

# Receptor binding sites for substance P, but not substance K or neuromedin K, are expressed in high concentrations by arterioles, venules, and lymph nodules in surgical specimens obtained from patients with ulcerative colitis and Crohn disease

(tachykinins/inflammatory bowel disease/autoradiography/inflammation)

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**ABSTRACT** Several lines of evidence indicate that tachykinin neuropeptides [substance P (SP), substance K (SK), and neuromedin K (NK)] play a role in regulating the inflammatory and immune responses. To test this hypothesis in a human inflammatory disease, quantitative receptor autoradiography was used to examine possible abnormalities in tachykinin binding sites in surgical specimens from patients with inflammatory bowel disease. Surgical specimens of colon were obtained from patients with ulcerative colitis ( $n = 4$ ) and Crohn disease ( $n = 4$ ). Normal tissue was obtained from uninvolved areas of extensive resections for carcinoma ( $n = 6$ ). In all cases, specimens were obtained <5 min after removal to minimize influences associated with degradation artifacts and were processed for quantitative receptor autoradiography by using <sup>125</sup>I-labeled Bolton–Hunter conjugates of NK, SK, and SP. In the normal colon a low concentration of SP receptor binding sites is expressed by submucosal arterioles and venules and a moderate concentration is expressed by the external circular muscle, whereas SK receptor binding sites are expressed in low concentrations by the external circular and longitudinal muscle. In contrast, specific NK binding sites were not observed in any area of the human colon. In colon tissue obtained from ulcerative colitis and Crohn disease patients, however, very high concentrations of SP receptor binding sites are expressed by arterioles and venules located in the submucosa, muscularis mucosa, external circular muscle, external longitudinal muscle, and serosa. In addition, very high concentrations of SP receptor binding sites are expressed within the germinal center of lymph nodules, whereas the concentrations of SP and SK binding sites expressed by the external muscle layers are not altered significantly. These results demonstrate that receptor binding sites for SP, but not SK or NK, are ectopically expressed in high concentrations (1000–2000 times normal) by cells involved in mediating inflammatory and immune responses. These data suggest that SP may be involved in the pathophysiology of inflammatory bowel disease and might provide some insight into the interaction between the nervous system and the regulation of inflammation and the immune response in human inflammatory disease.

Neurons with cell bodies located in dorsal root ganglia (DRG) are known to convey specific features of somatic sensory information from peripheral tissues to the central nervous system. Recently, several neuropeptides have been

identified within a subpopulation of these sensory neurons (1). The most extensively characterized of these sensory neuropeptides is substance P (SP), a member of the mammalian tachykinin family (Fig. 1), which also includes SK and NK. Though it is clear that SP and SK are expressed by sensory neurons (2) this remains uncertain for NK and derives from difficulty in generating highly specific antibodies for each of the tachykinins since they all share the highly antigenic carboxyl terminus -Phe-Xaa-Gly-Leu-Met-NH<sub>2</sub>, where Xaa is an aromatic or branched aliphatic amino acid.

Several of these sensory neuropeptides, most notably SP, have been associated with neurons specifically implicated in the conduction of nociceptive information. Thus, intrathecal injection of SP produces biting and scratching behavior [consistent with a role for SP as a peptide neurotransmitter associated with primary afferent nociceptors (3)]; SP release in the spinal cord is inhibited by opiate analgesics (4); depletion of SP by capsaicin [a neurotoxin that is relatively selective for unmyelinated sensory neurons (5)], including those containing SP] is associated with a loss of specific nociceptive response; and release of SP in the spinal dorsal horn in response to normally innocuous stimuli is enhanced in polyarthritic rats (6).

