Inflammation-induced impairment of enteric nerve function in nematode-infected mice is macrophage dependent

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1Intestinal Diseases Research Programme and 2Gastrointestinal Division and Department of Medicine, Health Sciences Centre, McMaster University, Hamilton, Ontario, Canada L8N 3Z5; and 3Department of Cell Biology, Vrije Universiteit, Amsterdam, The Netherlands

Galeazzi, Francesca, Eric M. Haapala, Nico van Rooijen, Bruce A. Vallance, and Stephen M. Collins. Inflammation-induced impairment of enteric nerve function in nematode-infected mice is macrophage dependent. Am. J. Physiol. Gastrointest. Liver Physiol. 278: G259–G265, 2000.—Trichinella spiralis infection in rodents is associated with suppression of ACh release from myenteric plexus that can be mimicked by macrophage-derived cytokines. We verified the presence of a macrophage infiltrate in the intestine during T. spiralis infection and determined the extent to which this cell type is responsible for the neural changes. C57BL/6 mice were infected with 375 T. spiralis larvae by gavage, and the presence of macrophages (F4/80 positive) in the jejunum was determined immunohistologically. In another experiment, infected mice were treated intravenously with liposomes containing dichloromethylene diphosphonate (clodronate, Cl2MDP), which causes apoptosis of macrophages, and killed at postinfection day 6, and jejunal tissues were evaluated for the presence of F4/80-positive cells and for [3H]ACh release from the myenteric plexus. Infection caused an infiltration of F4/80-positive cells into the intestinal mucosa, muscle layers, and myenteric plexus region and a significant suppression of ACh release (50%). Depletion of F4/80-positive macrophages using Cl2MDP-containing liposomes prevented the suppression in [3H]ACh release, identifying macrophages as the cell type involved in the functional impairment of enteric cholinergic nerves.

inflammatory bowel disease; enteric nervous system; acetylcholine; liposomes

INTESTINAL MOTILITY is under the control of several integrated systems, including the neurogenic, myogenic, and endocrine systems as well as the interstitial cells of Cajal. During inflammatory processes, this complex regulation is disrupted, as reflected in reports of motility disorders in human inflammatory bowel disease (IBD) and in animal models of IBD (1, 22).

Structural changes of enteric nerves have been described in IBD (12), and animal models have allowed the investigation of functional alterations of enteric nerves during inflammation. To evaluate the impact of intestinal inflammation on gut physiology, we have used a model of nematode infection in rats and mice. During the intestinal phase of Trichinella spiralis infection in rodents, mucosal inflammation is accompanied by a suppression of neurotransmitter release, such as ACh and norepinephrine, by enteric nerves (8, 14). Studies in rats and mice have shown that these alterations are not dependent on the presence of T lymphocytes and can be prevented by treatment with steroids (8, 13). Potential mediators of the functional alterations in enteric nerves have also been identified: administration of exogenous proinflammatory cytokines interleukin (IL)-1β and tumor necrosis factor (TNF-α) to control tissue mimics the impairment of neurotransmitter release from longitudinal muscle-myenteric plexus (LM-MP) observed during T. spiralis infection in animals (18, 14), and a synergistic effect of IL-6 and IL-1β has been described (24). Furthermore, increased mRNA expression for these cytokines has been shown in preparations from LM-MP during T. spiralis infection (15). Together, these data indicate that disruption of enteric nerve function during T. spiralis infection is a consequence of the inflammatory process and may involve locally expressed cytokines. However, although the potential mediators have been identified, the cell type responsible for these changes is unknown.

Several cell types share the ability to produce IL-1β (10), TNF-α (33), and IL-6 (32). In particular, macrophage-like cells with morphological signs of activation have been found in the myenteric plexus in T. spiralis-infected rats (9, 23). Thus macrophages may play a pivotal role in inducing functional disturbances in enteric nerves in T. spiralis infection. Study of the influence of macrophages in in vivo models has now been made possible by the development of a technique for depleting these cells, using liposomes encapsulating dichloromethylene diphosphonate (clodronate, Cl2MDP), which induces the apoptosis of macrophages on phagocytosis (28, 31).

