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SENSORY NEURON AND SUBSTANCE P INVOLVEMENT IN SYMPTOMS OF A ZYMOSAN-INDUCED RAT MODEL OF ACUTE BOWEL INFLAMMATION

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Abstract—Intestinal inflammation is a painful syndrome with multiple symptoms, including chronic pain. This study examined the possible role of sensory neurons and substance P in symptoms of an animal model of acute intestinal inflammation. The model was induced by injecting ethanol and zymosan into the colon of anesthetized male rats. Three hours later, sections of the colon were stained with hematoxylin and eosin. To determine the role of substance P, 5 mg/kg of the neurokinin-1 receptor (NK-1r) antagonist, CP-96,345, or 300 μg/kg of an antisense oligonucleotide targeted at NK-1r mRNA was administered. Spinal cord sections were examined for internalization of NK-1r, as an indicator of substance P release. Sections of colon revealed infiltration of inflammatory cells following ethanol and zymosan treatment. Plasma extravasation in rats given ethanol and zymosan was significantly greater than in controls given saline only ($P<0.0001$) or saline and ethanol ($P<0.001$). In ethanol- and zymosan-treated rats given CP-96,345, plasma extravasation was significantly less than in rats given ethanol and zymosan without the antagonist ($P<0.0001$). Administration of the antisense oligonucleotide also resulted in lower levels of plasma extravasation compared with controls ($P<0.01$). Internalization of the NK-1r was observed in neurons of lamina I in the T13–L2 and L6–S2 regions of the spinal cord, as well as in sympathetic preganglionic neurons at the L1 level. This internalization was observed in the absence of any other stimulus besides the inflammation itself. This study implicates substance P and its receptor, the NK-1r, in acute inflammation of the colon. © 2006 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: colon, neurogenic inflammation, visceral inflammation, NK-1 receptor, receptor internalization, antisense.

Chronic inflammation of the bowel characterizes inflammatory bowel disease (IBD), a collective term that includes ulcerative colitis and Crohn's disease, which are both life-changing disorders associated with persistent pain. While much of the research done on IBD has focused on genetic, molecular and systemic etiologies, the potential role of neurogenic inflammation has remained relatively underexplored. Through neurogenic mechanisms, neurotransmitters can be released from the terminals of sensory afferent fibers in the periphery or through increased calcium levels in peripheral terminals. Neurogenic inflammation typically consists of vasodilatation, increased vascular permeability and edema along with activation of mast cells and lymphocytes (Lembeck and Holzer, 1979; Holzer, 1998a; Richardson and Vasko, 2002).

Prominent among the neurochemicals released from central and peripheral terminals of primary sensory neurons is substance P. Substance P has been implicated as a neuromodulator of the pain signal (Otsuka and Yoshioka, 1993), and thus, through its release in the spinal cord may contribute to the pain originating from a region of gut inflammation (Perry and Lawson, 1998). Yet, through its release from the peripheral terminals of the same sensory fibers in the gut, substance P might also contribute to peripheral pathology. Although one source of substance P is enteric neurons (Costa et al., 1980), most substance P in the gut originates from visceral primary afferents (Ribeiro-da-Silva and Hokfelt, 2000). Substance P is known to induce intestinal smooth muscle contraction (von Euler and Gaddum, 1931; Holzer and Petsche, 1983) both through substance P release from sensory fibers and from enteric neurons (for review see Holzer and Holzer-Petsche, 1997). However, substance P can also inhibit gut motor activity through either stimulation of inhibitors of gut pathways or interrupting excitatory activity (for review see Holzer and Holzer-Petsche, 1997). Lastly, substance P induces plasma extravasation in the gastrointestinal tract by acting via its preferential receptor, the neurokinin-1 receptor (NK-1r) (Figini et al., 1997). In intestinal tissue of patients with IBD there is a pronounced upregulation of NK-1r (Mantyh et al., 1988, 1995a; Goode et al., 2000). Because substance P may play a role in the colonic inflammation associated with IBD, it seemed reasonable to explore its possible role in an animal model of acute bowel inflammation.

Specifically, this study sought to determine whether the administration of an NK-1r antagonist, or an antisense oligonucleotide targeted at NK-1r mRNA, would lessen peripheral symptoms in an acute animal model of gut inflammation. As internalization of the NK-1r in the spinal cord has been interpreted as an inducer of neuronal activation (Honore et al., 1999, 2002), we used NK-1r internalization as a marker of activation of peptidergic nociceptive primary sensory fibers, as well as an indirect way of inferring whether substance P could have been released from peripheral terminals of these fibers in the gut. This conjecture is based on Dale's (1935) principle that the same transmitter should be released from all terminals of a given neuron. Thereby, evidence of release of substance P from the central endings of the sensory neurons in the spinal cord would indirectly imply release from the peripheral endings, as per Holton and Holton (1954).

