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Development and Sequels of Intestinal Inflammation in Nematode-Infected Rats: Role of Mast Cells and Capsaicin-Sensitive Afferents

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Key Words
Mast cells · Sensory neurons · Inflammation · Nematode infection

Abstract
Objectives: To determine whether intestinal mast cells and capsaicin-sensitive afferent nerves are involved in the development and sequels of *Nippostrongylus brasiliensis*-induced intestinal inflammation in rats. ***Methods:*** Two series of experiments were performed. In the first series, six groups of 8 rats were used to study the effects of mast cell stabilization by ketotifen. In the second series, six groups of 6 rats were used to study the effects of gut extrinsic sensory neuron depletion by capsaicin. For each series, four groups of rats were infected with *N. brasiliensis* and two groups were not infected. ***Results:*** Infection with *N. brasiliensis* resulted in an increase of myeloperoxidase (MPO) activity and mast cell numbers at day 12 postinfection; MPO returned to preinfection levels by day 35 while mast cell numbers remained elevated at that time. In ketotifen-treated infected rats, the increase of MPO at day 12 was less pronounced, but MPO activity remained elevated and mast cell numbers were increased at day 35. In capsaicin-treated infected rats, the MPO increase at day 12 was augmented, and MPO was still not returned to preinfection values by day 35; in contrast, the increase of mast cell numbers at days 12 and 35 was not modified by afferent nerve depletion. ***Conclusion:*** Mast cell stabilization decreased jejunal inflammation during the acute stage (day 12), but prolonged the inflammatory process until at least day 35 postinfection. The data also confirmed the protective role of gut extrinsic sensory neurons against intestinal inflammation in a model of nematode infection and revealed that these afferent nerves do not seem crucial for the development of nematode-induced hypermastocytosis.

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Introduction

Both sensory neurons and mast cells have been found involved in intestinal inflammatory processes. Thus, while afferent nerves exert a protective role upon intestinal tissues [1–3], mast cells are generally implicated in the development of the inflammatory process [4, 5]. However, these roles are inferred from data obtained at a given time in experimental models of intestinal inflammation in animals. In contrast, inflammatory bowel disease (IBD) in humans is a chronic disease with intermingled periods of remission and relapse; the role of sensory neurons in the development of IBD has never been investigated.

The development of the intestinal inflammation induced by the nematode *Nippostrongylus brasiliensis* is well documented. *N. brasiliensis* induces an acute inflammation in the jejunum whose intensity peaks 12–14 days after infection [6]. This inflammation is characterized by mucosal lesions that consist of villus atrophy and crypt

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hyperplasia associated with mast cell activation and leukotriene generation [5–7]. Thirty days after infection, jejunal inflammation has resolved but mucosal mast cell hyperplasia and a profound enteric nerve remodeling persist for several weeks [8, 9].

Thus, a role of mast cells and their mediators has been found in some animal models of experimental inflammation [10–13], whereas some studies have shown that mast cells may not be essential in other experimental models such as trinitrobenzene sulfonic acid (TNBS)- or dextran sodium sulphate (DSS)-induced colitis in mice [14, 15]. In man, however, a greater number of intestinal mast cells has been reported in IBD patients in comparison with healthy subjects [16, 17] and mast cells may play a functional role in the inflammatory process of IBD [17] and its associated alterations of intestinal ion transport [16]. Several factors have been shown to promote and stimulate the development of mast cell hyperplasia such as T cells [18], stem cell factor secreted by fibroblasts and other stromal cells, epithelial and endothelial cells [19], interleukin-3 (IL-3), IL-4 and IL-10 produced by T cells and mast cells [20, 21].

The nervous system may also play a trophic role in the development of mast cells. Thus, truncal vagotomy or destruction of sensory afferent nerves by neonatal capsaicin significantly reduces the population of jejunal mucosal mast cells in rats [22]. This is anatomically supported by the close apposition between mast cells and sensory nerve fibers found both in normal and nematode-infected rats [8, 23, 24]. In addition, the number of jejunal mast cells in close association with neural processes is increased by 60% after *N. brasiliensis* infection in the rat [25], suggesting a dynamic interplay between the immune and nervous systems during inflammatory episodes in the gut.