Thus the purpose of the present study was to use liposome-mediated macrophage depletion to examine the role of macrophages in the functional impairment of enteric nerves during T. spiralis infection.

MATERIALS AND METHODS

Animals

C57BL/6 male mice (6–8 wk of age) were purchased from Taconic (Germantown, NY) and kept under specific pathogen-free conditions. All animals were housed in a conventional environment and had free access to food and water throughout the study. Animal experiments were conducted in accordance with the institutional animal care guidelines as approved by the Institutional Animal Care Committee of the University of Ottawa or the University of Waikato, Hamilton, New Zealand.

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free conditions at McMaster University central animal facilities. All the experiments were approved by the McMaster University Animal Care Committee and the Canadian Council on Animal Care.

Study Design

Mice were infected with 375 T. spiralis larvae by gavage and euthanized at postinfection day 6. For the immunohistochemical study, mice were euthanized on postinfection day 3, 6, 10, 21, or 28.

For the ACh release study, mice were infected using the same protocol and were studied on postinfection day 6. Animals received intravenous injections of liposomes containing Cl2MDP or PBS 4 h before infection and at postinfection days 1, 2, and 4. Myeloperoxidase (MPO) activity, as an index of granulocytic infiltration (5), was also evaluated at postinfection day 6. Uninfected mice, maintained under the same housing conditions, were used as controls for both studies.

Immunohistochemistry for Macrophages

Immunohistochemistry for macrophages was performed on cryopreserved sections of mouse jejunum using a monoclonal antibody recognizing F4/80 antigen, a glycoprotein expressed by mature murine macrophages (4). This antibody has already been used as a marker of macrophage viability in studies involving liposome-mediated macrophage depletion (20).

Mice were euthanized by cervical dislocation, and the jejunum was taken, gently washed in cold PBS, embedded in OCT compound, frozen using isopentane and liquid nitrogen, and stored at −70°C. Cross sections were cut at a thickness of 4 μm and fixed in ice-cold acetone for 20 min. To detect murine macrophages, a rat-anti-mouse monoclonal antibody directed against F4/80 antigen was used (clone Cl:A3-1; Serotec, Oxford, UK). Biotinylated polyclonal goat anti-rat antibody (Cedarlane Laboratories, Hornby, ON, Canada) was used in conjunction with Cl:A3-1. All antibodies were diluted in a 1% BSA solution in Tris-buffered saline (TBS). Immunohistochemistry was performed by using the streptavidin-biotin-peroxidase technique. Sections were washed three times in TBS, and endogenous peroxidase was blocked by submerging the slides in 10% hydrogen peroxide for 30 min. Sections were then washed three times with TBS and incubated with 1% BSA in TBS for 30 min. BSA was then washed off with TBS (3 washes). Cl:A3-1 was then added to the slides at a 1:50 dilution and left overnight at room temperature. The slides were then washed three times with TBS and goat anti-rat antibody, previously incubated in 10% mouse serum, was added at a 1:200 dilution for 1 h. The slides were again washed with TBS (3 times) and streptavidin-horseradish peroxidase (GIBCO BRL Life Technologies, Burlington, ON, Canada) was added at 1:300 dilution for 1 h. After three washes with TBS, antibodies were visualized by using 3-amino-9-ethylcarbazole. Tissues were then counterstained with Mayer’s hematoxylin and examined under a light microscope. Control experiments involved incubation of the sections with a rat IgG2b isotypic control (Clone LO-DNP-57, Cedarlane Laboratories) instead of the primary antibody or incubation with TBS, without adding the primary antibody.

