Nematode-Induced Jejunal Inflammation in the Ferret Causes Long-Term Changes in Excitatory Neuromuscular Responses

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ABSTRACT

Enteric infections in animals and humans have proven the link between mucosal inflammation and gastrointestinal motor dysfunction. The goal of the present investigation was to study the long-term effects of mucosal inflammation on the neuromuscular functions of the small intestine in a ferret model of primary Trichinella spiralis infection. Myeloperoxidase activity and isometric contractions of isolated jejunal muscles were studied on days 8, 30, and 60 postinfection (PI). The peak increase in myeloperoxidase activity seen on day 8 PI returned to normal levels by day 60 PI. Contractions of the longitudinal and circular muscles evoked by electrical field stimulation of enteric nerves on day 8 PI showed no difference when compared with uninfected controls. However, during this enteric phase of the infection, neurally mediated responses were characterized by a disturbance in the balance between cholinergic and nonadrenergic, noncholinergic (NANC) excitation with both a reduction of cholinergic and a reciprocal enhancement of NANC neurotransmission. On days 30 and 60 PI the amplitude of neurally mediated responses and the balance between cholinergic and NANC excitation were restored in the circular but not in the longitudinal muscle. In addition, there were changes in the effector function involving smooth muscle hyperresponsiveness to high K⁺ or carbachol on days 8, 30, and 60 PI. However, a significant reduction in E₉₀ for carbachol was found only on day 60 PI. The results demonstrate that T. spiralis infection results in alterations of muscle contractility and enteric neurotransmission that persist after the resolution of mucosal inflammation.

The outcome of an inflammatory reaction depends on the ability of the organism to react to the pathogen, eliminate it, and regain its normal functions. The onset of an inflammatory reaction and its development and resolution depend on a variety of factors, some genetically maintained, others extrinsic to the organism. Enteric infections are characterized by a close relationship between the presence of antigen-induced mucosal inflammation and disturbances in gastrointestinal motor function. Animal models have proven to be useful in studying the correlation between mucosal inflammation and changes in neuromuscular functions, however most experiments have been restricted to the period when the inflammatory response is at its height. The current literature lacks data on long-term effects following the recovery from inflammatory mucosal injury, with the exception of a recent study in a mouse model of T. spiralis-induced inflammation, which demonstrated that the increase in jejunal longitudinal muscle contractility persists after complete mucosal restitution and as late as 42 days postinfection (PI) (Barbara et al., 1997). In a previous in vivo study using a ferret model of T. spiralis infection, we reported a vagotomy-resistant increase in motility clusters and fluctuations in electrogenic ion transport on days 8–12 PI (Greenwood and Palmer, 1996) and postulated that such changes may contribute to the increased secretion and motor abnormalities during intestinal inflammation. The vagotomy-resistant activity was abolished by atropine and was associated with a decrease in intestinal Substance P and vasoactive intestinal peptide content (Palmer and Greenwood, 1993), suggesting changes at the level of enteric cholinergic and peptidergic nonadrenergic, noncholinergic (NANC) mechanisms. In the present study we took an in vitro approach to focus on the mechanisms essential for motility changes in response to enteric nematode infection and to confine the neuromuscular dysfunction to the longitudinal (LM) and/or circular muscle (CM) of the small intestine. Another goal of our study was to define whether inflammation-induced abnormalities in smooth muscle functions are completely reversed after eviction of the parasites from the gut and the healing of mucosal

ABBREVIATIONS: CM, circular muscle; LM, longitudinal muscle; PI, postinfection; EFS, electrical field stimulation; NANC, nonadrenergic, noncholinergic; MPO, myeloperoxidase.
injury. Specifically, our aim was to examine the responsiveness of the LM and CM isolated from the same region of the jejunum at three different PI periods: 1) at day 8 PI, a period that coincides with the enteric phase of the parasite infection characterized by inflammation and mucosal injury; 2) at day 30 PI, when the parasites have already been expelled from the gut and histopathological changes of the mucosa are reversed to normal; and 3) at day 60 PI, a time period long enough for any significant changes to be considered permanent. Changes that are neurally mediated and occur at a prejunctional site of the neuromuscular junction were characterized by investigating the cholinergic and NANC contractions evoked by electrical stimulation of enteric nerves. The changes in contractility taking place at the level of the smooth muscle were characterized by changes in the responses to direct activation of muscarinic cholinceptors or receptor-independent depolarization of the smooth muscle membrane.