Zymosan, a yeast cell wall polysaccharide, was injected into the bowel to induce an acute intestinal inflammation. Zymosan activates the alternative complement pathway (Pillemer et al., 1954), causes the release of histamine from mast cells and basophils (Van Arman et al., 1965), and evokes the synthesis and release of pro-inflammatory cytokines (Sanguedolce et al., 1992; Au et al., 1994). Zymosan causes multiple pathological changes in the gut comparable with those seen in IBD, such as large multi-focal areas of inflammation, thickening of the colon wall, luminal lesions, infiltration of inflammatory cells and damage to the crypts and epithelial lining (Coutinho et al., 1996). Traub et al. (1999) used zymosan to induce colonic inflammation, which resulted in a decrease in substance P–labeled cells in the T13–L2 and L6–S2 dorsal root ganglia, suggesting that zymosan increases substance P release from sensory neurons.

Some of these data have been reported in abstract form (Landau et al., 2002).

EXPERIMENTAL PROCEDURES

Animals

Male Sprague–Dawley rats weighing 200–225 g (Charles River, St. Constant, QC, Canada) were housed in pairs at a constant room temperature with 12-h light/dark cycles and fed standard rodent chow and water *ad libitum*. Prior to induction of the model, rats were fasted for 24 h to minimize the amount of food residues in the colon during the experiment. The animals were housed and used experimentally according to the Guide to the Care and Use of Experimental Animals, Vols. 1 and 2, of the Canadian Council on Animal Care and the guidelines of the International Association for the Study of Pain. All experimental protocols were reviewed and approved by the McGill Animal Care Committee. All measures were taken to minimize the number of animals used and their suffering.

Induction of inflammation

To induce bowel inflammation, rats were anesthetized with 60 mg/kg sodium pentobarbital (Abbott Laboratories, Montreal, QC, Canada) given i.p. The distal colon was carefully rinsed with 1 ml of saline via intrarectal perfusion at a distance of 2 cm from the anus; in pilot studies 1 ml was found to be the minimum volume required to void the colon of any material. This was

followed by the intrarectal administration (via a syringe inserted 8 cm into the anus) of 3 ml of 30% ethanol to compromise the mucous barrier (30 s). In pilot studies it was found that zymosan had inconsistent access to the gut tissue, presumably because of the mucous barrier, and this was rectified by administration of 3 ml of 30% alcohol. Ethanol solution was followed by 1 ml saline, again to rinse the colon, and 25 mg of zymosan in 1 ml saline (Sigma Chemical Company, Oakville, ON, Canada) to induce an inflammatory response (see also Coutinho et al., 1996; Honore et al., 2002). One control group consisted of naïve rats receiving only intracolonic saline and another control group received 3 ml of 30% ethanol followed by 1 ml saline. The rats were then placed prone, inclined head down at 30° for 30 min and kept anesthetized for the duration of the 3-h study; measurements were done after 3 h because the effects of zymosan are maximum at this time (Coutinho et al., 1996). Anesthesia was maintained with 15 mg/kg of pentobarbital per hour, i.p.

Histological processing of colon sections

Three hours following intracolonic injection of either ethanol and zymosan (*n*=3) or saline as a control (*n*=3), rats were administered 0.05 ml heparin (10 USP units/ml) i.p. and perfused transcardially for 30 min with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB), pH 7.4. Colon tissue samples proximal to the area of injection were then removed. The tissue was postfixed in 4% formaldehyde for 2 h at 4 °C and then infiltrated overnight in 30% sucrose in 0.1 M PB. A Reichert-Jung 2800 Frigocut N cryostat (Reichert-Jung, Wein, Austria) was used to cut 50-μm-thick transverse tissue sections. Tissue sections were then stained in Harris Hematoxylin Solution (Fisher Scientific Ltd., Whitby, ON, Canada) for 2 min, rinsed in water for 1 min before being dipped twice in differentiating solution with 0.25 ml HCl in 100 ml 70% ethanol, and then rinsed in water for 1 min. This was followed by immersion for 30 s in Scott's tap water substitute bluing solution (Fisher Scientific) and a 30 s rinse in 95% ethanol. Alcoholic eosin yellowish solution (Fisher Scientific; 0.25% w/v solution) was then used to rinse the slides for 30 s, followed by 1 min in 95% ethanol and two 1-min changes in 100% ethanol. Sections were then immersed in xylene for 5 min (Fisher Scientific) and coverslipped with Permount (Fisher Scientific). The sections were assessed under a Leitz Dialux 22 photomicroscope using 10× and 40× Plan Fluotar objectives (Leica Microsystems, Richmond Hill, ON, Canada) by investigators blinded to the experimental groups. Colon sections were assessed for ulcerations, thickening of lamina propria and presence of neutrophils.

Plasma extravasation studies

To detect the presence or absence of plasma protein extravasation, a solution of Evans Blue dye (Sigma Chemical Company), at a concentration of 25 mg/ml of saline and at a dose of 0.1 mg/kg of body weight, was injected via the tail vein into rats anesthetized with pentobarbital. Thirty minutes after the Evans Blue dye injection, rats were perfused transcardially with 500 ml saline. Tissue samples removed included a 1 cm-long portion of the colon collected at a distance of 8 cm from the anus (lower colon) and 6 cm proximal to the lower colon (upper colon), a 1 cm-long portion of the duodenum adjacent to the stomach and one ear. These samples were weighed, dried for 24 h in an oven at 60 °C, reweighed and then placed back in the oven in 2 ml of formamide for 24 h. Plasma extravasation (measured as albumin bound to Evans Blue dye) was then measured in tissue samples of the colon, duodenum and ear by measuring the optical density of each at 620 nm by spectrophotometry and dividing this value by the respective dry weight.