The aim of this study, therefore, was to investigate the involvement of both resident mast cells and capsaicin-sensitive afferent nerves in the modulation of the intensity and development of jejunal inflammation and mast cell hyperplasia in rats infected with the nematode *N. brasiliensis*.

Material and Methods

Animals and Nematode Infection

Male Wistar rats weighing 250–300 g were used for these experiments. Animals were housed in polypropylene cages with a standard diet (A04, Usine d'Alimentation Rationnelle, Epinay-sur-Orge, France) and tap water provided ad libitum. Experiments were conducted in control and *N. brasiliensis*-infected rats. Infection was achieved via subcutaneous (s.c.) injection of 2,500 third-stage (L3) infective larvae of *N. brasiliensis* in 0.5 ml saline (NaCl 0.9%) into the flank of the rats (day 0). Controls received a single s.c. injection of 0.5 ml saline at day 0. The dose and route of administration of infective larvae were consistent with previously described techniques [26, 27].

Myeloperoxidase Activity

The activity of the enzyme myeloperoxidase (MPO), a granulocyte-associated enzyme mainly present in polymorphonuclear neutrophils, was determined in jejunal tissues according to Bradley et al. [28]. Full-thickness segments of jejunum (2 cm long) were suspended in potassium phosphate buffer (50 m*M*, pH 6.0) and homogenized in ice using a Polytron (3 × 10 s at maximal speed setting). Three cycles of freezing and thawing were done. Suspensions were then centrifuged at 10,000 *g* for 15 min at 4 °C. Supernatants were discarded and pellets were resuspended in hexadecyl trimethylammonium bromide buffer (0.5% W/V in potassium phosphate buffer), a detergent inducing a release of MPO from neutrophils. These suspensions were sonicated on ice and again centrifuged at 10,000 *g* for 15 min at 4 °C. Supernatants were then diluted in potassium phosphate buffer containing 0.67 mg/ml of *o*-dianisidine dihydrochloride and 0.0005% of hydrogen peroxide. MPO from human neutrophils (Sigma, St. Louis, Mo., USA; 0.1 U/100 µl) was used as a standard. Changes in absorbance at 450 nm were recorded every 10 s over 2 min with a spectrophotometer (Uvikon 860, Kontron Instruments, St Quentin-en-Yvelines, France). Protein concentration (g/ml) was determined by the modified method of Lowry (Detergent Compatible Assay, BioRad, Ivry-sur-Seine, France), and MPO activity was expressed as MPO units/gram protein, 1 MPO activity unit being defined as the quantity of MPO degrading 1 µmol hydrogen peroxide/min/ml at 25 °C.

Histology

Full-thickness pieces of jejunum (1 cm long), collected 10 cm distal to the ligament of Treitz, were fixed in Carnoy's solution, cleared in toluene and embedded in paraffin blocks. Transverse sections (5 µm thick) were stained with Alcian blue-safranin O according to Roberts et al. [29], for identification of intestinal mast cells, both in the mucosa and submucosa. Three sections per animal and three views per section were examined in a blind fashion. Numbers of mast cells per square millimeter of jejunal mucosa and submucosa were determined with an image grabbing program (Neotech, Paris, France) and an image analysis software package (Optilab Pro, Graftek, Paris, France) running on an Apple Macintosh 7100/80 (Cupertino, Calif., USA). Other slices (5 µm thick) from each intestinal sample, were stained with hemalum and eosin, and examined by light microscopy in a blinded manner for histological analysis.

Quantification of Worms in the Small Intestine

Eight groups of 4 rats were used for quantification of adult (L5) *N. brasiliensis* worms in the small intestine of infected rats. Four groups were treated with ketotifen and four groups with its vehicle as previously described. Rats were euthanized by cervical dislocation under anesthesia 4, 7, 12 or 21 days after infection. The entire small intestine was removed and divided into five segments corresponding to duodenum, three identical segments of jejunum, and ileum. Each segment was cut longitudinally, attached with a cotton thread and placed in a plastic tube containing 30 ml of NaCl 0.9%. Tubes were then placed in water at 37 °C for 2 h. Worms were quantified at the bottom of the tubes (method adapted from D'Inca et al. [30]).

