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ALTHOUGH THE PHARMACOLOGICAL therapy for chronic ulcerative colitis (CUC) requiring colectomy; however, persistent episodes of ileal pouch inflammation, or pouchitis, may result in debilitating postoperative complications. Because considerable evidence implicates substance P (SP) as an inflammatory mediator of CUC, we investigated whether SP participates in the pathophysiology of pouchitis. With the use of a rat model of IPAA that we developed, we showed that ileal pouch MPO levels and neurokinin 1 receptor (NK-1R) protein expression by Western blot analysis. In situ hybridization and immunohistochemistry showed that the increase in NK-1R protein expression was localized to the lamina propria and epithelia of pouch ileum. The intraperitoneal administration of a neurokinin 1 receptor (NK-1RA) is efficacious in reducing experimentally induced colitis. Several lines of clinical experimentation also provide evidence suggesting that SP may play a role in regulating inflammation in the ileal pouch. Keranen et al. (14) showed that the number and intensity of SP-containing immunoreactive nerve fibers were significantly increased in the inflamed ileal pouch.

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compared with ileum from either uninflamed pouches or control. SP-containing immunoreactive nerve fibers also were found to be significantly elevated in patients with clinically asymptomatic pouch inflammation, and despite remission, these levels remained high and correlated with histological indications of persistent inflammation (13). Although these data do not establish a causal linkage, they suggest that SP participates in the pathophysiology of pouchitis. Therefore, it would appear from this clinical and experimental evidence that SP may play a key pathophysiological role in the development of ileal pouch inflammation; however, there are no data thus far, especially regarding functional changes in the NK-1R in the pouch, to support this hypothesis.

Prior studies in animal models of colitis (43) and ileitis (28) have administered an NK-1RA either shortly before or concurrent with the proinflammatory stimulus. However, there are no reports demonstrating the effectiveness of an NK-1RA in ameliorating an ongoing or chronic gastrointestinal inflammation. In this report, we investigated whether the NK-1RA, CJ-12,255 (Pfizer), is efficacious in ameliorating an ongoing inflammatory process in the ileal pouch as well as blunting the additional inflammatory response imposed on an animal already exhibiting ileal inflammation.

MATERIALS AND METHODS

Materials. All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise noted. The highly specific, nonpeptide, NK-1RA, CJ-12,255 (3R,4S,5S,6R)-6-diphenylmethyl-5-(5-isopropyl-2-methoxybenzylamino)-1-azabicyclo[2.2.2]octane-3-carboxylic acid (CJ-12,255; Pfizer) was used in this study. This antagonist is a structurally related analog of the parent compound CJ-11,974 (Ezlopitant, Pfizer) (29, 45). To determine the efficacy of CJ-12,255 in interfering with the binding of SP with the NK-1R, we compared its ability to inhibit salivation in rats with another well-characterized NK-1RA, CP-96,345, and its inactive enantiomer CP-96,344 using the sialogogic assay. All data are expressed as means ± SE. Values not designated by the same letter are significantly different (P < 0.05).

Animals. Male Sprague-Dawley (Charles River Laboratories, Wilmington, MA) rats weighing between 300 and 325 g were housed individually in metabolic cages at a constant room temperature (RT) with 12:12 light-dark cycles and fed standard rodent chow (Purina #5001) and water ad libitum. The Institutional Animal Care and Use Committee at the Boston University School of Medicine approved this study, and all procedures described were performed in accordance with recommendations outlined in the National Institutes of Health Guide for the Care and Use Laboratory Animals (NRC 1996).

Surgical model. On arrival, all animals were placed in stainless steel metabolic cages and provided with standard rat chow and water ad libitum for 5–7 days before surgery.

Fig. 1. The highly specific, nonpeptide, neurokinin 1 receptor (NK-1R) antagonist (NK-1RA) CJ-12,255 (Pfizer) was used in this study. To determine the efficacy of this antagonist in interfering with the binding of substance P to the NK-1R, we compared its ability to inhibit salivation in rats with another well-characterized NK-1RA, CP-96,345, and its inactive enantiomer CP-96,344 using the sialogogic assay. All data are expressed as means ± SE. Values not designated by the same letter are significantly different (P < 0.05).

