A Neurokinin 1 Receptor Antagonist Reduces an Ongoing Ileal Pouch Inflammation, and the Response to a Subsequent Inflammatory Stimulus

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Abstract

Ileal pouch-anal anastomosis (IPAA) is an excellent surgical option for patients with chronic ulcerative colitis (CUC) requiring colectomy; however, persistent episodes of ileal pouch inflammation or pouchitis may result in debilitating postoperative complications. Since considerable evidence implicates substance P (SP) as an inflammatory mediator of CUC, we investigated whether SP participates in the pathophysiology of pouchitis. Using a rat model of IPAA that we developed, we showed that ileal pouch myeloperoxidase (MPO) levels and neurokinin 1 receptor (NK-1R) protein expression by Western blot analysis were significantly elevated 28 days after IPAA surgery. 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 the neurokinin 1 receptor antagonist (NK-1RA), CJ-12,255 for 4 days, starting on day 28, was effective in reducing MPO levels. Starting on day 28, animals with IPAA were given 5% dextran sulfate sodium (DSS) in their drinking water for 4 days, which caused histological and physical signs of clinical pouchitis concomitant with significant increases in ileal pouch MPO concentrations as well as NK-1R protein expression by Western blot analysis. In situ hybridization and immunohistochemistry showed that the increase in NK-1R protein expression was especially to crypt epithelia of pouch ileum. When the NK-1RA was administered 1 day before starting DSS, and continued for the duration of DSS administration, the physical signs of clinical pouchitis, and the rise in MPO were prevented. These data implicate SP in the pathophysiology of pouchitis, and suggest that NK-1RA may be of therapeutic value in the management of clinical pouchitis.

Key Words: Pouchitis, neurokinin 1 receptor antagonists, substance P.
Introduction

Although the pharmacological therapy for chronic ulcerative colitis (CUC) has improved significantly, it has been estimated that approximately 20 - 30% of patients with CUC will eventually become refractory to treatment, and require total proctocolectomy (1) to control the inflammatory process (2). The recent advent of the sphincter-sparing total colectomy with mucosal proctectomy and endorectal ileal pouch-anal anastomosis (IPAA) has become an excellent option for these patients (3).

Although the majority of patients report a favorable functional and socially acceptable outcome (4), inflammation of the ileal pouch or pouchitis has become the most common late postoperative complication in patients having undergone the procedure for CUC (5). Pouchitis is an idiopathic, non-specific inflammation of the ileal pouch characterized clinically by high stool frequency, diarrhea, and rectal bleeding (6). In some series, pouchitis has been reported to occur in up to 59% of patients who undergo IPAA for CUC by 10 years (7-10). While there has been considerable speculation regarding the pathophysiology of pouchitis, attempts to resolve these debilitating inflammatory episodes have been hampered significantly by a poor understanding of its etiology (11), the lack of widely accepted diagnostic criteria (12), and more so by the lack of a relevant small animal model with clinical correlates. In the present study, we utilize our recently developed rat model of IPAA (13) to explore the role of substance P (SP) in this inflammatory process.

Substantial evidence supports a role for SP in the pathophysiology of colitis (14-16). Stucchi et al (17) and others (18,19) have shown that the early administration of a neurokinin 1 receptor (NK-1R) antagonist (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 (20) showed that the number and intensity of SP-containing immunoreactive nerve fibers were significantly increased in the inflamed ileal pouch compared with
ileum from either uninflamed pouches or control. Substance P-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 (21). 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 pathophysiologic 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 (17) and ileitis (22) have administered an NK-1RA either shortly before or concurrent with the pro-inflammatory 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, Inc.), 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 Chemical Co. (St. Louis, MO) unless otherwise noted. The highly specific, non-peptide, NK-1RA (3R,4S,5S,6S)-6-diphenylmethyl-5-(5-isopropyl-2-methoxybenzlyamino)-1-azabicyclo[2,2,2]octane-3-carboxylic acid (CJ-12,255) (Pfizer, Inc) was utilized in this study. This antagonist is a structurally related analogue of the parent compound CJ-11,974 (Ezlopitant, Pfizer, Inc.) (23,24). To determine the efficacy of CJ-12,255 in interfering with the binding of SP with the NK-1 receptor, we compared its ability to inhibit salivation in rats with another well characterized NK-1RA, CP-96,345 (Pfizer), using the sialogogic assay as described by Leeman (25). Briefly, anesthetized rats were injected IP with 2.5 mg/kg of either saline, CJ-12,255, CP-96,345, or CP-96,344, 30 minutes prior to an IV injection of SP (0.1 µg/100 g body wt). Saliva was collected immediately with a Pasteur pipette, and placed into pre-weighed vials. One milligram of saliva represented approximately microliter of saliva. Salivation in animals injected with CJ-12,255 thirty min prior to an IV bolus of SP was significantly reduced compared with control, CP-96,345 and the inactive enantiomer CP-96,344 (Figure 1).