Experimental Procedures

Two series of experiments were undertaken, each using six groups of 8 or 6 rats, for the study of the effects of ketotifen and capsaicin, respectively. For both series, four groups of rats were infected with *N. brasiliensis* ('Nb' groups) and two groups were not infected ('controls'). Animals were sacrificed at the end of their respective treatment. For ketotifen studies, three groups (two Nb, one control) received ketotifen (Sigma, 1 mg/kg, orally, b.i.d.) from 4 days before to 12 (one Nb) or 35 days (one Nb and controls) after infection (Nb groups) or saline injection (control group). The three other groups received saline (0.5 ml, orally, b.i.d.) instead of ketotifen for 12 (one Nb) or 35 days (one Nb and controls). A previous study showed that ketotifen pretreatment of rats with 1 mg/kg twice daily for 4 days prevented the release of mast cell mediators [31]. We chose the same dose of ketotifen in our study. A similar protocol was followed for capsaicin studies. Three groups (two Nb, one control) received capsaicin 1 week before infection (Nb groups) or saline injection (control group). Thus, increasing doses of capsaicin (Fluka Chemie, Buchs, Switzerland), dissolved in 10% ethanol, 10% Tween 80, 80% NaCl (referred to as 'vehicle'), were injected s.c. twice daily for 4 days, to reach a total dose of 100 mg/kg according to the following schedule: 5, 5; 5, 15; 15, 15; 20, 20 mg/kg. Capsaicin was administered via the systemic route, as indicated by Holzer [32]. The effectiveness of capsaicin treatment was tested by means of the eye-wiping test, which consists of impaired chemosensitivity of corneal afferents to one drop of 1% NH_4OH instilled into the eye [33]. The other three groups (two Nb, one control) received the vehicle (0.5 ml, s.c., b.i.d.) instead of capsaicin.

Data Analysis

MPO activity, mast cell counts and worm counts were expressed as the mean ± SEM for each group of rats. Statistical analysis was performed using analysis of variance (ANOVA) and the Tukey post hoc test. $p < 0.05$ was considered statistically significant.