Animals were fasted 24–36 h before surgery, during which time their regular drinking water was replaced with water containing 5% dextrose to reduce the hypermetabolic effects associated with the prolonged postoperative fast required for anastomotic healing. Colectomy and proximal proctectomy were performed followed by the surgical construction of a J pouch in the terminal ileum and anastomosis of the pouch to the distal rectum, as we have previously described (37). This model provides a unique opportunity to assess the effectiveness of the NK-1RA in reducing an ongoing ileal pouch inflammation as well as preventing a further exacerbation. The inflammation that occurs as a consequence of the ileal pouch surgery is localized to the suture line at early time points. By 28 days postoperatively, the inflammation at the suture line had subsided, but a generalized inflammation of the ileal pouch persists, as we have previously described (37). Therefore, we chose this time point to begin our experiments studying the effectiveness of the NK-1RA to ameliorate this ongoing inflammatory process as well as to blunt the effect of an additional inflammatory stimulus [dextran sulfate sodium (DSS)] imposed at this time. In a series of time course and dose-response studies (37), it was shown that 4 days of 5% DSS gave an optimal inflammatory response in the pouch without compromising the health of the animals. Further DSS treatment results in the rapid exacerbation of ulcerations, severe rectal bleeding, and animal death. To keep all other factors equal, we treated animals with the NK-1RA for an equivalent length of time.

It is also important to note that the inflammation is localized strictly to the pouch, proximal ileum was unaffected, and no other chemical or dietary manipulations were used to initiate inflammation other than the resumption of solid food and water.

Experimental design. Colectomy with IPAA was performed on 42 Sprague-Dawley rats. Twenty-eight days postoperatively, animals were randomized into two experimental groups. In group I, to test whether the NK-1RA was effective in reducing the ongoing inflammation found 28 days after IPAA surgery, animals were further divided into two groups: those receiving 200 μl of saline or those receiving CJ-12,255 at 2.5 mg/kg ip bid. The antagonist was administered beginning on day 28 after IPAA surgery and continuing twice daily interperitoneally for 4 days until death.
In group 2, to test whether the NK-1RA was effective in preventing the additional inflammatory response to 5% DSS, animals were divided into two groups: those receiving 5% DSS plus 200 μl of saline or those receiving 5% DSS plus CJ-12,255 at the same dose starting in the evening of day 27 and continuing twice daily for 4 days. Animals in the nonoperated control group were age-matched and housed under similar conditions.

Our earlier studies in this IPAA model have shown that the administration of 5% DSS, commonly used to induce acute colitis in rodents (10), also produced acute ileitis limited to the pouch (37). Most importantly, this inflammatory response was accompanied by a pouchitis-like syndrome that included clinical correlates such as weight loss, diarrhea, and rectal bleeding as well as histological changes associated with pouchitis such as mucosal friability and ileal ulcerations. In this pouchitis model, we used the disease activity index proposed by Cooper et al. (7) to grade stool consistency. Therefore, normal stools are well formed pellets, loose stools are pasty and semiformed and do not stick to the anus, and diarrhea is liquid, unformed stools that stick to the anus.

A fraction of both control animals were killed daily, and the ileal pouch was removed for histology, mucosal MPO activity, immunohistochemistry for NK-1R, and continuing twice daily for 4 days. Animals in the nonoperated control group were age-matched and housed under similar conditions.

In previous experiments (28), the specificity of the NK-1R antibody was demonstrated by showing that preincubation of the antibody with an excess (10 μM) of the COOH-terminal 15-amino acid peptide of the NK-1R that was used to generate the NK-1 receptor prevented the immunohistochemical signal.

Nonradioactive in situ hybridization. After death, ileal tissues were removed immediately and rinsed in cold PBS. Three- to five-micrometer tissue sections were fixed in 4% paraformaldehyde (PFA) for 30 min at 4°C, washed 3× in cold PBS, then cryopreserved overnight at 30°C sucrose at 4°C. After being embedded in optimum cutting temperature (OCT), 5-μM sections were cut and placed onto superfrost plus microscope slides (Fisher) and stored at −80°C for immunohistochemistry.

Sections were air dried briefly, and after two 10-min washes in 1× TBS (DAKO, Carpinteria, CA), were incubated for 20 min in normal donkey serum [1% in TBS and 0.1% Tween 20 (TBST)], and then for 2 h with a rabbit NK-1R antibody (1:200 in TBST and 0.5% donkey serum) at RT. Ileal sections were washed in TBST and incubated for 30 min with FITC-conjugated goat anti-rabbit IgG (1:50 in PBS; Jackson ImmunoResearch Laboratories, West Grove, PA). After multiple washing in TBST, sections were mounted with an antifading mounting solution (90% glycerol in 1× PBS containing 1 mg/ml p-propyl gallate). The images were viewed without knowledge of experimental groupings by confocal microscopy (MRC 1024; Bio-Rad Microsciences, Cambridge, MA) through a ×20 Plan-Neofluar objective and stored in Bio-Rad COMOS software.