Thirty minutes prior to the induction of the model and at each hour for the duration of the 3-h study, each rat received s.c. injections of either 5 mg/kg of the substance P receptor antago-

nist, CP-96,345 (a generous gift from Pfizer, Global Research and Development, Groton, CT, USA), or saline as a control. This dosing schedule was followed because in earlier studies on nociceptive mechanisms in the Sprague–Dawley rat it was found that the effects of CP-96,345 peaked at 30 min and had diminished markedly by 1 h (Yashpal et al., 1993).

As a parallel to the experiments run administering the substance P receptor antagonist, further studies were done in which the NK-1r was depleted by administration of an antisense oligonucleotide directed against the NK-1r mRNA. Thus, 300 μg/kg of a fully thio-substituted antisense sequence 5′-CCCTGCCTC-CATTTCCTT-(S-OLIGO)-3′ (Biosource International, Camarillo, CA, USA) which targets NK-1r mRNA, was injected intraperitoneally twice daily for the 7 days prior to the induction of the model with zymosan. The model was induced in antisense-treated rats as outlined above and plasma extravasation levels were compared with rats not receiving any injections prior to zymosan administration.

Plasma extravasation studies were performed in the following animal groups: saline alone ($n=7$), ethanol ($n=7$), zymosan ($n=8$), CP-96,345+zymosan ($n=7$) or antisense oligonucleotide+zymosan ($n=7$) +four controls.

Data are expressed as mean±standard error of the mean. Comparisons of differences among groups were made with the unpaired *t*-test, with significance level set at $P<0.05$.

Processing of spinal cord segments for immunocytochemistry

Immunocytochemical experiments were carried out in zymosan-treated ($n=4$) and in saline-treated (control) rats ($n=4$). For this, rats were anesthetized with 0.4 ml/kg of equithesin (6.5 mg chloral hydrate and 3 mg sodium pentobarbital in a volume of 0.3 ml, i.p., per 100 g body weight) and injected with 0.1 ml heparin to prevent blood coagulation. They were then transcardially perfused with 4% paraformaldehyde in 0.1 M PB (pH 7.4) for 30 min. Subsequently, spinal cord segments T13–L2 and L6–S2 were collected, as these segments represent those involved in the innervation of the colon through the hypogastric and pelvic nerves, respectively (Traub et al., 1999). Tissue was postfixed in 4% paraformaldehyde in 0.1 M PB for 2 h at 4 °C and then infiltrated overnight in 30% sucrose in 0.1 M PB. Transverse sections were cut at 50 μm on a freezing sledge microtome (Leica Microsystems) and collected as free-floating sections in 0.01 M phosphate-buffered saline (PBS) with 0.2% Triton X-100 (PBS+T). They were then placed in 1% sodium borohydride in PBS for 15 min and then washed extensively in PBS+T. Sections were next incubated with 10% normal horse serum (NHS, Vector Laboratories, Burlington, ON, Canada) and 10% normal goat serum (NGS, Vector Laboratories) in PBS+T at room temperature for 60 min. Subsequently, the tissue was incubated with a mixture of rabbit anti-NK-1r antibody (1:200, a generous gift from Dr. James E. Krause) (Ardelt et al., 1996) and a rat anti-substance P monoclonal antibody (code NC1-34; diluted 1:10; Abcam Inc., Cambridge, MA, USA) (Cuello et al., 1979) in 5% NHS+5% NGS in PBS+T, at 4 °C for 48–72 h. The tissue samples were then washed for 5 min with PBS+T four times and then treated with a goat anti-rabbit biotinylated IgG obtained from a Vector Elite kit (BA-1000; 1:200; Vector Laboratories) for 2 h at room temperature. Following washing in PBS+T, the tissue was incubated in the dark, with a mixture of donkey anti-rat IgG conjugated to Rhodamine Red X (1:50; Jackson Laboratory, Bar Harbor, MN, USA) and Streptavidin conjugated to Alexa 488 (1:150, Molecular Probes, Eugene, OR, USA). Finally, the sections were washed with PBS and mounted onto gelatin-coated slides using Vectashield mounting medium (Vector Laboratories). The slides were stored at −20 °C until examined under a Zeiss LSM 510 laser scanning confocal microscope. Images were processed using Photoshop version 7.0 (Adobe Systems Inc.) on a Windows-based computer.