MPO Activity

At postinfection day 6, full-thickness specimens from jejunum were taken for evaluation of MPO activity, as an index of granulocytic infiltration. Tissues were snap frozen in liquid nitrogen and then stored at −70°C. MPO activity was measured within 7 days, using the method described previously (6,7). Briefly, tissues were homogenized in hexadecyltrimethylammonium bromide buffer and centrifuged. The supernatant was then added to a solution of O-dianisidine (Sigma, St. Louis, MO) and hydrogen peroxide. The absorbance of the color reaction was then measured using a spectrophotometer. MPO is expressed in units per milligram of wet tissue, 1 unit being the quantity of enzyme able to convert 1 μmol of hydrogen peroxide to water in 1 min at room temperature. Liposome-Mediated Macrophage Depletion

Administration of liposomes containing Cl2MDP is a widely used technique to deplete macrophages. Cl2MDP is not, in itself, a toxic drug, but once released into macrophages from phagocytosed liposomes, it induces apoptosis (20, 31). Liposomes were prepared as previously described (30) and then were administered by serial intravenous injections 4 h before infection with T. spiralis and at postinfection days 1, 3, and 4. Previous studies optimized the dose of 0.2 ml of liposome suspension as effective for macrophage depletion in the mouse (28); therefore, this dose was used in the experiments on macrophage depletion. Mice then received 0.2 ml of a suspension of liposomes containing Cl2MDP (a gift from Boehringer Mannheim, Mannheim, Germany) or PBS as control, by tail vein injections under anesthesia. The efficacy of macrophage depletion was evaluated by immunohistochemistry of the cryopreserved section as described above.

[3H]ACh Release

[3H]ACh release was measured using LM-MP preparations. LM-MP were obtained by using a modification of a previously described technique (8).

LM-MP preparations. The entire length of jejunum was removed beginning from the ligament of Treitz and then placed in oxygenated (95% O2-5%CO2) Krebs buffer (in mM: 120.9 NaCl, 2.5 CaCl2, 1.2 MgCl2, 15.5 NaHCO3, 1.2 NaH2PO4, and 11.1 glucose). The jejunum was cut into 2-cm segments, and the mesentery was carefully removed. Each segment was then mounted onto a ½-in. Acrylic rod, and the serosal surface was scored using a dull scalpel blade. The LM-MP was then carefully peeled using a cotton swab. LM-MPs were tied at each end in the longitudinal axis and incubated in Krebs buffer containing 0.5 μM [3H]choline (specific activity 81 Ci/mmol) (DuPont) for 45 min. Temperature was maintained at 37°C by a water bath.

[3H]ACh release studies. Tissues were suspended in a water-jacketed bathing chamber inside an inner chamber lined with electrode rings of opposite polarity and then superfused with Krebs buffer containing 10 μM hemicolinium-3 (Sigma) by a peristaltic pump (Masterflex 7518-10, Cole Parmer) at a rate of 1 ml/min for 80 min. The superfusate was collected every 5 min using a fraction collector (Ultrorac 7000). Tissues were allowed to equilibrate for 40 min and then were stimulated with 50 mM KCl. At the end of the experiments, LM-MPs were weighed and then solubilized with 1 ml of NCS tissue solubilizer (Amersham, Oakville, ON, Canada). After 4 ml of aqueous counting scintillant (Amer sham) were added to each 2-ml sample, the tritium content was analyzed using a liquid scintillation counter (LS 5801, Beckman). Tritium release was calculated as in previous studies (8). Briefly, a baseline of spontaneous outflow of tritium from the tissue over the 80-min interval was obtained by fitting a linear regression line of log dpm of the five samples before the stimulation (KCl) and the final five samples of the experiment. The total tritium content of each tissue was calculated by the summation of tritium released during the course of the experiment before the stimulation and the counts remaining in the tissue after solubilization.
The evoked tritium release was obtained from the difference of total tritium released at each stimulation and the calculated baseline and expressed (in %) as the fractional release of the total tritium incorporated by the tissue during incubation.

Data Analysis

Immunohistochemistry was performed on tissues from four animals per group. Twenty villi per section and four nonadjacent sections from each animal were examined. Sections were coded, and the examination was conducted by a blinded observer. The number of positive cells was counted per villus crypt unit (VCU). Tissues from animals from several different infections were evaluated, and the experiments were repeated four times.

[3H]ACh release studies as well as the evaluation of MPO activity involved six mice per group. The experiments involved several different infections and were repeated six times. Statistical analysis was performed by using ANOVA and Bonferroni’s test. Statistical significance was inferred for P values < 0.05.