**Materials and Methods**

**Animals.** Adult male ferrets *Mustela putorius furo* weighing 1000–1200 g (Marshall Ferrets, North Rose, NY) were housed in individual cages at standardized conditions (12:12 h light/dark cycle and 37°C). The ferrets were fed a carnivore diet and had free access to water throughout the course of the study. The use of ferrets for this study was approved by Oklahoma City, Veterans Affairs Medical Center Animal Care Committee.

**Infection with *T. spiralis*.** The strain of *T. spiralis* used in the experiments has been maintained at our laboratory by serial passage of infections in CF-1 mice. Muscle stage *T. spiralis* larvae were isolated using pepsin-hydrochloric acid digestion of muscle homogenates. The ferrets were randomly assigned to either uninfected control or *T. spiralis*-infected groups. In the *T. spiralis*-infected groups, each of the host animals was orally inoculated with 8000 *T. spiralis* muscle stage larvae suspended in a bolus of 0.5 ml saline and infused (Greenwood and Palmer, 1996). Uninfected control ferrets received only 0.5 ml of saline. The ferrets infected with *T. spiralis* were divided into three subgroups and euthanized at days 8, 30, and 60 PI. Age-matched uninfected control ferrets were studied in parallel with each of the three *T. spiralis* infected groups and the data from the individual experiments were summarized in one control group.

**Preparations of Jejunal Muscle Strips.** On the day of the experiment ferrets were anesthetized with urethane (1.75 g/kg body weight) and segments of the jejunum (approx. 10–15-cm long) were removed and placed in oxygenated modified Krebs bicarbonate solution. The muscularia externa layers were separated from the mucosa-submucosal layer and muscle strips were excised parallel to the CM. LM-myoenteric plexus strips were obtained from intestinal segments by gently peeling off the muscle in longitudinal direction.

**Contractile Activity.** The CM and LM strips were mounted vertically in organ baths with one end fixed and the other attached to isometric force transducers (Radnoti Glass Technology, Inc., Monrovia, CA). Tension was measured as the load (g) applied to the muscle to maintain the isometric condition. The muscles were continuously bathed in modified Krebs bicarbonate saline, maintained at 37°C and aerated with 95% O₂ and 5% CO₂. The tissues were allowed to equilibrate at Li (the initial length at which resting tension is zero) for 30 min, and then gradually stretched by 0.25g load increments to Lo (the length at which maximal active tension was generated in response to 80 mM KCl). Strips were allowed an additional 20-min equilibration before starting the experimental program. All experiments were performed at Lo and isometric contractions were recorded on a Grass 7D polygraph (Grass Instrument Co., Quiney, MA).

**Frequency-Response Curves to Electrical Field Stimulation (EFS).** Experiments were designed to study the effects of intestinal inflammation on neurally mediated responses to stimulation of the enteric nerve terminals. EFS was delivered by a Grass S88 stimulator (Grass Instruments) and applied by pairs of platinum wire electrodes laying parallel to the muscle strips in 10-ml organ baths. The muscle strips were stimulated with rectangular pulses of 0.5-ms pulse duration and voltages of 40 to 60 V. In each strip a submaximal response to 0.5-Hz stimulation was defined by gradually increasing the voltages until the contraction amplitude reached a plateau and then adjusting the voltage to induce a response of 85 to 95% of the maximum. Frequency-response curves were obtained for pulse frequencies ranging from 0.5 to 16 Hz, applied in trains of 5 s to the LM and 10 s to the CM strips.

**Concentration-Response Curves.** A second series of experiments was designed to investigate the effect of intestinal inflammation on the smooth muscle M₃-cholinoreceptor-mediated response. Concentration-response curves were constructed in muscle strips isolated from *T. spiralis* infected and control animals by addition of increasing concentrations of carbachol until a maximal contractile response was achieved in each strip. Carbachol was added to the bathing solution in a cumulative fashion, adding up to concentrations of 3, 6, 10, 30, 60, 100, 300, 600, and 1000 nM.