It has become increasingly evident in the last decade that a specific class of DRG neurons conveying *afferent* somatosensory information from peripheral tissues to the spinal cord is involved in the *efferent* regulation of the peripheral tissues they innervate. Thus, SP-containing DRG neurons have been implicated in the *afferent* central transmission of nociceptive information and in the *efferent* regulation of inflammation and sensitization of joint sensory endings in a chronic pain state—e.g., arthritis (7, 8). Support for this concept includes observations that the bulk of the SP synthesized by DRG neurons is transported to the peripheral terminals rather than to the spinal cord (9); SP is a potent vasodilator in several peripheral tissues (10); terminals of SP-containing sensory neurons are observed in association with blood vessels (11); electrical stimulation of these peripheral nerves at intensities that release SP in peripheral tissues reproduces many of the physiological changes seen in

Abbreviations: DRG, dorsal root ganglia (ganglion); SP, substance P; SK, substance K; NK, neuromedin K; IBD, inflammatory bowel disease; H&E, hematoxylin/eosin; GI, gastrointestinal; BH, Bolton–Hunter.

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<u>Substance P</u>	H-Arg-Pro-Lys-Pro-Gln-Gln-PHE-Phe- <u>GLY-LEU-MET</u> -NH <sub>2</sub>
<u>Substance K</u>	H-His-Lys-Thr-Asp-Ser-PHE-Val- <u>GLY-LEU-MET</u> -NH <sub>2</sub>
<u>Neuromedin K</u>	H-Asp-Met-His-Asp-Phe-PHE-Val- <u>GLY-LEU-MET</u> -NH <sub>2</sub>

FIG. 1. Structure of mammalian tachykinins. Sequence homologies are noted by underlining, and invariant residues are indicated by uppercase script. Neurokinin  $\alpha$ , neurokinin A, and neuromedin L are alternative names for substance K (SK). Neuromedin  $\beta$  and neurokinin B are alternative names for neuromedin K (NK).

acute inflammation (12), including plasma extravasation (7); and in experimental animals SP and SK are expressed by several tissues involved in the inflammatory and immune responses (13).

What emerges from these observations is the hypothesis that sensory neurons containing SP and/or other neuropeptides are involved in conveying nociceptive information to the spinal cord and in regulating the inflammatory and immune responses in the peripheral tissues they innervate. Thus, the same sensory neuropeptide released by a sensory neuron may signal tissue damage in the spinal cord and participate in regulating the inflammation, immune response, and, ultimately, wound healing, in the affected peripheral tissue.

To test whether this hypothesis is applicable to human inflammatory disease we have examined the changes in tachykinin neuropeptide receptor binding sites in chronic inflammatory bowel disease (IBD) patients, where surgical removal of the affected tissue is used to ameliorate the disease in severe cases. IBD is a generic term that refers to chronic inflammatory diseases of the intestine that are of unknown etiology, principally ulcerative colitis and Crohn disease. Ulcerative colitis is an inflammatory, ulcerating process of the colon; Crohn disease is an inflammation of the intestine characterized by nodular granulomatous inflammatory lesions throughout the entire wall that may involve any part of the intestine but primarily attacks the distal small intestine and colon (14). The present report is based on an exploration of tachykinin receptor binding sites expressed in IBD and whether perturbation of this expression is correlated with these diseases.

## EXPERIMENTAL PROCEDURES

**Radioligands.** The radioligands used in the present study were <sup>125</sup>I-labeled tachykinins purified by reverse-phase HPLC to essentially quantitative specific activity ( $\approx 2000$  Ci/mmol; 1 Ci = 37 GBq). The figures are derived from autoradiograms by using the <sup>125</sup>I-labeled (mono-iodo) Bolton-Hunter (BH) conjugates of SP, SK, and NK. {BH reagent = 3-(*p*-hydroxy-*m*-[<sup>125</sup>I]iodophenyl)propionyl}. BH-SP was a gift from Amersham or was synthesized in our laboratory, whereas BH-SK and BH-NK were synthesized in our laboratory by conventional methods (13).