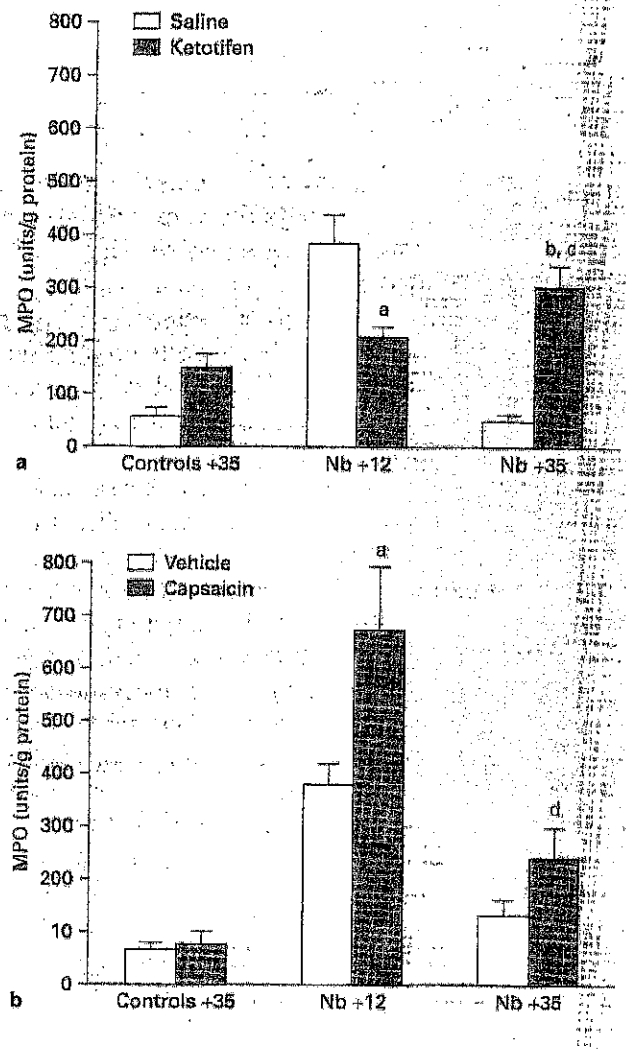

Fig. 1. Effects of ketotifen treatment (1 mg/kg/day, orally) or saline (0.9% NaCl, 0.5 ml/day, orally) (**a**) and of pretreatment with capsaicin (100 mg/kg, s.c.) or its vehicle (ethanol-Tween 80-saline, 0.2 ml, s.c.) (**b**) on MPO activity in the jejunum of *N. brasiliensis*-infected rats (12 and 35 days postinfection) and controls (35 days). Values are means ± SEM (n = 6–8). **a** [a] $p < 0.05$ vs. saline-treated Nb (12 days); [b] $p < 0.05$ vs. saline-treated Nb (35 days); [c] $p < 0.05$ vs. controls (35 days) treated with ketotifen. **b** [a] $p < 0.05$ vs. vehicle-treated Nb (12 days); [d] $p < 0.05$ vs. capsaicin-treated controls (35 days).

Results

MPO Activity

N. brasiliensis-infected rats treated with saline presented an acute jejunal inflammation at 12 days after infection, corresponding to an increase of MPO activity (380.0 ± 55.3 vs. 54.7 ± 14.1 MPO units/g protein in controls), which returned to normal preinfection values by day 35 postinfection (44.1 ± 10.7 MPO units/g protein) indicating that acute tissue inflammation had resolved. Ketotifen treatment reduced the increase of MPO activity at day 12 postinfection (207.3 ± 18.3 MPO units/g protein) compared to that observed in saline-treated infected rats; however, MPO activity remained elevated at day 35 postinfection in ketotifen-treated infected rats (298.8 ± 40.9 MPO units/g protein) indicating that jejunal inflammation persisted (fig. 1a). In control noninfected rats, ketotifen treatment for 35 days increased jejunal MPO activity but this increase did not reach statistical significance (fig. 1a).

In *N. brasiliensis*-infected rats treated with capsaicin vehicle, MPO activity levels were similar to those described above in saline-treated rats (fig. 1b). *N. brasiliensis*-infected rats treated with capsaicin showed an increase