**MPO Assay.** MPO is a granule-associated peroxidase primarily contained in polymorphonuclear neutrophils and is considered a specific enzymatic marker of neutrophil infiltration. Full-thickness jejunal tissue samples (100–150 mg) were isolated and immediately frozen in liquid nitrogen. The tissues were stored at −70°C and MPO activity was assayed upon completion of the experiments in each group. Tissue homogenization and the extraction of MPO from the homogenate were performed using the procedure previously described by Castro et al. (1974) and modified for ferret intestinal samples (Greenwood and Palmer, 1996). MPO activity was tested in 10-μl samples using a 3.3,5,5′-tetramethylbenzidine (TMB) Microwell Peroxidase Substrate System (Sigma Chemical Co., St. Louis, MO.) and horseradish peroxidase (HRP) as a relative standard. MPO activity was expressed as equivalent to the activity of the amount of HRP (in nanograms) that converts the same amount of substrate for 10 min at room temperature. Total solubilized protein in the samples was measured using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). All data were expressed as nanograms normalized per milligrams of protein.

**Solutions and Drugs.** The modified Krebs bicarbonate solution contained: 120 mM NaCl, 6 mM KCl, 1.2 mM MgCl₂, 1.2 mM NaH₂PO₄, 2.5 mM CaCl₂, 14.4 mM NaHCO₃, and 11.5 mM glucose. The solution was continuously gassed with 95% O₂ and 5% CO₂ (vol/vol) and the pH ranged from 7.2 to 7.3. The following drugs were obtained from Sigma Chemical Co.: carbamylcholine chloride, atropine sulfate, guanethidine sulfate, and tetrodotoxin. Tetrodotoxin was purchased as a powdered substance containing 1 mg tetrodotoxin and 5 mg citrate buffer (pH 4.3). After dilution, aliquots were stored frozen at −20°C and used at the day of the experiment. All drugs were dissolved in distilled water and were added to the baths in volumes less than 1% of the total bath volume.

**Data Analysis and Statistics.** Contractions induced by the addition of KCl or carbachol or by EFS were measured through visual analysis of the chart records. The changes in basal tension (g) were normalized per millimeter squared of cross-sectional area (CSA) for each muscle strip. The CSA was calculated using the following equation: CSA (mm²) = tissue wet weight (mg)/tissue length (mm) × tissue density (mg/mm³), where, tissue length was measured at Lo at the beginning of the experiment, tissue wet weight was measured upon completion of the experiment, and smooth muscle tissue density is assumed to be 1.05 mg/mm³ (Gordon and Seigman, 1971). Concentration-response curves were plotted as percentages of the maximal response against the -log of the molar concentration of carbachol. The concentration of carbachol producing 50% of the maximal effect (EC₅₀) was calculated for each muscle strip by regression.
analysis of the linear portion (20–80% of maximal effect) of the concentration-effect curve. The results are expressed as mean ± S.E. for each experimental group. The number of muscle strips and experimental animals is denoted in the figure legends. Statistical significance of the difference was assessed using one-way ANOVA followed by Bonferroni’s t test for multiple comparisons to define the significance of differences between groups showing normal distribution and equal variances. If the data failed to pass the test for equal variances, the significance of differences was tested using Kruskal-Wallis one-way analysis on ranks followed by Dunn’s test for multiple comparisons. Differences were considered significant at *p* < .05.

## Results

**T. spiralis-Induced Changes in Jejunal Muscle.** The enteric stage of *T. spiralis* infection in the ferret begins with ingestion of the parasite and lasts 14 to 21 days (Campbell et al., 1982). In our experiments adult *T. spiralis* worms were retrieved from scrapings of jejunal mucosa on day 8 PI. On days 30 and 60 PI the intestinal phase of trichinosis was concluded and the mucosa was completely free of worms. However, samples of striated muscle taken from the diaphragm of infected animals showed the presence of encysted *T. spiralis* larvae. Macroscopic assessment of the small intestine after laparotomy showed hyperemia on day 8 PI, whereas on days 30 and 60 PI the intestine looked similar to uninfected animals. It is known that although the infiltration of inflammatory cells into muscularis externa during acute mucosal inflammation is insignificant, the muscle layers in the inflamed intestine show considerable thickening (Blennerhassett et al., 1992). In the *T. spiralis* model of jejunal inflammation in the ferret, we found a significant increase in the cross-sectional area of the muscle strips isolated from infected animals when compared with the uninfected controls. The cross-sectional area of strips dissected in longitudinal or circular direction was significantly increased on day 8 PI and remained increased as late as days 30 and 60 PI (Table 1).