RESULTS

Time Course of Macrophage Infiltration Into Jejunum During T. spiralis Infection

In uninfected animals, F4/80-positive cells were present in the mucosa but not in the muscle layers (Fig. 1A). By postinfection (T. spiralis) day 3, an increased number of F4/80-positive cells was found in the mucosa, and few cells were seen in the muscle layers (Fig. 1B). Between postinfection days 6 and 10, numerous positive cells were evident in the muscle layers, with distribution clustered mainly in the circular muscle and in the myenteric plexus regions (Fig. 1, C and D). These findings are consistent with the onset of the functional neural changes in this model, which became evident by postinfection day 6 (B). In the late phases of the infection (day 21) few cells were still present in the muscle regions, but were no longer evident by postinfection day 28 (Fig. 1E).

Effect of Liposome Treatment

Infection with T. spiralis did not cause death in any animals studied. Intravenous injections with liposomes caused the same mortality rate whether the liposomes contained PBS or Cl2MDP (2 of 10 mice/group injected), and it was likely associated with the intravenous injection itself, because it was observed during the procedure. No deaths were observed in mice that could be related to macrophage depletion concomitant to the infection.

Macrophage Infiltrate

As shown in Fig. 1C, infection with T. spiralis caused a 240% increase in the number of F4/80-positive cells identified in the jejunum, with 8.1 ± 0.57 cells/VCU in uninfected mice rising to 19.8 ± 0.5 cells/VCU at postinfection day 6 (P < 0.05). In infected mice, repeated injections of PBS-containing liposomes did not modify the extent of macrophage infiltration at postinfection day 6 and numerous F4/80-positive cells remained evident in the mucosa, with numbers similar to those seen in untreated infected mice (19.3 ± 1.8 cells/VCU). Furthermore, F4/80-positive cells remained evident within the external muscle layers, with a clustered distribution within the myenteric plexus region, similar to the pattern seen at postinfection day 6 in the absence of liposome treatment (Fig. 2A).

In contrast, jejunal sections from mice treated with Cl2MDP-containing liposomes showed a significant reduction in the number of F4/80-positive cells in the mucosa (12.5 ± 1.4 cells/VCU), compared with the tissues from PBS-containing liposome-treated infected mice and infected mice not submitted to the treatment (P < 0.05) (Figs. 2B and 3). This value was not significantly different from that seen in uninfected mice. The patchy distribution of macrophages in the muscle layers and myenteric plexus made it unsuitable to assess the effect of liposome treatment by simply counting the positive cells in the muscle layers. Instead, the number of F4/80-positive cells/VCU was used to assess the efficacy of liposome mediated depletion.

MPO Activity

MPO activity is a commonly used marker of granulocytic infiltration. As expected, infection with T. spiralis led to a significant (P < 0.05) increase in myeloperoxidase activity, from 0.5 ± 0.1 U/mg tissue in uninfected mice to 3.3 ± 0.8 U/mg tissue at postinfection day 6. As expected, a similar MPO value was found in infected mice treated with PBS-containing liposomes (3.0 ± 0.7 U/mg). However, a statistically insignificant reduction in MPO activity was seen in infected animals after treatment with Cl2MDP-encapsulating liposomes (2.3 ± 0.4 U/mg; Fig. 4).

[3H]ACh Release

Treatment with liposomes encapsulating either PBS or Cl2MDP did not affect ACh release in uninfected mice, with values similar to those found in uninfected animals not submitted to liposome treatment (2.3 ± 0.3, 1.5 ± 0.2, and 1.8 ± 0.05%, respectively; P > 0.05).

We next investigated ACh release after infection with T. spiralis. There was a 50% suppression of KCl-evoked ACh release from the LM-MP of mice infected 6 days previously, as values dropped from 1.8 ± 0.05% in uninfected controls to 0.9 ± 0.1% at postinfection day 6 (P < 0.05; see Table 1). A similar value (1.2 ± 0.1%) was found in infected mice after treatment with PBS-containing liposomes.