**Human Specimens.** Specimens were obtained within 5 min after removal, embedded in Tissue-Tek (Miles), and placed on dry ice to minimize postsurgical degradation artifacts. In all cases, the diagnosis was independently determined by a pathologist to be either ulcerative colitis, Crohn disease, or histologically normal at the site of resection. The tissue was then blocked, placed on a brass microtome chuck, frozen on dry ice, and processed for quantitative autoradiography as described (14). The tissue was serially sectioned (30  $\mu$ m), thaw-mounted onto gelatin-coated microscope slides, and stored at  $-20^{\circ}\text{C}$  in boxes of desiccant for up to 3 months.

**Receptor Binding Protocols.** *SP.* For SP receptor binding, slide-mounted tissue sections were brought to room temperature and placed in a preincubation medium (19°C for 10 min) consisting of 50 mM Tris-HCl (pH 7.4) containing 0.005% (vol/vol) polyethylenimine (Sigma). The slide-mounted sec-

tions were then incubated at 19°C for 1 hr in a solution of 50 mM Tris-HCl (pH 7.4) containing 3 mM MnCl<sub>2</sub>, 200 mg of bovine serum albumin (Sigma) per liter, 2 mg of chymostatin (Sigma) per liter, 4 mg of leupeptin (Sigma) per liter, 40 mg of bacitracin (Sigma) per liter, and 100 pM radioligand. This was done by placing the slides on a flat surface and covering the sections with 1.5 ml of incubation medium. For estimating nonspecific binding, paired serial sections were incubated as described above except that 1  $\mu$ M unlabeled peptide was added to the incubation solution. Following this incubation, the slides were rinsed with four washes of 50 mM Tris-HCl (pH 7.4) (4°C, 2 min each) and two washes of distilled H<sub>2</sub>O (4°C, 5 sec each) and then quickly dried in the cold room by using a stream of cold air. Sections were then left for 3 hr to dry in the cold and stored in desiccant-filled boxes overnight at room temperature.

*SK and NK.* For labeling SK and NK receptor binding sites, the same protocol was employed except that incubation and wash conditions were slightly altered. The incubation solution for SK was the same as for SP except that the pH was 8.0. The wash conditions were the same as for SP except that time was increased to 5 min for each wash step. The incubation solution for NK was the same as for SP except that the pH was 8.5. The wash conditions were the same except the time was increased to 8 min for each step. Polyethylenimine was omitted from the preincubation medium for SK and NK and the incubation times were 2 hr in each case. Nonspecific binding was determined in the presence of 1  $\mu$ M SK or NK, respectively.

**Analysis of Autoradiograms.** Quantitative autoradiographic analysis of the tachykinin binding sites was performed by placing the dried, labeled slide-mounted tissue sections in apposition to <sup>3</sup>H-sensitive film (Ultrafilm, LKB or Hyperfilm, Amersham) along with iodinated brain mash or commercially available standards (Amersham). After 1–4 wk, the <sup>3</sup>H-sensitive film was developed in Kodak D-19 developer, fixed, and washed. In selected sections where a higher degree of histological resolution of the binding sites was sought, the tissue slices were overlaid with emulsion-coated coverslips (15) or treated with paraformaldehyde vapors (16) to fix the radioligand to its binding site; these slide-mounted tissue sections were then processed for standard emulsion-dipped autoradiography. After these autoradiograms were developed, the sections were placed in Carnoy's fixative for 3 hr, stained with hematoxylin/eosin (H&E), and mounted with Histoclad. Dark-field or bright-field photomicrographs were then taken of the silver grains or counterstained sections, respectively. By using this approach three complementary images were generated: autoradiograms that were analyzed by quantitative densitometry, the autoradiograms of the emulsion-dipped slides that provided detailed histological resolution of the binding sites, and the counterstained section that allowed identification of the cell type expressing a specific binding site. Controls for chemographic artifacts were generated by performing the binding exactly as described except that the radioligand was omitted from the incubation medium.