**MPO Activity.** The activity of MPO in the jejunal tissue of *T. spiralis*-infected ferrets was compared with MPO activity in the jejunal tissue of uninfected controls. On day 60 PI, MPO values were reduced to control levels.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Effects of <em>T. spiralis</em> infection on cross-sectional area (CSA) of jejunal muscle strips isolated in longitudinal and circular direction</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of Animals</td>
<td>CSA (mm²)</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
</tr>
<tr>
<td>Day 8 PI</td>
<td>6</td>
</tr>
<tr>
<td>Day 30 PI</td>
<td>5</td>
</tr>
<tr>
<td>Day 60 PI</td>
<td>6</td>
</tr>
</tbody>
</table>

*Statistically significant difference from respective control value (p < .05).*

Neurally Mediated Contractions. EFS (0.5 ms, 0.5–16 Hz) evoked frequency-dependent contractions of the LM and CM strips isolated from *T. spiralis*-infected and uninfected ferrets. The responses were completely abolished by tetrodotoxin (1 μM).

Responses of LM Strips to EFS. In the LM contractions developed as an immediate response to EFS and achieved maximum at a stimulus frequency of 16 Hz. On day 8 PI the amplitudes of EFS-evoked contractions to all frequencies of stimulation did not prove to be significantly different from the responses in uninfected controls (Fig. 2A). On days 30 and 60 PI the amplitude of EFS-evoked contractions was significantly (p < .05) increased for all frequencies of stimulation (Fig. 2A). Despite the increase in amplitude, responses were not shifted to lower stimulus frequencies. To distinguish between the cholinergic and noncholinergic components of the neurally mediated contractions, LM strips were treated with atropine (1 μM). In the presence of atropine (Fig. 2B) the contractile responses to stimuli at frequencies ranging from 0.5 to 4 Hz were virtually abolished (<90% inhibition), revealing a low-amplitude relaxation in some strips. Under the conditions of our experiments, inhibitory responses were masked by the low basal tension (0.1–0.3 g/mm²). To maintain an accurate estimation of the cholinergic component of EFS-evoked contractions, no spasmogens were applied to increase the tone and unmask inhibitory responses. High frequencies of stimulation (8 Hz and 16 Hz) evoked noncholinergic contractions. The amplitudes of the atropine-resistant contractions were significantly (p < .05) increased on days 8, 30, and 60 PI (Fig. 2B). Addition of guanethidine (30 μM) to the bathing solution did not change the amplitude or the pattern of the responses evoked by EFS-evoked (data not shown).

Responses of CM Strips to EFS. In the CM strips contractile responses to EFS were preceded by a brief inhibition, which occurred during the first 2 to 5 s of stimulation and
was rapidly replaced by an excitatory contractile response. The neurally mediated relaxations were insignificant because of the low basal tension of the CM strips. The amplitudes of contraction were frequency-dependent and achieved a maximum at 16 Hz. On day 8 PI, the amplitudes of the contractile responses did not differ significantly from uninfected controls, whereas the responses to stimulation at 16 Hz were significantly increased on day 30 PI (Fig. 3A). On day 60 PI the contractile responses to EFS returned to normal showing values similar to the ones in uninfected controls (Fig. 3A). The increase in amplitude at day 30 PI was not accompanied by a shift to lower stimulus frequencies. In the presence of atropine (1 μM) the contractions to low stimulus frequencies (0.5–2 Hz) were abolished and a low-amplitude relaxation remained the only component of EFS-evoked responses. At higher frequencies of stimulation (4–16 Hz) this inhibition was followed by contractions (Fig. 3B). The amplitudes of atropine-resistant contractions were significantly higher in CM strips isolated on days 8 PI (p < .05) and 30 PI (p < .05) compared with the uninfected controls. Treatment with guanethidine (30 μM) had no effect on the contractile responses evoked by EFS (data not shown).

**Cholinergic/NANC Ratio.** In a separate series of experiments maximal responses to EFS (0.5 ms, 16 Hz) were elicited before and after the consecutive addition of atropine (1 μM) and guanethidine (30 μM). The cholinergic (atropine-sensitive) and NANC (atropine- and guanethidine-resistant) components of excitatory responses were studied as relative

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**Fig. 2.** Effect of *T. spiralis* infection on the contractile responses of jejunal LM strips to EFS (0.5 ms, 0.5–16 Hz for 5 s) in the absence (A) and presence (B) of atropine (1 μM). Frequency-dependent contractions were measured as changes in the resting tension of LM strips isolated on days 8 PI (○, n = 8), 30 PI (▲, n = 7), 60 PI (■, n = 9), and from uninfected controls (▲, n = 9). Values shown are mean ± S.E.; n is the number of muscle strips isolated from at least six ferrets in each group. Comparison with controls was made using Kruskal-Wallis ANOVA on ranks followed by Dunn’s test for multiple comparisons. A, responses to EFS at days 30 and 60 PI were significantly increased (p < .05) at all frequencies of stimulation. B, on days 8 and 30 PI the atropine-resistant responses to EFS at frequencies of 4 to 16 Hz were significantly increased (p < .05). On day 60 PI only the responses to 16 Hz stimulation were significantly increased (p < .05).