In contrast, treatment with Cl2MDP-encapsulating liposomes completely prevented the suppression of ACh release in infected mice, with values similar to those obtained in uninfected controls (1.83 ± 0.3 and 1.8 ± 0.05%, respectively; P = not significant; Fig. 5), implicating macrophages as the cell type responsible for the functional impairment of cholinergic nerves in this model (see Table 1).

DISCUSSION

The purpose of the present study was to examine the role of macrophages in the development of enteric
cholinergic nerve dysfunction during intestinal inflammation in a model of nematode infection in the mouse. Previous studies have shown that during the intestinal phase of *T. spiralis* infection enteric nerves exhibit an impairment of their ability to release ACh after stimulation and that a similar suppression of ACh release can be induced in tissues from uninfected animals by several proinflammatory cytokines. In this study, we have demonstrated that macrophages infiltrate the muscle layers and myenteric plexus region and that depletion of these macrophages prevents the functional neural changes.

We first investigated whether macrophages are present within the myenteric plexus during the enteric phase of *T. spiralis* infection. In normal mice, no F4/80-positive macrophages were evident in the muscle layers and myenteric plexus area. Previous data have shown the presence of macrophage-like cells within the...
myenteric plexus in normal rodents (23, 19); this discrepancy may be caused by the housing conditions of the animals, because in our experiments mice were kept in a specific pathogen-free environment. In contrast, T. spiralis infection was accompanied by a conspicuous infiltration of macrophages in the muscle layers and myenteric plexus, reaching a maximum by postinfection day 6 and decreasing in the late stages. These results are consistent with previous findings, because electron microscopy demonstrated the presence of activated macrophage-like cells in the myenteric plexus in infected rats (23).

To verify whether macrophages were the cell type responsible for the impairment in ACh release during T. spiralis infection, we used a liposome-mediated technique to deplete them. Liposomes are selectively ingested by phagocytic cells and are currently used as vehicles for foreign molecules. In our procedure Cl2MDP, a substance able to induce apoptosis of the cells (20, 31), was delivered into the cells by using liposomes as carriers. Previous studies have shown that this technique allows a selective depletion of macrophages, whereas other cell types are not affected (20, 30, 31). This procedure has been extensively used to selectively deplete macrophages from specific targeted organs. The site of the depletion depends on the route of administration of liposomes, because intravenous administration affects mainly splenic and hepatic macrophages (29), whereas intraperitoneal injection depletes peritoneal cells (5). Depletion of intestinal macrophages has been more difficult to achieve and has only been described in one study after oral administration of liposomes; however, this strategy only allowed a depletion limited to macrophages in Peyer’s patches of the terminal ileum (3). Our study is the first showing a protocol effective in depleting macrophages at an inflamed intestinal site.
Previous studies in T. spiralis-infected mice have shown an increased blood flow in the inflamed gut in the early stages of the infection (21). Thus, by giving serial administrations of liposomes into the bloodstream, we could target the inflamed intestine, which is an area of increased cell migration and blood flow. Another possibility is that the liposome treatment affected the cells arising from the bone marrow and thus prevented them from infiltrating the inflamed gut. We have shown by immunohistochemistry that this strategy was effective in decreasing the number of macrophages present in the inflamed tissue. In mice treated with Cl2MDP-containing liposomes, the number of macrophages in the inflamed intestine was similar to that seen in uninfected animals.

In normal conditions, liposome treatment did not influence cholinergic enteric nerve function, as measured by ACh release, because we found no differences in this parameter in uninfected mice injected with either PBS- or Cl2MDP-containing liposomes. However, in animals infected with T. spiralis, although PBS-encapsulating liposomes did not influence the suppression in ACh release caused by the infection, treatment with Cl2MDP liposomes completely prevented the impairment in cholinergic nerve function. As already mentioned, this treatment decreased the number of macrophages present in the inflamed tissue, with values similar to those seen in uninfected mice. These data show that the impairment in ACh release caused by the infection was prevented by depletion of macrophages, and we therefore conclude that macrophages are the cells responsible for the suppression in ACh release seen during T. spiralis infection. This is also in keeping with the finding of a temporal relationship between the presence of macrophages in the myenteric plexus and altered nerve function and with previous studies showing that functional alterations of enteric nerves are inducible in vitro, in tissues from uninfected rats, by macrophage-derived proinflammatory cytokines (14, 18).