Animals. Male Sprague-Dawley (Charles River Labs, Wilmington, MA) rats weighing between 300-325 g were housed individually in metabolic cages at a constant room temperature with 12-hr light and 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.** Upon arrival, all animals were placed in stainless steel metabolic cages and provided with standard rat chow and water ad libitum for 5 – 7 days prior to surgery. Animals were fasted 24 to 36 hours prior to surgery, during which time their regular drinking water was replaced with water containing 5% dextrose in order 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 (13). This model provides a unique opportunity to assess the effectiveness of the NK-1R antagonist 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 (13). 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 (DSS) imposed at this time. In a series of time course and dose response studies (13), 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 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 and proximal ileum was unaffected, and that no other chemical or dietary manipulations were utilized to initiate inflammation other than the resumption of solid food and water.
Experimental design: Colectomy with ileal J pouch-anal anastomosis (IPAA) was performed on 42 Sprague-Dawley rats. Twenty-eight days postoperatively, animals were randomized into two experimental groups. In Group 1, 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 IP for 4 days until sacrifice.

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 non-operated 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 (26), also produced acute ileitis limited to the pouch (13). 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 (27) to grade stool consistency. Therefore, 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.

At the conclusion of both studies, animals were sacrificed by CO₂ exposure, and ileal pouch was removed for histology, mucosal MPO activity, immunohistochemistry for NK-1 receptor protein, and in situ hybridization for NK-1 receptor mRNA by confocal fluorescence.
microscopy as described below. Throughout all studies, body weights were recorded daily, and animals were monitored daily for indices of pouchitis activity such as diarrhea and rectal bleeding.

**Myeloperoxidase Activity.** MPO activity has been widely accepted as an enzyme marker to quantitate the degree of inflammation and is indicative of the accumulation of neutrophils or polymorphonuclear cells in tissues (28). Ileal pouch mucosa was assayed for MPO activity in duplicate according to Barone et al (29) with modifications as described by Shebani et al (30). One unit of MPO activity was defined as that degrading 1 µmol of hydrogen peroxide per minute.

**Histology.** Following the removal of the distal ileum from either non-operated animals or animals with an ileal J-pouch, two sections were fixed immediately in 10% neutral buffered formalin and embedded in paraffin for histological analysis. Full thickness sections were stained with hematoxylin and eosin and examined by a pathologist blinded to the groupings for evidence of neutrophil infiltration of the mucosa and submucosa, crypt microabscesses, blunting of intestinal villi, increased crypt depth, and ulcerations.

**Preparation of cytosolic extracts for Western blotting.** Approximately 100 mg of distal colonic mucosal tissue was ground with a mortar and pestle in liquid nitrogen, suspended in 500 µl homogenization buffer (HB) (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 50 mM sucrose, 1 mM DTT) supplemented with either 0.5 mM PMSF, 5 µg/ml leupeptin and 5 µg/ml aprotinin or a mammalian protease inhibitor cocktail used at 100X (Sigma), and homogenized in a small hand-held tissue grinder (size 21; Kontes) with ~40 strokes. Fifty microliters of adjusting buffer (same as HB except 1 M KCl) were then added to the tissue to increase the salt concentration to 100 mM. The homogenate was centrifuged at 850
x g for 15 min at 4°C and the supernatant (cytosolic fraction) recovered for Western blot analysis.

**Western blot analysis of the Neurokinin-1 receptor.** Cytosolic extracts (20 µg per sample) were separated on 4-12% Bis-Tris mini-gels (InVitrogen) and transferred to Hybond ECL membrane (Amersham Pharmacia Biotech). The membranes were blocked for 1 h at RT in blotto (1X TBS [25 mM Tris-HCl, pH 7.4, 2.5 mM KCl, 125 mM NaCl], 0.1% Tween 20, 5% non-fat dry milk) and incubated with a polyclonal anti-NK-1 receptor antibody (Santa Cruz Biotechnology; cat# sc-15323), diluted 1:5000 in blotto, overnight at 4°C. Membranes were then subjected to vigorous washes, 4 x 15 min in TBS/ 0.1% Tween 20 at RT, and incubated for 1 h at RT in 2° antibody, peroxidase-conjugated goat anti-rabbit IgG (Pierce Chemical) diluted 1:10,000 in blotto. Membranes were then vigorously washed again, 4 x 15 min in TBS/ 0.1% Tween 20 at RT. The NK-1 receptor was detected by chemiluminescence with the ECL Plus Western Blotting Detection System (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. Western blots were scanned and quantified using Scion Image software (Scion Corporation). Mean values were determined and expressed as a percent of non-operated controls.