**Analysis of Data.** To estimate quantitatively the density of radiolabeled tachykinin binding sites, microdensitometry with <sup>3</sup>H-sensitive film was performed (17). In all cases,

specific binding was obtained by subtracting total binding from nonspecific binding. Nonspecific binding was defined as that binding sustained in the presence of 1  $\mu$ M unlabeled peptide.

**Statistical Analysis.** The results were expressed as mean  $\pm$  1 SEM and examined for statistical significance by using the Student's *t* test for independent samples.

## RESULTS AND DISCUSSION

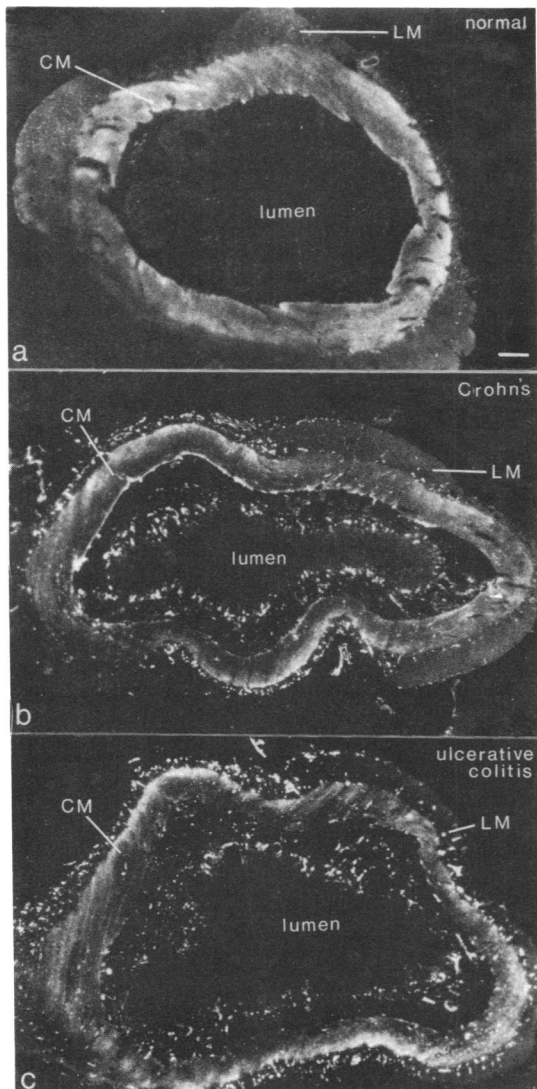
**Expression of SP and SK Binding Sites in Normal Tissues.** In the histologically normal colon moderate concentrations of SP receptors are expressed by the external circular muscle and by smooth muscle cells comprising the tunica media of the large arteries that are just outside of the submucosa (Fig. 2*a*). In addition, a low concentration of SP

binding sites is expressed by arterioles and venules in the submucosa. SK receptors are expressed in low concentrations by the external circular and longitudinal muscle, whereas specific NK binding sites were not observed in any area of the human colon. To control for possible degradation artifacts, one Crohn colon was obtained immediately after removal and pieces were frozen at various times to assess the effects of postsurgical degradation at room temperature. By using this material it was determined that significant degradation had not occurred within the first 5 min after removal, suggesting that postsurgical artifacts should be minimal in this material since all were obtained within this time period.