The observation that the number of macrophages in mice treated with Cl2MDP-containing liposomes was similar to that seen in uninfected controls suggests that the depletion selectively affected the infiltrating cells and did not alter the number of resident macrophages. Thus we speculate that the macrophages infiltrating the intestine during the infection, rather than the resident macrophages, are the cells involved in the development of the neural changes. This is consistent with data from studies performed in humans, because it has been shown that, although resident intestinal macrophages from normal intestinal biopsies do not express TNF-α mRNA, this is detectable in infiltrating macrophages present in biopsies from patients with severe enterocolitis (26). Studies in IBD patients have also shown that mononuclear cells from normal colonic mucosa produce less IL-1β and are less sensitive to lipopolysaccharide than mononuclear cells from inflamed mucosa, indicating that the cytokine likely derives from a newly recruited cell population (17).

Myeloperoxidase activity is commonly used in in vivo models of inflammation as an index of granulocytic infiltrate (6, 7). In our study, as expected, the infection resulted in an increase of MPO activity. After macrophage depletion MPO values were lower than those seen in infected animals not treated with Cl2MDP-containing liposomes. This is not surprising, because macrophages produce several chemotaxitants, such as platelet-activating factor, leukotriene B4, (3, 25), and macrophage inflammatory protein 1 and 2 (11). However, after macrophage depletion, MPO activity remained higher in infected mice than in normal controls, indicating that depletion of macrophages did not completely prevent the infiltration of granulocytes, which are the likely source of the enzyme. Thus our results indicate that macrophages are directly involved in causing the cholinergic nerve alterations in this model. This is also consistent with the previous demonstration that the mediators of enteric nerve dysfunction in the rat are macrophage-derived cytokines.

The impairment of the ability of enteric nerves to release neurotransmitters is shared by several inflammatory conditions, and it does not depend on either the initiating stimulus or the intestinal district involved. We have previously shown (16) a suppression in norepinephrine release in colonic tissues during experimental colitis induced either by a hapten or by parasitic

Table 1. Effect of treatment with PBS- or Cl2MDP-containing liposomes on KCl-stimulated [3H]ACh release from LM-MP after T. spiralis infection

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<tr>
<th>Experimental Group</th>
<th>[3H]ACh Release, %</th>
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<tr>
<td>Controls</td>
<td>1.8 ± 0.0</td>
</tr>
<tr>
<td>Day 6</td>
<td>0.9 ± 0.1*</td>
</tr>
<tr>
<td>Day 6 + PBS</td>
<td>1.2 ± 0.0†</td>
</tr>
<tr>
<td>Day 6 + Cl2MDP</td>
<td>1.8 ± 0.3</td>
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Values are means ± SE for [3H]ACh release in longitudinal muscle-myenteric plexus (LM-MP) from noninfected C57BL/6 mice (controls, n = 8) and C57BL/6 mice infected with Trichinella spiralis measured 6 days after infection. Day 6, mice infected but not treated with liposomes (n = 6); Day 6 + PBS, mice infected and treated with PBS-containing liposomes (n = 6); Day 6 + Cl2MDP, mice infected and treated with dichloromethylene diphosphonate (Cl2MDP)-encapsulating liposomes. * P < 0.05 vs. controls; † P < 0.05 vs. Day 6 + Cl2MDP.
infection. Thus it seems that enteric nerves present a unique response during inflammation. In our model, this response is macrophage dependent, because it is prevented by the depletion of infiltrating macrophages. These findings not only further demonstrate a direct interaction between inflammatory cells and nerve function but also open the possibility of new therapeutic approaches for the treatment of neuromotor disturbances during inflammation.

Finally, because the changes in muscle contractility observed in this model are T cell dependent (27), our results showing that nerve functional changes are instead macrophage dependent indicate that intestinal inflammation alters nerve-muscle function by distinct and separate pathways.

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