**Immunohistochemistry:** Following sacrifice, ileal tissues were removed immediately and rinsed in cold phosphate buffered saline (PBS). Three to five micron tissue sections were fixed in 4% paraformaldehyde (PFA) for 30 min at 4°C, washed 3X in cold PBS, then cryopreserved overnight in 30% sucrose at 4°C. Following embedding in 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 1X tris-buffered saline (TBS) (Dako Co., Carpinteria, CA), were incubated for 20 min in normal donkey serum (1% in TBS and 0.1% Tween 20: TBST), and then for 2 hours with a rabbit NK-1 receptor antibody (1:200 in TBST and 0.5% donkey serum) at room temperature. Ileal sections were washed in TBST and incubated for 30 min with FITC conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Labs, West Grove PA) (1:50 in TBS). After multiple washing in TBST, sections were mounted with an anti-bleaching mounting solution (90% glycerol in 1X PBS containing 1mg/ml n-propyl gallate). The images were viewed without knowledge of experimental groupings by confocal microscopy (Md. MRC1024; BioRad Microsciences, Cambridge, MA) through a 20X Plan-Neofluar objective and stored in BioRad COMOS software.

In previous experiments (22), the specificity of the NK-1R antibody was demonstrated by showing that preincubation of the antibody with an excess (10 µM) of the carboxy-terminal 15-amino acid peptide of the NK-1 receptor that was used to generate the NK-1R antiserum prevented the immunohistochemical signal.

**Non-Radioactive In Situ Hybridization.** Following sacrifice, ileal tissues were removed immediately and rinsed in cold PBS. Three to five mm² tissue sections were immediately flash frozen in liquid nitrogen. Following embedding in OCT, 5 µM sections were cut and placed onto superfrost plus microscope slides (Fisher) and stored at -80°C for in situ hybridization.

A riboprobe (588bp) complimentary to the NK-1 receptor gene (pBSrNK-1R) was labeled with digoxigenin (DIG) and quantified following the manufacturers instructions (Roche Inc., Indianapolis, Indiana). Five µM rat ileal sections were fixed in 4% PFA in diethyl pyrocarbonate (DEPC) treated 1X PBS), and acetylated for 15 min in freshly made TEA buffer (0.1M triethanolamine, HCl, pH 8.0 plus 0.25% acetic anhydride). In situ hybridization was performed in a moisture chamber overnight at 53°C using 50ng/ml DIG-NK1-R probe in hybridization buffer (50% formamide, 10% dextran sulfate, 4X
SSC, 1X Denhardt’s solution, 0.25mg/ml yeast tRNA, and 50 mM DTT). After hybridization, sections were incubated for 30 min with 20 µg/ml of RNaseA at 37°C and washed for 30 min with 2X SSC and 0.1X SSC at 60°C. The localization of NK-1 receptor was observed after incubating sections with a fluorescein (FITC) conjugated sheep anti-DIG antibody (Roche, Inc., Indianapolis, Indiana) for 2 hours at a 1:5 dilution in blocking solution (1% donkey serum in TBST). After several washings, sections were mounted with anti-bleaching solution, and again examined without knowledge of experimental groupings by confocal microscopy as described above.

**Statistics:** Group differences were determined by analysis of variance (ANOVA) followed by a post hoc Tukey’s multiple mean separation tests. Data were plotted and statistical analyses were performed using Origin (OriginLab, Northampton, MA) and SigmaStat (SPSS Inc., Chicago, IL), respectively. All data are expressed as mean ± SEM.
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 grams per day), eventually exceeding their preoperative weight by at least 15% prior to commencing any studies. Non-operated 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: The 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 non-operated controls (Fig. 2, 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. 2, Group 1). Although all IPAA animals have loose stools following the resumption of solid food (3 –5 days post-operatively), 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 non-operated 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 non-operated controls (Fig. 3A). There are neutrophils and other white blood cells scattered throughout the mucosa of 28-day IPAA animals that accounts for the elevations in mucosal MPO. Despite the elevation in MPO activity in the IPAA controls which is indicative of sub-clinical inflammation, there are no corresponding clinical or physical signs typically associated with pouchitis.
Group 2: The 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 nearly 80% (p < 0.05). When the NK-1RA was administered 1 day prior to 5% DSS (5% DSS + NK-1RA), the inflammatory response was completely prevented (P < 0.001) compared with animals receiving 5% DSS + Saline (Fig. 2, 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 prior to DSS consumption had loose stools but not 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.