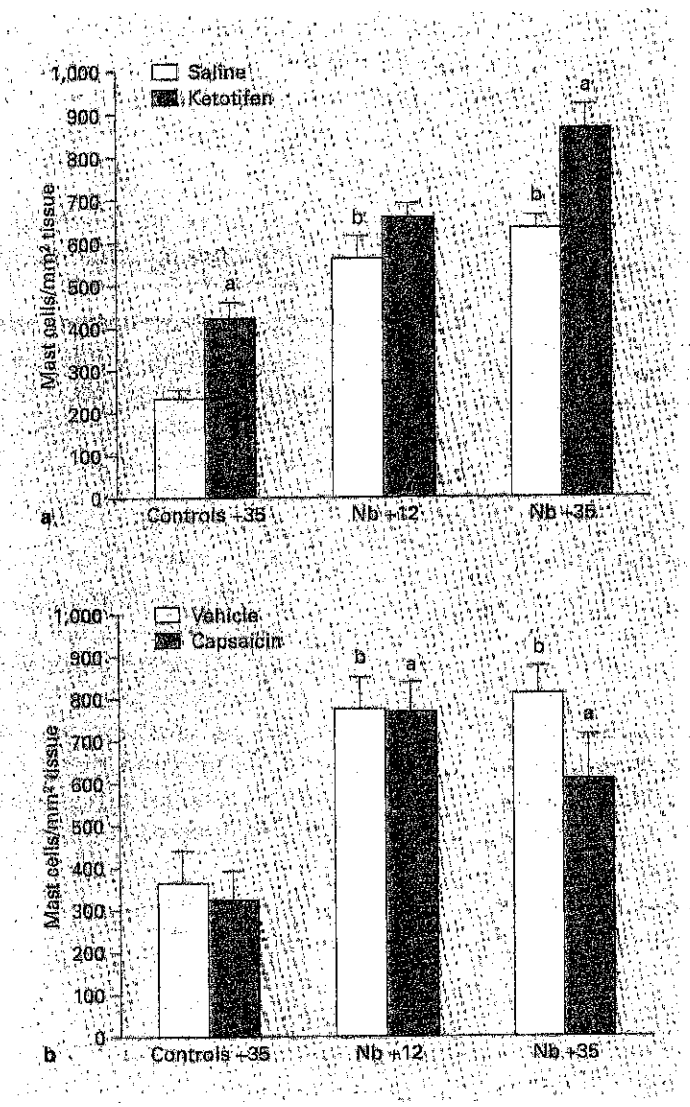

Fig. 2. Effects of ketotifen treatment (1 mg/kg/day, orally) or saline (0.9% NaCl, 0.5 ml/day, orally) (**a**) and of pretreatment with capsaicin (100 mg/kg, s.c.) or its vehicle (ethanol-Tween 80-saline, 0.2 ml, s.c.) (**b**) on mast cell numbers in the jejunum of *N. brasiliensis*-infected rats (12 and 35 days postinfection) and controls (35 days). Values are means ± SEM (n = 6–8). **a** [a] $p < 0.05$ vs. their respective saline-treated group; [b] $p < 0.05$ vs. saline-treated controls (35 days). **b** [a] $p < 0.05$ vs. capsaicin-treated controls (35 days); [b] $p < 0.05$ vs. vehicle-treated controls (35 days).

of MPO activity compared with vehicle-treated rats at day 12 (667.1 ± 121.5 vs. 374.2 ± 39.6 MPO units/g protein, respectively, $p < 0.05$) and at day 35 (234.4 ± 55.6 vs. 123.6 ± 30.7 MPO units/g protein, respectively, $p < 0.05$) (fig. 1b). Capsaicin treatment had no effect on MPO activity in noninfected rats (fig. 1b).

Histological Analysis

At day 12, infected animals treated with saline were characterized by several morphological changes. A separation of the epithelium from the core of the lamina propria was consistently seen at the villus tips at this stage but not in control tissues. In most animals, a mucus cell hyperplasia and a thickness of the submucosa were observed. Some sections demonstrated regions where chains of enterocytes were in the process of getting detached. These animals were also characterized by moderate ulcerations, an important infiltration with numerous inflammatory cells and dilated blood vessels compared with controls and 35 days' postinfected animals. Ketotifen abolished the severity of the lesions at day 12 postinfection and only a few blood vessels were still dilated in three animals.

In animals treated with the vehicle of capsaicin, 12 days postinfection, an infiltration of numerous inflammatory cells was observed but only two parameters, ulceration and infiltration, were augmented in most capsaicin-pretreated rats, 12 days postinfection. In vehicle-pretreated rats, 35 days postinfection, all the parameters were normal and not different from controls. In capsaicin-pretreated rats, 35 days postinfection, vascular dilation was still increased compared with vehicle-pretreated rats.

Mast Cell Numbers

In control noninfected rats, the number of stained mast cells in the jejunum was 239 ± 19/mm^2. This number was significantly increased ($p < 0.05$) at day 35 in ketotifen-treated rats (fig. 2a) but not in capsaicin-treated rats (fig. 2b). In *N. brasiliensis*-infected rats, the number of mast cells was significantly increased ($p < 0.05$) on days 12 and 35 after infection (fig. 2a). Neither ketotifen nor capsaicin modify significantly ($p > 0.05$) the number of mast cells on the 12th day after infection. On the 35th postinfection day, the number of mast cells was significantly ($p < 0.05$) higher in postinfected rats treated with ketotifen (fig. 2a) and not affected in capsaicin-treated rats (fig. 2b).

Worm Counts

Adult worms were found in the small intestine on days 4 and 7 after infection. They were localized mainly in the upper third of the jejunum and nearly absent in the duodenum and ileum. No worm was found in any of the intestinal segments studied at days 12 and 21, suggesting that all worms were expelled from the small intestine by the 12th day after infection. Ketotifen treatment did not modify the number of worms at 4 and 7 days after infec-

Table 1. Number of *N. brasiliensis* adult worms at 4, 7, 12 and 21 days postinfection in intestinal segments of infected rats treated orally with ketotifen (1 mg/kg/day) or saline (0.9% NaCl, 0.5 ml/day)

	Saline				Ketotifen			
	day 4	day 7	day 12	day 21	day 4	day 7	day 12	day 21
Duodenum	ND	4±4	ND	ND	15±9	10±5	ND	ND
Jejunum 1	499±106	626±62			470±135	600±105		
Jejunum 2	60±18	176±63	ND	ND	65±30	95±25	ND	ND
Jejunum 3	11±7	15±15			10±5	5±5		
Ileum	4±4	4±4	ND	ND	ND	ND	ND	ND

Values are means ± SEM (n = 4). ND = Not detected.

tion and did not affect the time of expulsion, i.e. absence of adult worms at 12 and 21 days (table 1).