RESULTS

Hematoxylin and eosin staining of histological colon sections

Compared with samples taken from untreated colon (Fig. 1a), those removed 3 h after zymosan administration demonstrated obvious signs of inflammation of the colonic mucosa (Fig. 1b). There was an infiltration of the lamina propria with inflammatory cells, such as neutrophils, lymphocytes and macrophages, although there were no signs of bleeding. Such infiltrations were noticeable when comparing the space between the crypts of Lieberkühn, which was not only more cellular but also narrower than in controls. Furthermore, there was a thickening of the colonic mucosa (Fig. 1b). The overall thickening of the mucosa in Fig. 1b can be assessed by the fact that the crypts of Lieberkühn do not touch the muscularis mucosae, and are separated from it by a gap which is absent in Fig. 1a.

Plasma extravasation

A common feature of neurogenic inflammation is discontinuities in the vascular endothelium and leakage of plasma proteins into the extravascular space. Therefore, to determine whether plasma extravasation occurs in this model of acute inflammation, we injected Evans Blue. In the lower colon, close to the site of the intrarectal injections, the level of plasma extravasation was significantly higher in rats that received saline+ethanol compared with those who received intrarectal saline ($P<0.0001$; Fig. 2). Rats that received ethanol+zymosan had significantly higher levels of plasma extravasation in the lower colon than those treated with saline ($P<0.0001$) or with saline+ethanol ($P<0.01$).

When it became apparent that extravasation of plasma proteins was occurring in this model, experiments were done to identify whether substance P played a role by administering a substance P receptor antagonist before the induction of inflammation, and measuring plasma extravasation as above. Three groups were run to test the effects of administration of the NK-1r antagonist, CP-96,345. In one group CP-96,345 was given 30 min prior to induction of the model and at each hour afterward for 3 h; plasma extravasation was significantly decreased in the lower colon, compared with zymosan-treated rats which received no other injection ($P<0.0001$) or saline injections ($P<0.0001$; Fig. 3). In fact, ethanol+zymosan-treated rats given CP-96,345 also had significantly lower levels of plasma extravasation than rats treated with saline and ethanol only. CP-96,345 had no effect on values of plasma extravasation in the upper colon, duodenum or ear.

As a parallel to the experiments run administering the substance P receptor antagonist, further studies were done in which the substance P receptor was depleted by administration of an antisense oligonucleotide directed against the NK-1r mRNA. In the group of ethanol+zymosan-treated rats given the antisense oligonucleotide, lower levels of plasma extravasation were found than in the ethanol+zymosan-treated rats that received no antisense injections ($P<0.01$; Fig. 4).
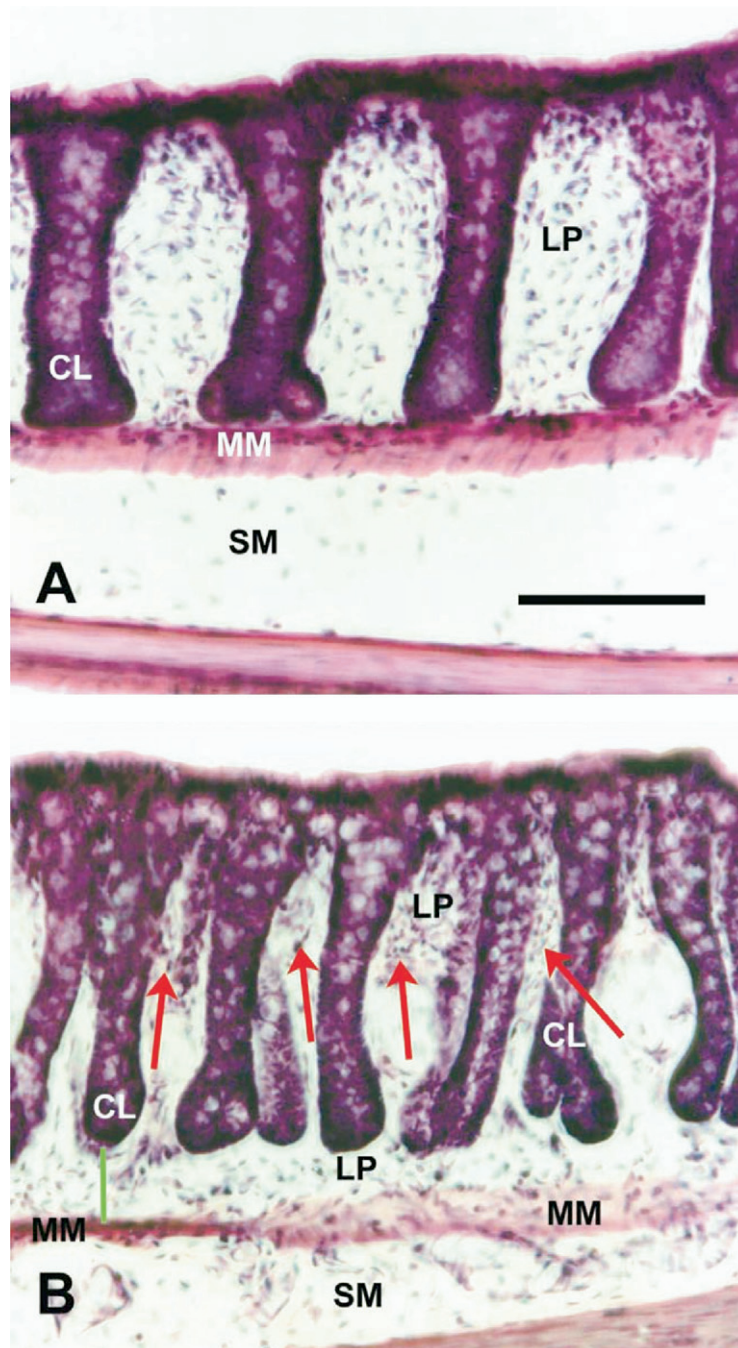
Fig. 1. Histological sections of the colon stained with hematoxylin and eosin: (a) untreated rat colon. (b) Rat colon 3 h after intrarectal injection of saline, ethanol+zymosan. Note the presence of inflammatory cells (indicated by red arrows), the diminished space in between the crypts of Lieberkühn (CL) and the thickening of the mucosa in (b). The overall thickening of the mucosa can be easily assessed in (b) by the fact that the CL do not touch the muscularis mucosae (MM), and are separated from it by a gap (represented by a green line) which is absent in (a). LP, lamina propria; SM, submucosa. Scale bar=100 μm (for both micrographs).