In situ hybridization was performed in a moisture chamber overnight at 53°C using 50 ng/ml DIG-NK1-R probe in hybridization buffer (50% formamide, 10% dextran sulfate, 4× SSC, 1× Denhardt’s solution, 0.25 mg/ml yeast tRNA, and 50 mM DTT). After hybridization, sections were washed in 1× SSC and 0.1× SSC at 60°C. The localization of NK-1R was observed after incubating sections with FITC-
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NEUROKININ 1 RECEPTOR ANTAGONISTS AND POUCHITIS

Table 1. Effects of administration of NK-1RA on %weight change, diarrhea, occult and gross rectal bleeding, and ileal ulcerations in IPAA Sprague-Dawley rats in the presence or absence of 5% DSS

<table>
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<th>Group 1</th>
<th>Group 2</th>
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<tr>
<td></td>
<td>(n = 11)</td>
<td>(n = 11)</td>
</tr>
<tr>
<td>%Weight change</td>
<td>+7%</td>
<td>+6%</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>0/11</td>
<td>0/11</td>
</tr>
<tr>
<td>Rectal bleeding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Occult</td>
<td>0/11</td>
<td>0/11</td>
</tr>
<tr>
<td>Gross</td>
<td>0/11</td>
<td>0/11</td>
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<td>Ileal ulcerations</td>
<td>0/11</td>
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</table>

NK-1RA treatment was started 28 days postoperatively and continued for 4 days. DSS was administered for 4 days commencing 28 days postoperatively. The antagonist treatment was administered 1 day before commencing DSS. %Weight change is over the 4-day experimental treatment period. Stool consistency: normal stools are well-formed pellets; loose stools are pasty and semi-formed and do not stick to the anus; and diarrhea is liquid, unformed stools that stick to the anus. NK-1RA, neurokinin 1 receptor antagonist; IPAA, ileal pouch-anal anastomosis; DSS, dextran sulfate sodium.

RESULTS

Animals tolerated the IPAA procedure well, with few postoperative complications. Although weight loss was noted through day 10 postoperatively, nearly all animals gained weight steadily thereafter (5–8 g/day), eventually exceeding their preoperative weight by at least 15% before commencing any studies. Nonoperated controls gained, on average, an additional 7% body weight during the experimental period and had no physical signs of intestinal inflammation such as diarrhea or rectal bleeding (Table 1).

Group 1: NK-1RA reduces an ongoing ileal pouch inflammation. In this rat model of IPAA, a spontaneous ileal pouch inflammation develops by 28 days postoperatively. Ileal pouch mucosal MPO activity is significantly elevated (P < 0.0001) when compared with ileum obtained from nonoperated controls (Fig. 2A, group 1). Administration of the antagonist for 4 days at this point significantly reduced MPO activity (NK-1RA) compared with saline-injected (saline; P < 0.001; Fig. 2A, group 1). Although all IPAA animals have loose stools following the resumption of solid food (3–5 days postoperatively), there is no evidence of diarrhea or rectal bleeding in the treated animals from group 1. Animals in this group gained weight at a normal rate during the therapeutic phase compared with nonoperated controls (Table 1, group 1). Histologically, the villi at 28 days postoperatively appeared normal in both the saline (Fig. 3B) and NK-1RA (Fig. 3C) of group 1, although the tips of some villi showed blunting compared with ileum from nonoperated controls (Fig. 3A).
There are neutrophils and other white blood cells scattered throughout the mucosa of 28-day IPAA animals that account for the elevations in mucosal MPO. Despite the elevation in MPO activity in the IPAA controls, which is indicative of subclinical inflammation, there are no corresponding clinical or physical signs typically associated with pouchitis.

**Group 2: NK-1RA reduces the additional ileal pouch inflammatory response to DSS.** The addition of 5% DSS in the drinking water for 4 days, beginning at 28 days postoperatively (5% DSS + saline), exacerbated the existing ileal pouch inflammation by ~80% (*P* < 0.05). When the NK-1RA was administered 1 day before 5% DSS (5% DSS + NK-1RA), the inflammatory response was completely prevented (*P* < 0.001) compared with animals receiving 5% DSS + saline (Fig. 2B, group 2). However, even before any biochemical or histological data were obtained, it could be observed that the animals receiving 5% DSS plus the antagonist had significantly reduced physical signs of pouchitis. We found that 5% DSS consistently produced physical signs typically associated with clinical pouchitis such as diarrhea and significant gross rectal bleeding (Table 1, group 2, 5% DSS + saline). In contrast, rats administered the antagonist before DSS consumption had loose stools but no diarrhea and no gross rectal bleeding (Table 1, group 2, 5% DSS + NK-1RA). However, there was evidence of occult blood in the stools of all animals consuming 5% DSS and receiving the NK-1RA.