Discussion

This work was aimed at evaluating the role of mast cells and capsaicin-sensitive afferent neurons in the development and intensity of intestinal inflammation and mastocytosis induced by the nematode *N. brasiliensis* in rats, an animal model which has been extensively used to study postinfective neuroimmune alterations in the digestive tract. To investigate the role of mast cells and afferent innervation in *N. brasiliensis*-induced inflammation and subsequent mast cell hyperplasia, we used ketotifen as a mast cell stabilizer and capsaicin for its ability to cause degeneration of primary afferent fibers.

Our results are consistent with a possible involvement of mast cells in the genesis and/or development of the *N. brasiliensis*-induced inflammatory reaction since MPO activity was less increased at 12 days after infection following treatment with the mast cell stabilizer ketotifen than in infected rats treated with saline. Histological analysis indicated a suppression by ketotifen of the lesions at day 12 postinfection since no infiltration or ulceration was observed compared with saline-treated rats. A role of mast cells in inflammatory processes has been described in several models of inflammation [4, 5]. In addition, a protective effect of ketotifen in animal models of inflammation is well documented. Ketotifen prevents mucosal damage in experimental colitis induced by TNBS or acetic acid, an effect accompanied by a reduction in mucosal generation of platelet-activating factor (PAF), prostaglandin E_2, thromboxane B_2, and leukotrienes C_4 and B_4; MPO activity was reduced as well [34]. In a model of jejunal inflammation induced by the sulfydryl blocker, iodoacetamide, and mimicking Crohn's disease, the protective effect of ketotifen indicates the contribution of proinflammatory mediators of mast cells to the pathogenesis of the damage [35]. In another model of gastric mucosal damage induced by ethanol, ketotifen prevented the lesions, probably through a reduction of mast cell mediator release [36]. A beneficial effect of ketotifen has also been reported in patients with eosinophilic gastroenteritis [37]. These results highlight the important role of mast cells and their mediators in the pathogenesis of gastric and intestinal inflammatory lesions. For example, in a rat model of colitis, local mast cell hyperplasia was observed and the antagonism of a major mast cell mediator, histamine, significantly reduces the severity of inflammation in this model [38]. In our study, we observed a ketotifen-induced reduction of the intensity of inflammation at 12 days after infection, but the inflammatory state was prolonged 35 days postinfection. We may then suppose that some mediators secreted by mast cells may favor repair mechanisms during the late phase of inflammation. Another explanation for these results could be that, when the function of mast cells is dampened by ketotifen, other inflammatory cells may be recruited at the sites of inflammation to maintain the inflammatory state. Thus, mast cells could act not only as an active component of inflammation but also as an important modulator of inflammation in basal or stimulated states. Heyman et al. [39] demonstrated for the first time that ketotifen stimulated inducible nitric oxide synthase (iNOS) activity in rats and humans. Moreover, a recent study [40] suggests that leukocyte-derived iNOS ameliorates the early phase, but does not affect the chronic phase of TNBS-induced inflammation. Inhibition of NOS results in the activation of mast cells, increased epithelial permeability and leuko-

cyte adhesion [41, 42]. Consequently, our finding that ketotifen reduces or delays *N. brasiliensis*-induced inflammation 12 days after infection suggests that its anti-inflammatory properties may be not only due to mast cell stabilization but also to NO generation. Moreover, ketotifen may act on inflammatory cells other than mast cells. For example, it may reduce the functions of mobility and phagocytosis of polymorphonuclear cells [43], it may reduce the chemotaxis of neutrophils [44] or it may inhibit their metabolic responses [45]. It also inhibits leukotriene C_4 release and PAF-induced chemotaxis of eosinophils [46]. Thus, the effect of ketotifen on inflammation may consist of a direct action on inflammatory cells or more likely on mast cells by reducing the release of inflammatory and chemotactic factors.

The mastocytosis induced by ketotifen may serve as a compensatory mechanism to support mast cells which have undergone a complete or partial inhibition of their mediator release. Therefore, mast cell mediators such as cytokines may participate in the regulation of the number of mast cells in the tissues. It has been reported that mast cells differentiate and proliferate under the influence of an array of cytokines and growth factors, such as IL-3, IL-4 and IL-10 derived from T cells and possibly from mast cells [20, 21].