Confocal microscopy of spinal segments

When the results began to demonstrate the involvement of the substance P receptor in the vascular changes occurring in the acute model of colon inflammation it remained to determine whether the presumed ligand, substance P, originated from enteric neurons (for review see Holzer and Holzer-Petsche, 1997) or from the peripheral terminals of sensory neurons. This was a critical step for any conclusion that effects were due to neurogenic inflammation as outlined above.

In control rats administered only intrarectal saline, the NK-1r was located mainly on the plasma membrane of neurons in laminae I (Fig. 5a, c) and III–IV, as well as in lamina X in all the spinal cord segments studied. As pre-
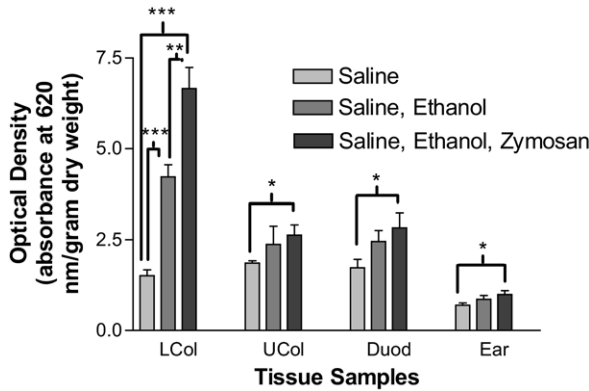
Fig. 2. Histogram showing plasma extravasation in tissues taken 3 h after treatment in rats injected intrarectally with saline alone ($n=7$), saline followed by ethanol ($n=7$) and saline and ethanol followed by zymosan ($n=8$). Tissue samples include lower colon (LCol) at the location of the intrarectal injections, upper colon (UCol), duodenum (Duod) and ear. Spectrophotometric readings were taken for each sample at 620 nm, which were then divided by the dry weight of the tissue. * $P<0.05$, ** $P<0.01$, *** $P<0.0001$.

viously described (Nakaya et al., 1994), NK-1rs were also detected on the plasma membrane of preganglionic sympathetic neurons of the intermediolateral column in spinal cord segments T13–L2 (Fig. 5e). There was no internalization seen in any of these neurons.

In contrast, in rats given zymosan extensive NK-1r internalization was observed in neurons of lamina I (Figs. 5 b, d) as well as in the preganglionic sympathetic neurons in segments T13–L2 and L6–S2 (Fig. 5f). In the zymosan-treated animals, virtually all neurons of lamina I that were NK-1 immunoreactive in spinal segment S1 showed massive receptor internalization. Also, in zymosan-treated rats, internalization occurred in virtually all NK-1r positive neurons in the preganglionic sympathetic neurons of segment L1; at this spinal level, there was also NK-1r internalization in lamina I neurons.
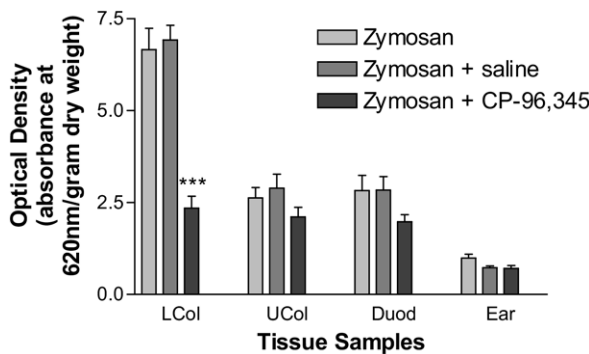

Fig. 3. Histogram displaying the effect of CP-96,345 on plasma extravasation at 3 h after zymosan administration only ($n=8$), in zymosan-treated rats when administered CP-96,345 ($n=7$) or in zymosan-treated rats injected with saline as a control ($n=7$). An s.c. injection of 5 mg/kg CP-96,345 was administered 30 min prior to zymosan administration and at every hour for the duration of the 3 h study. Tissue samples include lower colon (LCol) at the location of the intrarectal injections, upper colon (UCol), duodenum (Duod) and ear. Spectrophotometric readings were taken for each sample at 620 nm, which were then divided by the dry weight of the tissue. *** $P<0.0001$.