**Fig. 3.** Effect of *T. spiralis* infection on the contractile responses of jejunal CM strips to EFS (0.5 ms, 0.5–16 Hz for 10 s) in the absence (A) and presence (B) of 1 μM atropine. Frequency-dependent contractions were obtained in CM strips isolated on days 8 PI (○, n = 7), 30 PI (▲, n = 9), and 60 PI (■, n = 8) and from uninfected controls (▲, n = 8). Values shown are mean ± S.E.; n is the number of muscle strips isolated from at least six ferrets in each group. Comparison with controls was made using Kruskal-Wallis ANOVA on ranks followed by Dunn’s test for multiple comparisons. A, responses to EFS at day 30 PI were significantly different (p < .05) from uninfected controls at stimulation frequencies of 4 to 16 Hz. B, on day 8 PI the atropine-resistant responses to EFS at frequencies of 4 to 16 Hz were significantly increased (p < .05). On day 30 PI the increase was significant (p < .05) only at 16 Hz stimulation, whereas on day 60 PI there were no significant differences.
shares of the total contractile response before treatment. The ratio between cholinergic and NANC components of the contraction was used to characterize the mechanisms of *T. spiralis*-induced changes of neuromuscular transmission. On day 8 PI the cholinergic component was significantly reduced in both LM and CM, which resulted in a decrease of the cholinergic/NANC ratio (Fig. 4). In the LM from uninfected ferrets the cholinergic component represented 76.7 ± 9.2% (n = 6) of the total response, whereas on day 8 PI it decreased to 20.2 ± 1.3% (n = 6). In the CM, the cholinergic component was reduced from 73.5 ± 6.6% (n = 7) in uninfected controls to 27.8 ± 2.9% (n = 5) on day 8 PI. On days 30 and 60 PI, the cholinergic component was completely restored and even increased above control level in the CM (Fig. 4B), whereas in the LM the cholinergic component remained significantly (p < .05) inhibited (Fig. 4A).

**Cholinergic Smooth Muscle Contractions.** To characterize changes involving smooth muscle M2-cholinoreceptors, responses to carbachol were studied in jejunal muscles isolated from *T. spiralis*-infected ferrets and uninfected controls. Carbachol (1 nM–100 μM) evoked concentration-dependent contractions in the LM and CM strips. In both layers, tetrodotoxin (1 μM) caused no change in the amplitude of maximal contractions and no shifts in the concentration-response curves (data not shown).

**Responses of LM Strips to Carbachol.** The concentration-dependent increase in the amplitude of contractions induced by carbachol in LM strips isolated from uninfected and

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**Fig. 4.** Effect of *T. spiralis* infection on the cholinergic and NANC components of maximal contractile responses to EFS (0.5 ms, 16 Hz) in jejunal LM (A) and CM (B) strips. The atropine-sensitive component of the contractile response to EFS at 16 Hz was defined as cholinergic (■). NANC responses (□) were obtained in the presence of 1 μM atropine and 30 μM guanethidine. Cholinergic/NANC ratios, calculated between the mean values of cholinergic and NANC components, are indicated at the top of each bar for days 8, 30, and 60 PI and in uninfected controls. The number of observations in each group was n = 7 strips isolated from at least six ferrets. Statistical significance of the difference from untreated controls was tested by subjecting the raw data (g/mm²) for the cholinergic and NANC components to Kruskal-Wallis ANOVA on ranks followed by Dunn’s test for multiple comparisons. *Statistically significant differences at p < .05.