Upon 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 non-operated controls (Non-op Control). In animals administrated 5% DSS starting 28 days after IPAA (IPAA with 5% DSS), receptor expression remains significantly elevated (Fig. 4A and B). In these rats, since the expression of NK-1R protein in pouch ilea is not further elevated, suggests that IPAA alone may have caused maximal receptor protein expression.

**Neurokinin 1 receptor 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 non-operated 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 non-operated 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 were 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-1R antagonist is effective in reducing a chronic, ongoing ileal pouch inflammation that develops spontaneously after IPAA surgery. Other studies, for example Pothoulakis et al (31), in which NK-1R antagonists 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 one day prior to the imposition of an additional inflammatory stimulus, the consumption of DSS, prevents the exacerbation of the inflammatory response. This result was somewhat surprising since 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, sub-clinical 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 (6,10). This may be due, in part, to the morphologic (32), histologic (33), and metabolic adaptations (34) that occur in the functioning ileal pouch. Whether these adaptations increase susceptibility to inflammation remains unclear. While these adaptive changes may include an increase in SP-containing immunoreactive nerve fibers in the ileal pouch (21), the findings presented here demonstrate that compared with normal, control ilea from non-operated 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 lamina propria 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 substance P-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 (35). These increases may initiate a cascade of pro-inflammatory SP-mediated events orchestrated by the upregulation of nuclear factor kappa B that can compromise epithelial integrity and exacerbate inflammation (36). 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 prior to 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 substance P 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 (22).

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 colon-like 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 (32). All of these luminal contents have been implicated in underlying inflammatory changes in the ileal pouch (5). Since the
cumulative risk of pouchitis increases over time (6,10), 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 pathophysiologic stimuli underlying these inflammatory changes remain unclear, events related specifically to ileal pouch physiology such as stasis or delayed pouch emptying (37) and bacterial overgrowth (6), both of which occur in this model (13), 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 (13). Lipopolysaccharide (LPS) derived from non-indigenous, gram-negative bacteria overgrowing the ileal pouch can induce an inflammatory response. LPS has also been shown to facilitate SP-mediated neutrophil uptake (38), and since SP itself is chemotactic for neutrophils (39), this alone may explain the increase in MPO activity. Substance P (40) has also been shown to increase endothelial cell expression of vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1), which can further facilitate neutrophil extravasation. LPS can also directly induce the synthesis and release of SP from macrophages (41), and in concert with SP originating from increased SP-containing immunoreactive nerve fibers in the ileal pouch (21), 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 histologic evidence of chronic mucosal inflammation in the ileal pouch (33). Since 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-1R antagonists 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 (42), and not on maintaining remission between episodes. Currently, only probiotic therapy has shown efficacy in long-term maintenance of remission (43), purportedly by inducing a protective mucosal immune response (44). Since SP is a known pro-inflammatory immunomodulator (45,46), thus, NK-1R antagonists 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-1R antagonists may be of therapeutic value in resolving clinical pouchitis.
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Figure Legends

Figure 1. The highly specific, non-peptide, neurokinin 1 receptor antagonist (NK-1RA), CJ-12,255 (Pfizer, Inc) was utilized 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 Mean ± SEM. Values not designated by the same superscript letter are significantly different (p < 0.05).

Figure 2. **Group 1:** The therapeutic effectiveness of the NK-1RA in reducing MPO levels in the ileal pouch. MPO levels are significantly elevated 28 days after IPAA surgery (Saline) compared with non-operated controls (P < 0.001). The antagonist, when administered for 4 days to 28 day IPAA animals (NK-1RA) in the presence of this ongoing inflammation, significantly reduces MPO levels compared with Saline (P < 0.001). Values not designated by the same superscript letter are significantly different (p < 0.05).

**Group 2:** The prophylactic effectiveness of the NK-1RA in preventing the rise in MPO levels in the ileal pouch in response to the inflammatory stimulus of 5% DSS added to the drinking water for 4 days. The administration of 5% DSS to IPAA animals after 28 days significantly elevated MPO levels (5% DSS + Saline) compared with IPAA animals after 28 days alone (No DSS) (P < 0.001). When the antagonist was administered to IPAA animals (5% DSS + NK-1RA) one day prior to DSS consumption, the rise in MPO levels was prevented. All data are expressed as Mean ± SEM. Values not designated by the same superscript letter are significantly different (p < 0.05).
Figure 3. Representative cross sectional views of hematoxylin and eosin (H & E) stained ileum.