On gross examination, ileal pouch mucosa from animals in the group consuming 5% DSS (saline) was thickened, edematous, erythematous, and hyperemic, with mucosal ulcerations containing fibrinous exudates. DSS-induced ileal ulcerations were patchy and did not extend deeper than the mucosa (Fig. 3D). In contrast, gross examination of ileal pouch mucosa from animals consuming 5% DSS and administered the NK-1RA were thickened and slightly erythematous, but there were no gross ulcerations. Subsequent histological examination showed a dense neutrophil infiltrate with crypt abscesses, granulation, and microulcerations in the untreated animals (Fig. 3D) compared with the NK-1RA-treated animals (Fig. 3E).

**Neurokinin 1 receptor protein expression increases in inflamed ileal pouch tissue.** Western blot analysis showed that by 28 days (IPAA alone), the inflammation in the ileal pouch is associated with a significant increase in NK-1R protein expression compared with nonoperated controls (non-op control). In animals administered 5% DSS starting 28 days after IPAA (IPAA with 5% DSS), receptor expression remains significantly elevated (Fig. 4, A and B). In these rats, because the expression of NK-1R protein in pouch ilea is not further elevated, this suggests that IPAA alone may have caused maximal receptor protein expression.

**NK-1R mRNA expression increases in inflamed ileal pouch tissue.** In situ hybridization and immunohistochemistry showed that the expression of NK-1R mRNA (Fig. 5A) and NK-1R protein (Fig. 5D), respectively, is limited primarily to ileal crypt lamina propria (LP) of nonoperated controls (arrow). However, expression of both the receptor mRNA (Fig. 5B) and receptor protein (Fig. 5E) is markedly increased in the LP of inflamed ileal pouches at 28 days after IPAA surgery (arrow). There was also a low but observable level of mRNA and protein expression in the crypt epithelium (box) that was not observed in the nonoperated controls. The administration of 5% DSS also increases the expression of NK-1R mRNA (Fig. 5C, box) and NK-1R protein (Fig. 5F, box) not only in the LP, but also in the crypt epithelial cells. Localization of NK-1R mRNA and NK-1R protein was consistent for all specimens within each group.

**DISCUSSION**

In the present study, we report new findings in a rat model of IPAA supporting a role for SP in the pathophysiology of ileal pouch inflammation. The administration of a specific, nonpeptide NK-1RA is effective in...
Reducing a chronic, ongoing ileal pouch inflammation that develops spontaneously after IPAA surgery. Other studies, for example Pothoulakis et al. (27), in which NK-1RAs have been administered to inhibit inflammatory responses, have involved acute experiments where the antagonist has been administered approximately at the same time as the stimulus and for a short duration.

In a second series of experiments in IPAA animals, in which an ongoing inflammation had been present for nearly a month, administration of the antagonist 1 day before the imposition of an additional inflammatory stimulus, the consumption of DSS, prevents the exacerbation of the inflammatory response. This result was somewhat surprising, because previous studies in this lab (unpublished results) have shown that antagonist administration is ineffective in reducing the inflammatory response to DSS after it has begun. These findings highlight differences between the chronic, subclinical inflammation that occurs after IPAA surgery and that of DSS administration. These results are especially important when considering the clinical usefulness of this antagonist.

In clinical studies of patients who have undergone IPAA for CUC, the cumulative risk of pouchitis increases over time (35, 40). This may be due, in part, to the morphological (23), histological (9), and metabolic adaptations (15) that occur in the functioning ileal pouch. Whether these adaptations increase susceptibility to inflammation remains unclear. Whereas these adaptive changes may include an increase in SP-containing immunoreactive nerve fibers in the ileal pouch (13), the findings presented here demonstrate that compared with normal, control ilea from nonoperated animals, the expression of NK-1R protein, as shown by Western blot analysis, is significantly increased in pouch ilea of IPAA rats after 28 days. In situ hybridization and immunohistochemistry localize the increased expression of NK-1R mRNA and NK-1R protein to the LP and epithelia. These results raise the interesting question as to whether these other cell types, such as epithelial cells, in which receptor expression is increased play a role in the SP-dependent inflammatory response. The upregulation of the NK-1R in ileal epithelial cells has been shown to correlate with increased intestinal levels of TNF-α as well as MPO activity (6). These increases may initiate a cascade of proinflammatory SP-mediated events orchestrated by the upregulation of NF-κB that can compromise epithelial integrity and exacerbate inflammation (31). The increase in NK-1R mRNA in the epithelial cells of the mucosa found just 28 days after IPAA may, at least partially, contribute to the effectiveness of the antagonist just before the DSS stimulus.