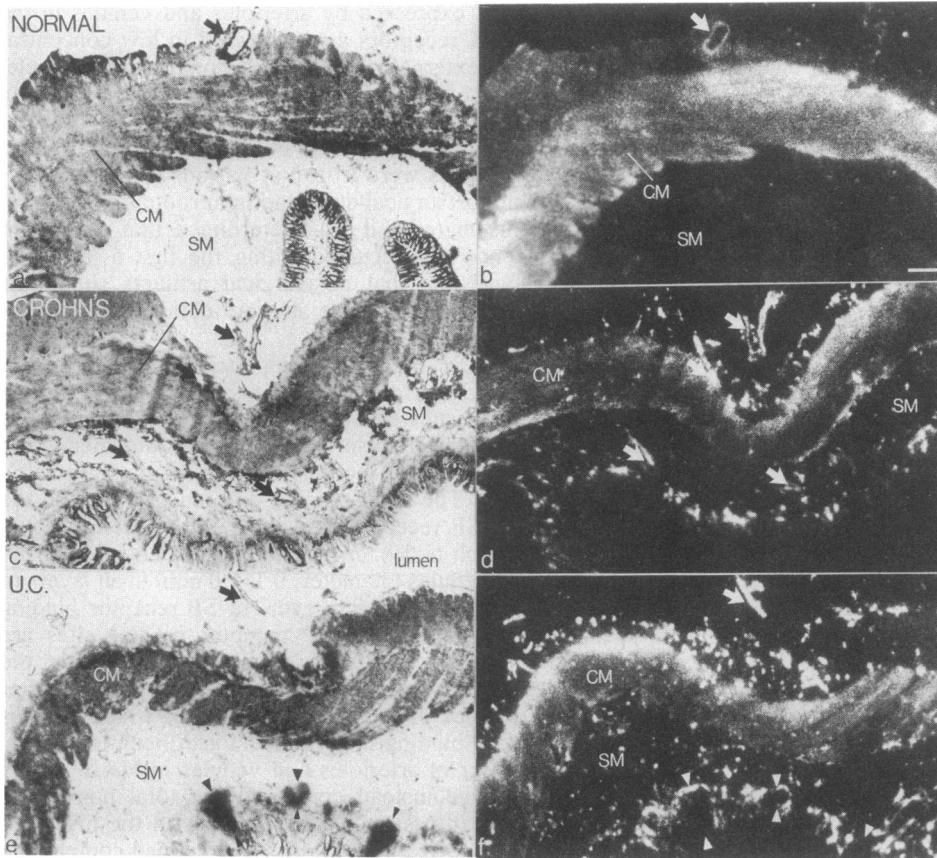
**Expression of SP and SK Binding Sites in IBD.** In surgical material from Crohn disease patients there was a striking change in the tissues expressing SP receptor binding sites compared to that seen in surgical specimens obtained at the margins of extensive resections for carcinoma "normals" (Figs. 2 and 3). The most notable difference is seen in the expression of SP receptor binding sites by arterioles, venules, and lymph nodes. In tissue from patients with IBD, arterioles and venules (diameter, 0.1–1.0 mm) in all layers of the colon express very high levels of SP receptor binding sites, whereas in normals, SP receptor binding sites are undetectable in blood vessels, with the exception of the occasional large arteries just inside the serosa or the arterioles and venules in the submucosa (all of which express low levels of SP binding sites). Since it is difficult to identify the total number of arterioles and venules in H&E-stained sections we have instead compared the total number of blood vessels expressing SP binding sites in the histologically normal vs. disease specimens in a defined comparable area (2.6 mm  $\times$  3.5 mm) of the tissue sections. Comparison of the average number of arterioles and venules expressing SP receptor binding sites in samples from six normal and four Crohn disease patients revealed  $6.50 \pm 2.38$  vs.  $14.5 \pm 4.95$  in the submucosa, 0 vs.  $14.0 \pm 1.4$  in the external circular muscle, and 0 vs.  $18.5 \pm 3.5$  in the external longitudinal muscle.

Concomitant with the ectopic expression of SP receptor binding sites by arterioles and venules is the expression of SP receptor binding sites by the lymph nodules that border the muscularis mucosa. Within each lymph nodule, only cells associated with the lightly stained (by H&E) germinal center expressed a detectable level of SP receptor binding sites. In a total of 36 lymph nodes found in tissue from surgical specimens obtained from patients with Crohn disease, all 36 expressed high concentrations of SP binding sites. This is in contrast to the 24 lymph nodules we could localize in histologically normal surgical specimens from the six patients with carcinoma resection in which the expression of SP receptor binding sites was undetectable. In IBD tissues the external circular muscle displays a slightly lower level of SP receptor binding than in normal (Fig. 4), but this decrease does not appear to be statistically significant.