**Group 1:**

A) Non-operated control (40X); B) Pouch ileum from an IPAA rat after 28 days (Saline) (40X); C) Pouch ileum from a rat administered CJ-12,255 (NK-1RA)(40X);

**Group 2:**

D) Pouch ileum from an IPAA rat after 28 days that received 5% DSS for 4 days (DSS + Saline) showing a dense neutrophilic infiltrate, granulation, and ulceration (100X); and, E) Pouch ileum from an IPAA rat after 28 days that received CJ-12,255 one day prior to the administration of 5% DSS for 4 days (DSS + NK-1RA) (40X).

Abbreviations: IPAA; ileal pouch-anal anastomosis; NK-1RA, neurokinin 1 receptor antagonist; DSS, dextran sulfate sodium; MPO, myeloperoxidase.

Figure 4. A) A representative Western blot of neurokinin-1 receptor protein expression from rat pouch ileum. Lanes 1-5 are non-operated controls; lanes 6-10 are 28 day IPAA pouch alone; and, lanes 11-14 are 28 day IPAA pouch plus 4 days of 5% DSS. The NK-1 receptor was identified by its migration position that corresponded to the NK-1R receptor from rat submaxillary salivary gland, a tissue rich in NK-1R in the rat (not shown). The molecular weight of the band shown corresponded to the known weight to the NK-1R receptor (approximately 50 kilodaltons). B) Quantification of neurokinin-1 receptor protein expression. Western blots were scanned and quantified using Scion Image software (Scion Corporation). Mean values were determined and expressed as a percent of non-operated controls. All data are expressed as Mean ± SEM. Values not designated by the same superscript letter are significantly different (p < 0.05). Abbreviations:
IPAA; ileal pouch-anal anastomosis; Non-op, non-operated; DSS, dextran sulfate sodium.

Figure 5. Top panels show representative cross sectional views of neurokinin-1 (NK-1) receptor mRNA by in situ hybridization: A) non-operated control (non-operated control); B) IPAA animal (IPAA alone) after 28 days; and, C) IPAA animal after 28 days plus 4 days of 5% DSS (IPAA with 5% DSS). Bottom panel shows representative cross sectional views of NK-1 receptor protein expression by immunohistochemistry: D) non-operated control (non-operated control); E) IPAA animal after 28 days (IPAA alone); and, F) IPAA animal after 28 days plus 4 days of 5% DSS (IPAA with 5% DSS). (Mag. 40X) Abbreviations: IPAA; ileal pouch-anal anastomosis; NK-1RA, neurokinin 1 receptor antagonist; DSS, dextran sulfate sodium.
Table 1. Effects of the administration of a neurokinin 1 receptor antagonist (NK-1RA) on percent (%) weight change, diarrhea, occult and gross rectal bleeding and ileal ulcerations in ileal pouch-anal anastomosis (IPAA) Sprague Dawley rats in the presence or absence of 5% dextran sulfate sodium (DSS).

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<tr>
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<th>Group 1</th>
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<tbody>
<tr>
<td></td>
<td>Non-Operated Controls (n = 11)</td>
<td>Saline (n = 11)</td>
<td>NK-1RA&lt;sup&gt;a&lt;/sup&gt; (n = 11)</td>
</tr>
<tr>
<td>% Weight change&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+7%</td>
<td>+6%</td>
<td>+6 %</td>
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<tr>
<td>Diarrhea&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>0/11</td>
<td>0/11</td>
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<tr>
<td>Rectal bleeding</td>
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<td></td>
<td></td>
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<tr>
<td>- Occult</td>
<td>0/11</td>
<td>0/11</td>
<td>0/11</td>
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<tr>
<td>- Gross</td>
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<tr>
<td>Ileal Ulcerations</td>
<td>0/11</td>
<td>0/11</td>
<td>0/11</td>
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<sup>a</sup>NK-1RA treatment was started 28 days postoperatively and continued for 4 days.

<sup>b</sup>DSS was administered for 4 days commencing 28 postoperatively. The antagonist treatment was administered 1 day prior commencing DSS.

<sup>c</sup>percent weight change over the 4 day experimental treatment period

<sup>d</sup>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 sticks to the anus.
Figure 1

μL Salivagm body wt

Control  CJ-12,255  CP-96,345  CP-96,344

n = 6  n = 6  n = 6  n = 6
Figure 5

In situ Hybridization (NK-1R mRNA)

Non-operated control

IPAA alone

IPAA with 5% DSS

Immuno-Histochemistry (NK-1R protein)