The rapid onset of the physical signs of ileal inflammation initiated by the oral administration of 5% DSS to IPAA rats may be analogous to the clinical condition of an acute flare-up of pouchitis. The accompanying physical signs, diarrhea and rectal bleeding, in concert with a significant elevation in mucosal MPO activity, further support the utility of this model to study the role(s) of SP in the pathophysiology of pouchitis. The increased expression of NK-1R in epithelial cells after DSS administration is reminiscent of our earlier findings that showed a dramatic increase in NK-1R mRNA and protein expression in intestinal epithelial cells of the ileal mucosa early in the course of Clostridium difficile toxin A-induced enteritis (28).

Although the mechanisms underlying the increase in NK-1R expression after IPAA remain unclear, there are several plausible explanations. As the pouch adapts to its new colonlike role, ileal enterocytes encounter significant changes in the luminal environment that include high concentrations of secondary bile acids, short-chain fatty acids, and unfamiliar colonic bacteria (23). All of these luminal contents have been implicated in underlying inflammatory changes in the ileal pouch (42). Because the cumulative risk of pouchitis increases over time (35, 40), it appears from the data presented in this study that the increased neutrophil insudation, in concert with the increased expression of NK-1R protein, may also reflect temporal adaptations that predispose the ileal pouch to subsequent inflammatory episodes. Although the specific pathophysiological stimuli underlying these inflammatory changes remain unclear, events related specifi-
cally to ileal pouch physiology, such as stasis or delayed pouch emptying (44) and bacterial overgrowth (35), both of which occur in this model (37), have been implicated in the subsequent development of pouchitis. In fact, our earlier studies in this model have shown that aerobic and anaerobic bacteria do overgrow the ileal pouch within 28 days, during which time the bacterial profile of the pouch becomes quite similar to that of the distal colon (37). LPS derived from nonindigenous, gram-negative bacteria overgrowing the ileal pouch can induce an inflammatory response. LPS has also been shown to facilitate SP-mediated neutrophil uptake (16), and because SP itself is chemotactic for neutrophils (33), this alone may explain the increase in MPO activity. SP (19) has also been shown to increase endothelial cell expression of VCAM-1 and ICAM-1, which can further facilitate neutrophil extravasation. LPS can also directly induce the synthesis and release of SP from macrophages (32) and, in concert with SP originating from increased SP-containing immunoreactive nerve fibers in the ileal pouch (13), may mediate the increased expression of the NK-1R in the pouch 28 days after IPAA.

Even when clinically asymptomatic, nearly all long-term IPAA patients have some histological evidence of chronic mucosal inflammation in the ileal pouch (9). Because the ongoing inflammation observed in rat pouch ileal mucosa at 28 days after IPAA can be reduced by administration of the NK-1RA, it seems reasonable to infer that NK-1RA may also be of benefit in maintaining remission between episodes. Patients with chronic pouchitis suffer from multiple inflammatory episodes, and treatment algorithms typically focus on ameliorating acute inflammatory flare-ups (4) and not on maintaining remission between episodes. Currently, only probiotic therapy has shown efficacy in long-term maintenance of remission (11), purportedly by inducing a protective mucosal immune response (12). Because SP is a known proinflammatory immunomodulator (17, 36), thus NK-1RA may invoke similar immunoprotective and anti-inflammatory mechanisms.

Chronic episodes of pouchitis continue to afflict a significant percentage of patients following IPAA for CUC. Collectively, these data implicate a role for SP in the pathophysiology of pouchitis and suggest that NK-1RAs may be of therapeutic value in resolving clinical pouchitis.

The authors thank J. Lowe for technical advice. The NK-1 antagonists CJ-12,255 and CP-96,345 and the inactive 2R, 3R enantiomer CP-96,344 were kindly provided by Pfizer Central Research (Groton, CT).

Fig. 5. Top shows representative cross-sectional views of NK-1R mRNA by in situ hybridization: nonoperated control (A), IPAA animal (B; IPAA alone) after 28 days, and IPAA animal after 28 days + 4 days of 5% DSS (C; IPAA with 5% DSS). Bottom shows representative cross-sectional views of NK-1R protein expression by immunohistochemistry: nonoperated control (D), IPAA animal after 28 days (E; IPAA alone), and IPAA animal after 28 days + 4 days of 5% DSS (F; IPAA with 5% DSS; magnification, ×40). Arrows, see NK-1R mRNA expression increases in inflamed ideal pouch tissue.
REFERENCES