**Fig. 5.** Effect of *T. spiralis* infection on the contractile responses to carbachol (10⁻⁹ to 10⁻⁶ M) in jejunal LM (A) and CM (B) strips. Concentration-response curves were obtained in strips isolated on days 8 PI (●), 30 PI (■), and 60 PI (■) and from uninfected controls (○). Values are mean ± S.E. The number of observations in each group was n = 8 strips isolated from at least six ferrets. Comparison with controls was made using Kruskal-Wallis ANOVA on ranks followed by Dunn’s test for multiple comparisons. A, in the LM the responses to carbachol at days 30 and 60 PI were significantly different (p < .05) from uninfected controls at concentrations of 10⁻⁸ to 10⁻⁶ M. B, in the CM the responses to carbachol at days 8 and 30 PI were significantly different (p < .05) from uninfected controls at concentrations of 10⁻⁸ to 10⁻⁶ M. At day 60 PI differences were significant (p < .05) for the entire concentration range.
TABLE 2
Effects of T. spiralis infection on maximal contraction and EC_{50} values for carbachol in LM and CM of ferret jejunum
Concentration-response curves were constructed in muscle strips isolated from T. spiralis-infected and control ferrets by cumulative addition of increasing concentrations of carbachol (1 nM–100 μM). Amplitude of maximal contraction and EC_{50} were calculated individually for each muscle strip. Values are mean from ≥8 muscle strips in each group. Comparisons were made using one-way ANOVA followed by Bonferroni's t test for pairwise multiple comparison.

<table>
<thead>
<tr>
<th>No. Animals</th>
<th>LM</th>
<th>CM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Max. contraction</td>
<td>EC_{50}</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>1.2 ± 0.2 g/mm²</td>
</tr>
<tr>
<td>Day 8 PI</td>
<td>6</td>
<td>1.6 ± 0.4 g/mm²</td>
</tr>
<tr>
<td>Day 30 PI</td>
<td>5</td>
<td>3.5 ± 0.4 g/mm²</td>
</tr>
<tr>
<td>Day 60 PI</td>
<td>5</td>
<td>2.7 ± 0.2 g/mm²</td>
</tr>
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* Statistically significant difference from respective control value (p < .05).

T. spiralis-infected ferrets is illustrated in Fig. 5A. On day 8 PI, no significant changes were found in the maximal response and EC_{50} value for carbachol (Table 2). On day 30 PI, responses in the concentration range of 10 nM to 1 μM carbachol were significantly (p < .05) increased, reaching a 3-fold increase in maximal tension. However, the EC_{50} for carbachol remained unchanged (Table 2). On day 60 PI, the responses to carbachol within the entire concentration range were significantly (p < .05) higher compared with controls. This significant increase in the amplitude of responses to low concentrations of carbachol resulted in a shift of the concentration-response curve to the left and a significant (p < .05) reduction of the EC_{50} for carbachol (Table 2). On day 60 PI, the maximal response was lowered compared with day 30 PI, but it was still significantly (p < .05) increased in comparison to the uninfected controls.

Responses of CM Strips to Carbachol. The concentration-response curves obtained for carbachol in CM strips isolated from T. spiralis-infected or uninfected ferrets are shown in Fig. 5B. The contractions induced by carbachol concentrations in the range of 30 nM to 1 μM were significantly (p < .05) higher on day 8 PI. Compared with uninfected controls, there was a more than 2-fold increase in the amplitude of maximal contraction. The changes persisted after the enteric stage of T. spiralis infection was over and similar concentration-response curves were obtained on days 30 and 60 PI. Despite the hyper-responsiveness of the muscle, EC_{50} for carbachol obtained on days 8 and 30 PI showed no alteration (Table 2). Only at day 60 PI was a shift of the concentration-response curve to lower carbachol concentrations established, yielding an EC_{50} that was significantly (p < .05) lower than the one in uninfected control ferrets.

Contraction Induced by High K⁺ Membrane Depolarization. Contraction induced by high K⁺ are considered a receptor-independent phenomenon caused by depolarization of the smooth muscle membrane. In our experiments smooth muscles were treated with increasing concentrations of KCl (20–80 mM) to induce a maximal contraction. The results obtained in LM and CM from T. spiralis-infected and uninfected ferrets are summarized in Table 3. The maximal contractions to high KCl were significantly increased in both LM and CM from T. spiralis-infected ferrets at each of the PI periods. In the CM a maximal 2.5-fold increase was observed during the enteric phase of T. spiralis infection at day 8 PI and tended to decrease on days 30 and 60 PI, whereas in the LM a maximal 2.8-fold increase was found on day 30 PI.

TABLE 3
Effects of T. spiralis infection on maximal contraction induced by high K⁺ depolarization in LM and CM of ferret jejunum
Values are mean from ≥8 muscle strips in each group. Comparisons were made using one-way ANOVA followed by Bonferroni’s t test for pairwise multiple comparison.

