as monocyte chemotractant protein-1 is generated in the region to promote the infiltration of monocytes, and 2) growth factors, such as granulocyte/macrophage colony-stimulating factor, stimulate proliferation of macrophages directly in an autocrine manner.

Rhythmic disorder and ICC disruption. In the present study, we observed that spontaneous rhythmic contractions in the ileums of ETB(−/−) rats were very irregular (Fig. 10). In addition, the frequency of the rhythmic contractions were significantly decreased in the ileums of ETB(−/−) rats (both constricted and dilated portions) in which the number of macrophages was also shown to have increased significantly (Fig. 5B). As reported by Mikkelsen et al. (22), myenteric macrophages are closely associated with enteric nerves and ICC. The latter are the pacemaker cells in the GI tract that generate rhythmic depolarizations known as slow waves (30, 31). In this study, we identified myenteric macrophages and ICC by electron microscopy in the ETB(−/−) rat ileum. As demonstrated in Fig. 9, the number of ICC (ICC-MY) was decreased in the ileal muscularis of ETB(−/−) rats, and close contacts between macrophages and ICC were occasionally observed. Macrophages are known to secrete many kinds of bioactive substances, including prostaglandins, leukotrienes, nitric oxide, active oxygen molecules, and cytotoxic cytokines (27, 29), and it is known that many of these compounds such as NO can affect the frequency of slow-wave generation (18). We therefore hypothesize that dysfunction of intestinal motility observed in the enterocolitis of Hirschsprung’s disease may be attributable to the substances originated from macrophages resident at the level of the MY and possibly also from secondary infiltrating inflammatory cells in the GI tract. Der et al. (5) have reported inflammation-induced alterations in the network of ICC of the small intestine in the Trichinella spiralis infected mice. Galeazzi et al. (9) also demonstrated an inflammation-induced impairment of nerve function in the same animal model and have suggested that these changes are due to F4/80 positive macrophages.

As described earlier (3, 20, 36), the increase and changes in intestinal microflora might enhance mucosal inflammation. Mucosal inflammation, once it begins, may impair intestinal motility by amplifying inflammatory reactions within the muscular layers. Because intestinal motility is considered to be an important factor for the maintenance of normal intestinal flora, there could be a “vicious cycle” created between changes in intestinal flora and motility in the intestine. This may result in the alteration of intestinal functions, which might lead to systemic diseases in advanced cases.

In conclusion, our results have described inflammation of the muscularis in the gut of ETB(−/−) rats. Muscularis macrophages may play a central role in motility disorders associated with inflammation of the GI tract. In the Hirschsprung’s disease gut, LPS, which can be derived from the gram-negative
aerobes, may activate muscularis resident and monocyte-de-
derived macrophages to release proinflammatory cytokines and
chemokines and upregulate the expression of LPS receptors.
Release of bioactive substances from the immune cells may
affect the number and functions of neighboring ICC that
generate the slow-wave pacemaker activity in the gut. These
changes may result in damage to ICC networks leading to
disordered intestinal rhythmicity in regions of the gut in which
myenteric ganglia are intact.

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