Surgical material from ulcerative colitis patients revealed a pattern and level of SP receptor binding sites similar to that observed in the specimens obtained from Crohn disease patients. In specimens from ulcerative colitis patients very high levels of SP receptor binding sites are expressed by small arterioles and venules located in the muscularis mucosa, submucosa, external circular muscle, external longitudinal muscle, and serosa. In samples of tissue obtained from six carcinoma resection patients and four patients with ulcerative colitis, the average number of arterioles and venules in the histologically normal and ulcerative colitis tissue in a 2.6 mm  $\times$  3.5 mm sample was  $6.50 \pm 2.3$  vs.  $13.3 \pm 1.5$  in the submucosa, 0 vs.  $15.0 \pm 6.2$  in the external circular muscle, and 0 vs.  $19.0 \pm 5.0$  in the external longitudinal muscle, respectively (Fig. 5).



**FIG. 2.** Dark-field autoradiograms showing the distribution of SP binding sites in transverse sections of colon obtained from the margins of extensive resection for carcinoma (*a*) and with patients with Crohn disease (*b*) and ulcerative colitis (*c*). In these dark-field autoradiograms the  $^3\text{H}$ -sensitive film was used as the negative; white silver grains represent areas of high concentration of SP binding sites. Note that whereas in normal colon (*a*) a moderate concentration of SP receptor binding sites is expressed by the external circular muscle (CM) and the tunica media of a large artery in the serosa, in Crohn disease (*b*) and ulcerative colitis (*c*), arterioles, venules, and lymph nodules express very high levels of SP receptor binding sites. LM, external longitudinal muscle. (Bar = 1.4 mm.)



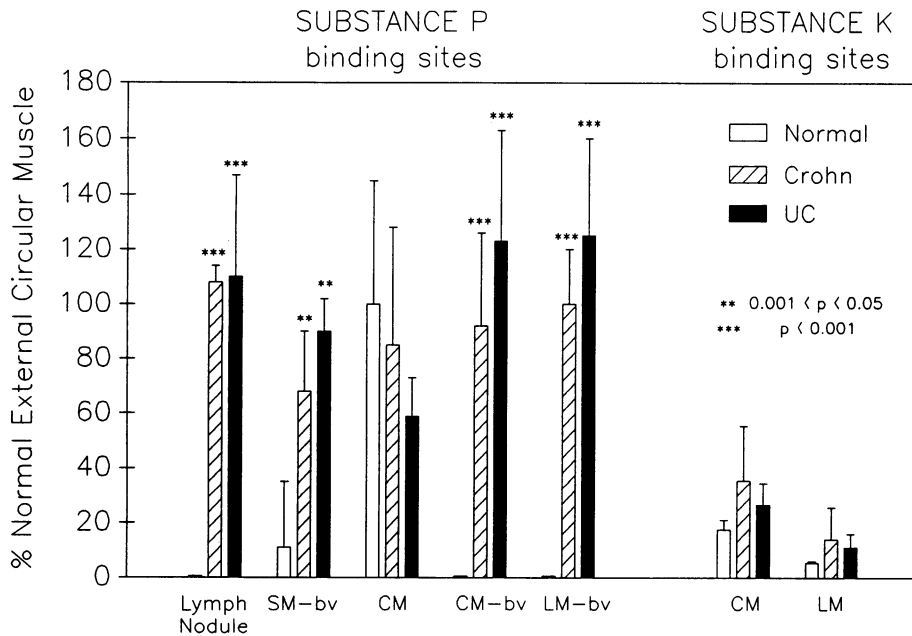
**FIG. 3.** Higher-power autoradiograms of transverse sections of colon shown in Fig. 2 demonstrating the localization of SP receptor binding sites in the histologically normal human colon (a and b), Crohn disease patients (c and d), and ulcerative colitis (U.C.) (e and f) patients. (a, c, and e) Bright-field photomicrographs of the human colon stained with H&E. (b, d, and f) Dark-field photomicrographs obtained by using <sup>3</sup>H-sensitive film that overlaid this tissue as the negative. In b, d, and f the white silver grains represent high concentrations of SP binding sites. Note that in the normal tissue a moderate concentration of SP binding sites is expressed by the external circular muscle (CM), whereas in the Crohn disease and ulcerative colitis tissue SP receptor binding sites are expressed by arterioles and venules (arrows) and the lightly stained outer rim of the lymph node (arrowheads), which corresponds to the germinal center. Control sections were treated identically to the sections shown in b and c except that 1 μM unlabeled peptide was added to the incubation medium. Unless otherwise noted, control sections were essentially blank. SM, submucosa. (Bar = 0.63 mm.)