<table>
<thead>
<tr>
<th>No. Animals</th>
<th>Max. Contraction</th>
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<td></td>
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</tr>
<tr>
<td>Day 8 PI</td>
<td>6</td>
</tr>
<tr>
<td>Day 30 PI</td>
<td>5</td>
</tr>
<tr>
<td>Day 60 PI</td>
<td>5</td>
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</table>

* Statistically significant difference from respective control value (p < .05).

Discussion
The present study was designed to evaluate the effects of nematode-induced intestinal inflammation on contractility of jejunal LM and CM. Alterations in neuromuscular functions were defined during a 60-day PI period that covers the enteric phase of an experimental T. spiralis infection, its resolution, and a period following recovery. Intestinal inflammation was assessed as changes in the activity of MPO, an enzyme used as a biochemical index of neutrophil infiltration because there is a close correlation between the increase in MPO activity and the extent of mucosal damage in the jejunum (Smith and Castro, 1978). Despite the inflammatory response being localized in the mucosa and submucosa, profound thickening of the smooth muscle layers occur due to hypertrophy and hyperplasia (Blennerhassett et al., 1992). In the current study the increase in cross-sectional area of jejunal muscularis externa on day 8 PI in the ferret confirms the structural changes observed as early as day 6 PI in the rat (Weisbrodt et al., 1994). We observed that the thickness of the external jejunal muscles remained increased even at day 60 PI when inflammatory infiltration was completely reversed to that in found in uninfected ferrets. This thickening of the muscle layers is consistent with some long-lasting changes in muscle function, such as the increased generation of tension in response to high K⁺ depolarization. Moreover, in the ferret smooth muscle, thickening is associated with hypercontractility in both LM and CM during the enteric phase of nematode infection and persists as late as day 60 PI.

During this enteric phase of T. spiralis infection in the ferret, the jejunal muscle displayed increased contractions in response to high K⁺ depolarization but changes in the contractile responses to intramural nerve stimulation were insignificant. Concurrently the LM and CM showed different...
responsiveness to activation of smooth muscle M<sub>2</sub>-muscarinic cholinoceptors: in the CM there was an increase in amplitude of the responses to carbachol, whereas in the LM there were no significant changes in contractions induced by carbachol. Taking into account the increase in contractions evoked by receptor-independent depolarization of the membrane induced by high K<sup>+</sup>, it is reasonable to assume that the enhanced responsiveness of the CM to carbachol reflects alterations in the muscle contractile apparatus rather than significant changes in M<sub>2</sub>-cholinoceptor expression or sensitivity. The functional neuromuscular alterations found by us in the LM during the enteric stage of <i>T. spiralis</i> infection are identical with the alterations described for the LM during the enteric stage of nematode infection in the rat (Vermillion and Collins, 1988; Collins et al., 1989; Grossi et al., 1993) and the mouse (Barbara et al., 1997). Although no data is available characterizing the effect of <i>T. spiralis</i>-induced inflammation in the CM, a decrease in the responsiveness of the this muscle layer has been seen in the rat model of enteric infection induced by <i>Nippostrongylus brasiliensis</i> (Crosthwaite et al., 1990) and a guinea pig model of trinitrobenzenesulfonic acid-induced ileitis (Martinolle et al., 1997). These findings differ from the increase in the contractile responses to high extracellular concentrations of K<sup>+</sup> or to carbachol observed in the CM from the <i>T. spiralis</i>-infected ferret. Despite the differences, which probably reflect species-dependent characteristics of the nematode parasite or the host, similarities were established between neurally mediated alterations during the acute inflammatory response in the rat and the ferret. Nerve stimulation in the presence of atropine induced noncholinergic excitation in the CM from <i>N. brasiliensis</i>-infected rats, whereas uninfected muscles respond with relaxation. This observation is compatible with the increase in the amplitude of NANC excitatory responses in the ferret. The reduction in inhibitory innervation to the CM proposed by Crosthwaite et al. (1990) in combination with the impairment of cholinergic neurotransmission may be a common mechanism contributing to the augmentation of NANC excitation during the enteric stage of nematode infection. Inhibitory responses were not evaluated in our experiments but we found a significant increase in the amplitude of noncholinergic contractions and an increased share of the NANC component in the response to high-frequency nerve stimulation in both LM and CM. These findings are in agreement with the data reported for ricin-induced inflammation in the rabbit ileum, where the CM generated larger noncholinergic excitatory junction potentials in response to nerve stimulation (Goldhill et al., 1995) and showed enhanced responses to tachykinin agonists in the presence of TTX or a combination of atropine and an inhibitor of NO synthesis (Goldhill et al., 1997). In the rabbit ileum, the increase in noncholinergic excitatory junctional potentials was reversed by Substance P autodesensitization and the enhanced contractile responses were due predominantly to activation of neurokinin 1 receptors. We would like to speculate that similar mechanisms may be involved in the enhanced NANC excitation during the enteric stage of nematode infection in the ferret. In addition, a more complicated smooth muscle mechanism, which involves rebound contraction following the effects of inhibitory neurotransmitters, may contribute to the long-lasting increase in NANC excitation. Similar findings in the dog (Ward et al., 1992) and cat (Venkova and Krier, 1994) colon, suggest that NO released by enteric nerves may play a role in infection-dependent hyper-responsiveness of the intestine.