Fig. 4. Histogram demonstrating the effect of an NK-1r antisense oligonucleotide administered twice daily for a week prior to induction of the model on plasma extravasation at 3 h after zymosan administration ($n=7$) compared with rats not receiving antisense injections prior to zymosan administration ($n=4$). Tissue samples include lower colon (LCol) at the location of the intrarectal injections, duodenum (Duod) and ear. Spectrophotometric readings were taken for each sample at 620 nm, which were then divided by the dry weight of the tissue. ** $P<0.01$.
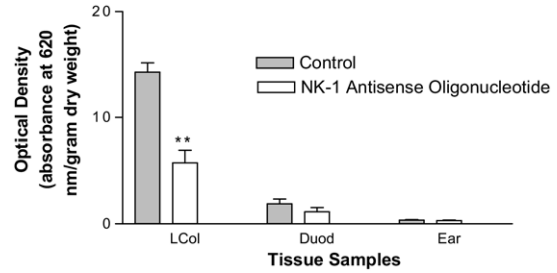
A constant factor in all neurons in zymosan-treated animals displaying extensive internalization was a high number of appositions from boutons immunoreactive for substance P (Figs. 5 b, d, f). For the purpose of this study, nerve cell bodies were considered as having receptor internalization if they displayed at least 10 endosomes that were positive for NK-1r immunoreactivity.

DISCUSSION

In this study, we have found that the administration of zymosan to the colon of rats induces a local inflammatory response that is consistent with what has been previously reported (Coutinho et al., 1996; Traub et al., 1999; Honore et al., 2002). This response consisted of mucosal swelling and infiltration of immune cells. The purpose of our study was to examine whether neurogenic inflammation, and peripheral substance P, released from sensory neurons, may play a role in the local inflammatory response following administration of zymosan. Our study also demonstrates a significant increase in plasma extravasation that accompanies the histological changes and that this increase is reduced by prior administration of an NK-1r antagonist, consistent with the results of a previous study (Figini et al., 1997). We also demonstrate that this increase in plasma extravasation is reduced by prior administration of an NK-1r antisense oligonucleotide. Overall, these results confirm that the NK-1r plays a role in the local inflammatory response in the gut.

Plasma extravasation that occurred in this model was an important indicator of the local inflammatory response. The observation that the groups treated with the NK-1r antagonist and with the NK-1r antisense oligonucleotide had lower levels of plasma extravasation indicated that this local inflammatory response was mediated at least partially via the local release of substance P. Our results are consistent with those of a previous study in which the NK-1r antagonist CP-96,345 was found to be effective in reducing colonic inflammation and oxidative stress in a dextran sulfate-induced model of gut inflammation in the rat (Stucchi et al., 2000).
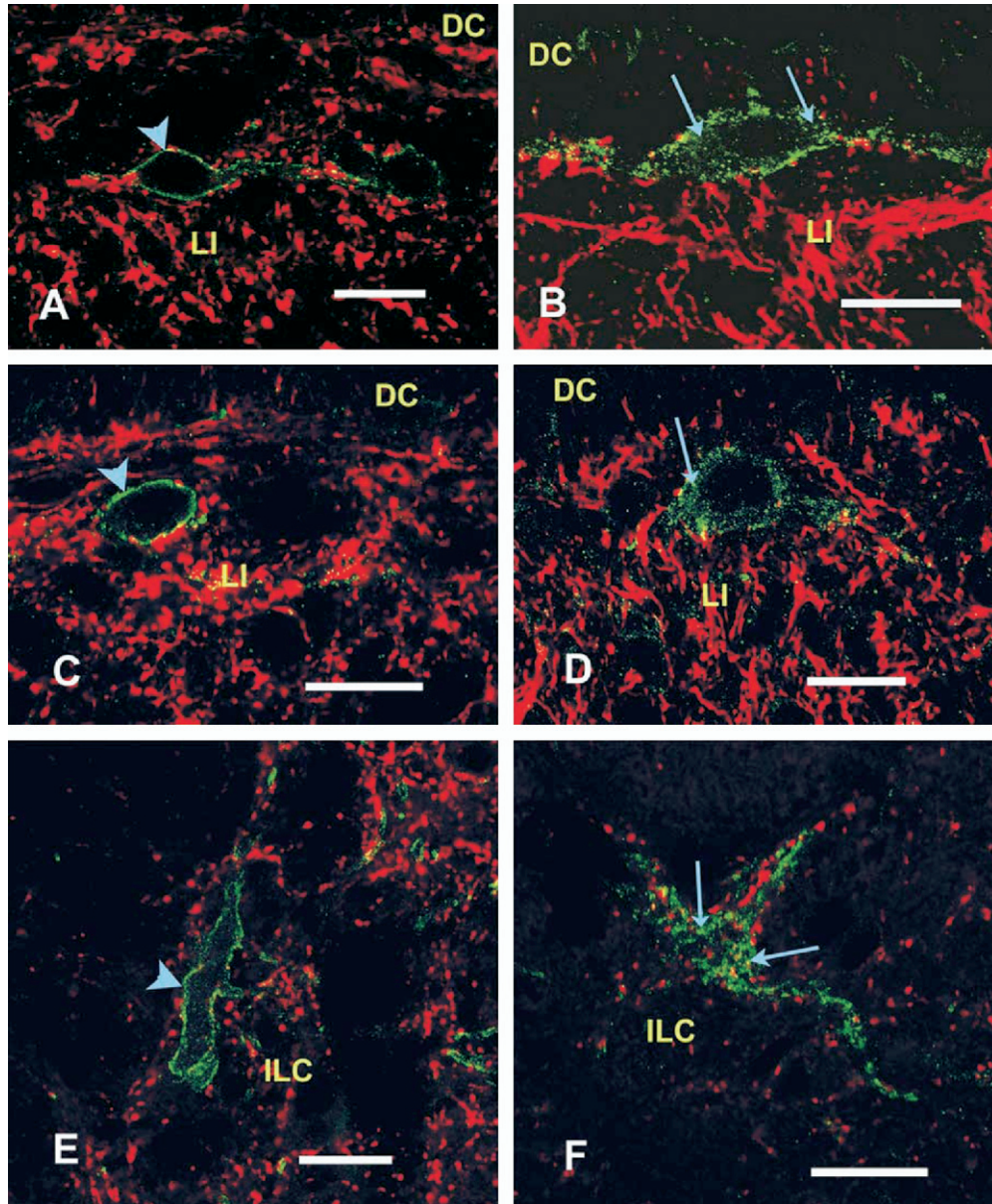
Fig. 5. NK-1r internalization in zymosan-treated rats. In these micrographs, substance P immunoreactivity is represented in red and NK-1r immunoreactivity in green. Micrographs in (a, c and e) were obtained from control animals, and those in (b, d and f) from zymosan-treated rats. In the superficial dorsal horn at the S1 spinal segment level, there was extensive internalization of the NK-1r (arrows) in animals treated with zymosan (see b and d), whereas in controls most of the immunostaining was restricted to the cell membrane (arrowheads), with only occasional endosomes (see a and c). Note the extensive innervation of the NK-1r immunopositive cells by varicosities immunoreactive for substance P. In the intermediolateral cell columns, at the L1 spinal level, note extensive internalization of the NK-1r in the preganglionic sympathetic neurons (see f), which was absent in controls (see e). Note also that at the level of the intermediolateral cell columns, the substance P innervation is still considerable, although not as extensive as in lamina I. DC, white matter of the dorsal columns; LI, lamina I; ILC, intermediolateral cell column. Scale bars=20 μm.