The germinal center of lymph nodules in tissue obtained from the ulcerative colitis patients also expresses very high levels of SP receptor binding sites compared to those found in normals (Fig. 4). Thus, of the 29 lymph nodes histologically localized in H&E-stained sections from the four cases of ulcerative colitis, 28 expressed very high levels of SP binding sites, whereas 24 nodules localized in normal colon failed to express detectable levels of SP binding sites.

**Involvement of SP in Inflammatory Diseases.** Although the etiology of IBD remains unknown, recent research into a variety of inflammatory diseases such as arthritis (18) and asthma (19) suggests that the nervous system may exagger-

ate associated immune and inflammatory responses in genetically predisposed individuals. The present findings reveal that in a disease state a specific tachykinin binding site for SP appears to be strategically expressed by specific cells that are involved in producing two of the salient symptoms of IBD. These include a hyperactive inflammatory and immune response, often accompanied by abdominal pain.

A key question is whether SP receptor binding site expression by arterioles, venules, and lymph nodules is an etiological factor in IBD or whether it is simply concomitant with the inflammation. One observation that suggests it is an etiological factor is that whereas corticosteroids are effica-



**FIG. 4.** Histogram showing changes in the location and concentration of SP and SK binding sites in surgical specimens of normal colon obtained from carcinoma resection (open bars), Crohn disease (hatched bars), and ulcerative colitis (UC) (dark bars). In this histogram, 100% specific binding is that concentration of specific SP binding sites expressed by the smooth muscle of the normal external circular muscle (CM). Note that whereas SP receptor binding sites are undetectable in blood vessels (bv) vascularizing the external circular muscle (CM) and external longitudinal muscle (LM) and in the lymph nodules of normal patients, in the Crohn disease and ulcerative colitis patients they reach levels that are the highest observed in the human gastrointestinal (GI) tract. Distinct binding sites for NK were not detected in any area of the human GI tract. bv, blood vessel (arteriole or venule); SM, submucosa.