The present study also shows that ketotifen does not modify the apparent migration of larvae, their number and final localization. Thus, we can conclude that the inflammation observed 35 days after infection in ketotifen-treated animals is not due to a prolonged worm colonization induced by mast cell stabilization. Such a hypothesis arises from studies which have clearly established that mucosal mast cells are functionally active during spontaneous expulsion of intestinal nematodes in rats [47] and that *N. brasiliensis* infection is prolonged in genetically mast cell-deficient mice [48].

Our study also indicates that sensory denervation with capsaicin results in increased MPO activity during the acute phase of inflammation, 12 days postinfection. MPO is a granulocyte-associated enzyme, and a direct relationship between MPO activity measured in tissue samples and the number of neutrophils has been shown [28]. Consequently, a higher MPO activity in response to *N. brasiliensis* in capsaicin-treated rats supports an increased attraction and activity of neutrophils in jejunal mucosa although other cells such as eosinophils and macrophages contain low amounts of MPO. Moreover, the histological analysis showed an aggravation of ulceration and infiltration in most animals pretreated with capsaicin. Our data agree with the protective role of sensory neurons shown in TNBS-induced models of colitis [1–3]. However, the role of sensory afferents on intestinal inflammation has been shown only with experimental models using TNBS, which is a Th1-predominant T cell-mediated inflammation [49] while the inflammatory response induced by *N. brasiliensis* involved preferentially Th2 T cell cytokines [50]. Another important finding in this study concerns the prolonged inflammatory state 35 days postinfection in capsaicin-treated rats, which suggests that sensory innervation might influence inflammation not only during the early phase but also at later stages in this model of nematode-induced inflammation. In contrast, Reinshagen et al. [1] indicated a role of sensory nerves during the early phase of colitis, providing mucosal protection, while other factors were likely to prevail at later stages of chronic inflammation. However, it seems difficult to compare nematode-induced and chemically induced inflammations that differ markedly not only in the timing of cellular activation and cellular components involved in the development and repair, but also in the role of the resident microflora.

Pretreatment with capsaicin does not influence the number of jejunal mast cells in controls or the intensity of mastocytosis in the jejunum of rats at 12 and 35 days after nematode infection, suggesting that capsaicin-sensitive afferent nerves are not essential in the development of nematode-induced mast cell hyperplasia. However, it cannot be excluded that sensory denervation affects the number of nematodes present in the intestine. Nevertheless, it seems unlikely that the worm burden may be affected by capsaicin pretreatment. A previous study [51] has revealed that rats pretreated with systemic capsaicin did not display particular changes of motility patterns in comparison with the controls. Our data showing that capsaicin does not modify the number of jejunal mast cells in both controls and infected rats were not expected because mast cells and nerves are in intimate contact and a structural basis exists in the rat gastrointestinal tract for communication between the immune and nervous systems [8]. It has been shown that vagal afferent nerve fibers come in contact with mast cells in the rat small intestinal mucosa [24] and are responsible for the mucosal protection by sensory nerves [3]. Such a physiological communication is observed in a model of stress where activation of sensory nerves participates in stress-induced histamine release from mast cells in rats [52]. On the basis of these results we suggest a trophic influence of afferent nerves on mucosal mast cell maturation and proliferation. However, our results are in agreement with a study in which prior ablation of sensory afferent nerves by subcutaneous

capsaicin administration 10 days before experiments did not significantly affect the numbers of mucosal mast cells within the gastric mucosa in either infected or noninfected rats [53]. On the other hand, neonatal treatment with capsaicin increased the number of rat mast cell protease II-immunoreactive mast cells in the dura mater [54]. It has also been reported that 3 weeks after vagotomy or 3 months after capsaicin administration, intestinal mucosal mast cells were almost 30% less numerous in capsaicin-treated rat jejunum than in untreated controls [22].

In summary, our study suggests that mast cell stabilization with ketotifen decreases jejunal inflammation during the acute stage (day 12) but prolonged the inflammatory state until at least day 35 postinfection in *N. brasiliensis*-infected rats. Our data also confirm the protective effect of intestinal sensory innervation against nematode-induced inflammation, whereas afferent nerves do not seem to be crucial in the development of intestinal mast cell hyperplasia.

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