However, it remained to be determined whether this release of substance P was from local enteric neurons or from the peripheral terminals of sensory neurons. If the latter, then the local response in the gut would be considered to have been carried out at least partially via a process known as neurogenic inflammation. Internalization of the NK-1r in the spinal cord confirmed that primary sensory neurons were activated in this acute model and that substance P was released from these neurons. Therefore, based on the Dale principle (Dale, 1935) we suggest that

substance P was also released from the peripheral terminals of these neurons. We suggest further that the zymosan-induced acute inflammatory response in the gut was manifested at least partially by neurogenic inflammation and that this was carried out by mechanisms including substance P release from primary sensory nerve terminals. This principle has been applied in other studies. As an example, Holton and Holton (1954) suggested that ATP is released from the central terminals of sensory nerve fibers projecting from the ear of the rabbit because they

measured release of ATP in the ear after stimulation of these fibers. It was subsequently found that ATP does indeed excite second order neurons (Salter and Henry, 1985) and this was interpreted to support the earlier suggestion of Holton and Holton (1954).

NK-1r internalization was used as a method to visualize signaling by substance P. Mantyh et al. (1995b) were the first to demonstrate that the NK-1r, which is normally confined to the cell membrane, is internalized upon noxious peripheral stimulation. This was found to occur in the superficial dorsal horn, in cell bodies and dendrites of lamina I neurons, and in the dorsally directed dendrites of neurons in lamina III. It is relevant that in a nematode-infected rat model, levels of substance P immunoreactive material in dorsal root ganglia and dorsal horn were increased (De Giorgio et al., 2001). In a previous study (Honore et al., 2002), a similar model of zymosan-induced colonic inflammation was used. These authors did not observe any spontaneous internalization of the NK-1r in lamina I neurons 3 h after zymosan injection into the colon, although such internalization was observed after colonic distension, even at levels which were non-noxious in the absence of inflammation. The novelty of our study is therefore that, contrary to Honore et al. (2002), we found a spontaneous activation by the inflammation itself of neurons expressing the NK-1r. Therefore, the present study expands previous work showing internalization of the NK-1r in the spinal cord in an animal model of visceral inflammation induced by zymosan, further implicating substance P signaling in the associated symptoms, by showing for the first time that the internalization can occur as a result of the inflammation itself. As substance P release from sensory neurons has been implicated in nociceptive mechanisms, we also suggest that the central release of substance P may be involved in the pain that is often associated with inflammation of the gut.