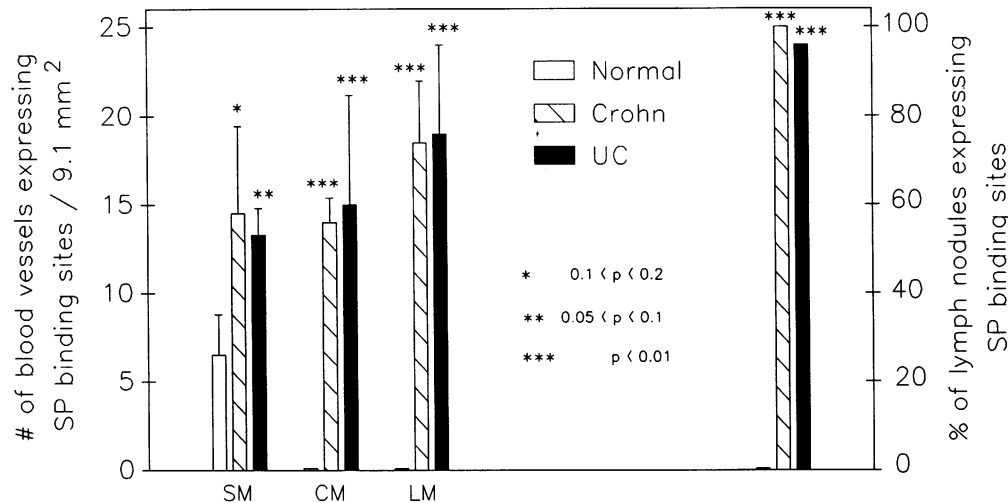


FIG. 5. Histogram showing changes in the number of blood vessels (arterioles and venules) and percentage of lymph nodes expressing SP binding sites in surgical specimens of normal colon obtained from the margins of carcinoma resection (open bars), Crohn disease (hatched bars), and ulcerative colitis (UC) (dark bars). Values for blood vessels are the average number of blood vessels in a  $2.6 \text{ mm} \times 3.5 \text{ mm}$  ( $9.1 \text{ mm}^2$ ) sample expressing SP receptor binding sites, whereas for the lymph nodes it is the percentage of lymph nodes that express SP binding sites. For abbreviations, see Fig. 4.

cious in ameliorating the symptoms of IBD (14) they do not appear to affect SP receptor binding expression in affected tissue. Thus, though corticosteroids suppress the inflammation and the immune response, withdrawal of corticosteroids results in a return of the symptoms, suggesting that the underlying pathology is still present. The location or levels of SP receptor binding expression do not appear to be significantly different in the two Crohn disease patients who received high doses of corticosteroid (Pregnesone) treatment as compared to two Crohn disease patients who had not received any corticosteroid treatment for >12 months.

Another suggestive link between tachykinins and IBD pathophysiology is that the SP/SK-containing axons that are known to innervate blood vessels and regulate the inflammatory response are the same sensory fibers hypothesized to transmit nociceptive input from the GI tract to the spinal cord (11, 13, 20, 21). Capsaicin, the active ingredient in red peppers, is known to release SP/SK from the central and peripheral ends of these sensory nerves (2, 12, 20, 21) and also appears to produce a set of responses in the GI tract of normal subjects similar to that seen in IBD patients. Thus, ingestion of capsaicin or a similar compound by a subject accustomed to bland food may elicit a crampy diarrhea, which although less severe, is similar to the symptoms presented in IBD patients. Capsaicin-sensitive sensory nerve fibers also appear to be involved in other pathophysiological states such as arthritis (8) and skin disorders, which are among the common extraintestinal manifestations of patients with IBD (14).

These data support speculation that in some cases of IBD, tachykinins and their receptors may be involved in the pathophysiology of the disease. We suggest that the tachykinin system is involved in the pathophysiology of IBD and that this involvement may be relevant to several critical features: (i) DRG neurons, which are known to innervate the submucosal blood vessels in the GI tract and express SP and SK, alter what they synthesize and release in the peripheral tissues during times of stress (such as the herpes simplex virus), possibly accounting for the increase in the severity of IBD during stress; (ii) in IBD, SP binding sites are expressed in high concentrations by small arterioles and venules that are known to be involved in the inflammatory response in the GI tract; (iii) SP receptor binding sites are expressed by cells in gut-associated lymph tissue that is involved in regulating the immune response; (iv) DRG neurons that release SP and SK in the periphery also are thought to be involved in conveying nociceptive sensory information to

the spinal cord, which may account for the accompanying abdominal pain in IBD; and (v) DRG neurons that express SP and SK have also been hypothesized to be involved in the pathophysiology of arthritis and several skin disorders, which are common extraintestinal manifestations of patients with IBD. Thus, alterations in tachykinin-containing sensory neurons and tachykinin receptors may contribute to the pathophysiology of IBD. If this hypothesis is correct, it might provide a rationale for the development of new therapies employing specific tachykinin antagonists to help combat what is now an inadequately understood group of diseases.

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