Despite the reversal of small intestinal muscle dysfunction demonstrated after resolution of the <i>T. spiralis</i> infection in rats (Vermillion and Collins, 1988), it is evident from the current findings that at least some inflammatory-induced abnormalities persist after resolution of the infection and may cause permanent neuromuscular dysfunction. A persistent intestinal neuromuscular dysfunction following nematode infection has been demonstrated in mice by Barbara et al. (1997). Our results in the ferret are consistent with the increased contractility and suppression of cholinergic nerve function in the LM found in the murine small intestine. We have advanced these observations showing that the increased muscle contractility is not confined only to the LM but involves also the CM layer. The long-lasting hypercontractility to carbachol is complicated because it reflects both the increased ability of the contractile apparatus to develop smooth muscle tension and an increased sensitivity to cholinergic agonist that developed as late as day 60 PI. This late change in cholinergic responses of the LM most likely reflects an adaptation to the decreased release of transmitter from cholinergic nerve terminals, through changes in the smooth muscle cell amplification system for cholinoceptor stimuli. Our data during the enteric phase of infection favor such a mechanism, because the atropine-sensitive component of the neurally mediated responses was inhibited, indicating suppression of myenteric cholinergic activity. At the same time the atropine-resistant component and the relative share of NANC excitation in the response to EFS were increased (i.e., cholinergic/NANC ratio is decreased) during the enteric phase of infection. After resolution of the infection, the atropine-resistant component remained enhanced in the LM, whereas in the CM it returned to levels in uninfected controls. These results demonstrate that the enteric neurotransmission responds to inflammation with dynamic changes in the balance between cholinergic and NANC excitatory neurotransmitters mediating the motor response to enteric nerve stimulation. The motor response in the jejunum is transformed from predominantly cholinergic in uninfected controls to predominantly NANC during the enteric stage of infection. In the CM the alterations are temporary and return to control levels upon recovery of the mucosa, whereas in the LM the increase in NANC excitatory component of neurally mediated responses appears to be permanent because it persists after the resolution of mucosal inflammation.

In summary, nematode-induced intestinal inflammation results in two different forms of neuromuscular alterations. The first involves the neuromuscular dysfunction that occurs during the inflammatory reaction of the host that is involved in the expulsion of the parasite from the intestine and is characterized by dramatic changes in motility, the release of inflammatory mediators such as prostaglandins (Kao and Zipser, 1988), cytokines, and nitric oxide (Collins et al., 1992; Lyons, 1995; Collins, 1996). The second type of neuromuscular dysfunction is long lasting and is apparent after resolution of the inflammation and healing of mucosal injury. At the level of the smooth muscle these changes involve a permanent increase of the muscle ability to develop active tension. At the level of the myenteric nerves there is a long-
lasting change in the balance between cholinergic and NANC excitatory neurotransmission.

In conclusion, we have shown that long-term changes in muscle contractility occur in response to a brief enteric infection. Although the mechanisms that generate the long-lasting changes in intestinal motility require additional investigation, in the current experiments we have clearly shown that they are in part due to abnormalities in the enteric neural regulation of the smooth muscle. Our results may provide an explanation for the clinical observation that following acute infectious gastroenteritis, the symptomatology of an irritable bowel persists in some patients for years after a complete recovery from the infection (McKendrick and Read, 1994; Gwee et al., 1996). Furthermore, our results may explain why some patients in remission from inflammatory bowel disease suffer the symptoms of an irritable bowel, in which painful gastrointestinal symptoms are present in the absence of mucosal inflammation (Isgar et al., 1983).

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References


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