It was considered important that the centrally-induced internalization of the NK-1r was observed in both T13–L2 and L6–S2 regions of the spinal cord. The fact that we observed such a widespread internalization of the NK-1r in the spinal cord, in all spinal segments known to be innervated by afferents from the colon allows us to suggest that the substance P release from sensory neuron terminals in the periphery should be extensive as well.

There have been several studies focusing on the possible involvement of sensory neurons in colitis. It was found that capsaicin-sensitive afferents play a protective role in experimental colitis and sympathetic nerves contribute to the development of colitis (McCafferty et al., 1997). Tomita et al. (1998) found that non-adrenergic non-cholinergic inhibitory afferents play a significant role in the impaired motility observed in the colon of ulcerative colitis patients. Through a mechanographic technique used to evaluate *in vitro* muscle responses, it was found that substance P containing afferents seem to act more weakly in the colon of patients with ulcerative colitis than in the normal colon (Tomita et al., 2000). Sensory neurons were found to have a protective function in the acute, but not in the chronic phases of inflammation (Reinshagen et al.,

1996), and this was attributed to the action of calcitonin-gene-related peptide (CGRP; Reinshagen et al., 1998). This and other transmitters have been suggested to regulate a variety of functions including mucosal hemostasis, resistance of tissue to injury and repair of damaged tissue (Holzer, 1998b). The present study employed CP-96,345 to determine the role of substance P in the acute inflammatory response in zymosan-induced inflammation, and it was found that the plasma extravasation was reduced. Thus, in addition to CGRP, substance P, released from peripheral terminals of sensory neurons may also play a role in regulation of gut tissue. Substance P has been implicated in neurogenic inflammation in other tissues (Wong et al., 2003; Lundy and Linden, 2004; Meyer-Siegler and Vera, 2004), and the proximity of substance P immunoreactive fibers to blood vessels and mast cells confirms the important role of substance P in neurogenic inflammation (Ruocco et al., 2001). The observation that CP-96,345 reduced the effects of zymosan suggests that substance P plays a destructive, rather than a protective, role in regulation of gut tissue and thus its role seems to be different from that of CGRP.

There has been controversy regarding neuropeptide involvement in intestinal inflammation. In one study, an increase in substance P immunoreactivity was found which was proportional to the degree of inflammation in human ulcerative colitis patients (Vento et al., 2001). As well, in a nematode-infected rat model there was a marked increase in substance P immunoreactive material in the jejunum gut wall (De Giorgio et al., 2001). Yet, Miampamba and Sharkey (1998) found that trinitrobenzene sulfonic acid (TNBS) caused a reduction in substance P immunoreactivity throughout the rat colon although these results may have been due to lower stores of endogenous substance P resulting from release and internalization. Evangelista et al. (1996) reported decreased levels of substance P immunoreactivity, which might also be accounted for by internalization, and decreased binding of exogenous substance P, which might be accounted for by occupation of receptors by endogenous substance P (Yashpal et al., 1994). Data obtained by Lee et al. (2002) revealed no evidence of differences in substance P distribution from surgical specimens from patients with ulcerative colitis compared with controls. CP-96,345 was found to be effective in reducing colonic inflammation and oxidative stress in a dextran sulfate–induced model of IBD in rats (Stucchi et al., 2000). SR140333, another NK-1r antagonist, was found to reduce inflammation and smooth muscle contractions in a TNBS rat model of IBD (Di Sebastiano et al., 1999). Kirkwood et al. (2001) determined that the deletion of endopeptidase, which degrades substance P in normal conditions, exacerbates intestinal inflammation induced by the activation of the NK-1r by *Clostridium difficile* toxin A. However, Wallace et al. (1998) found no benefit in treating TNBS-induced inflammation with the NK-1r antagonist, RP67580, over a 3-day period. Due to this controversy involving the role of the NK-1r in rat models of IBD and acute intestinal inflammation, further studies are required to elucidate the mechanisms of colonic inflammation, and

to understand mechanisms of acute vs. more persistent inflammation.

There have been several studies targeting molecules implicated in IBD with antisense oligonucleotides. The ISIS-2302 antisense oligonucleotide targeted against the intercellular adhesion molecule 1, which is upregulated in response to proinflammatory mediators, was found to be ineffective in reaching the primary endpoint of steroid free remission (Schreiber et al., 2001). In the current study, it was found that an antisense oligonucleotide targeted against NK-1r mRNA attenuated plasma extravasation in zymosan-treated rats compared with zymosan-treated rats that did not receive these injections.

CONCLUSION

In summary, it is suggested that neurogenic inflammation is an important contribution to the acute local response in the gut to irritants. This neurogenic inflammation appears to be mediated at least partially by substance P release from primary sensory nerve terminals and the subsequent activation of the NK-1r. Our data also indicate that substance P and the NK-1r may also be involved in mediation of the pain associated with acute colonic inflammation. Thus, it is possible that novel interventions based on interfering with the substance P/NK-1r system may be beneficial in treatment of the pain that is often associated with these disorders.

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