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Changes in Substance P-Immunoreactive Innervation of Human Colon Associated with Ulcerative Colitis

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The amount of colonic substance P and substance P-receptors is increased in ulcerative colitis, which may denote that substance-P is involved as a neurogenic mediator in the inflammatory process of ulcerative colitis. We studied the anatomical distribution of elevated colonic substance P in ulcerative colitis and assessed morphometrically whether the changes in substance P correlate with alterations in colonic innervation. Full-thickness specimens of colonic wall were obtained from normal human colons ($N = 9$) and the most and least affected regions of ulcerative colitis colons ($N = 10$) and immunostained for substance P. Substance P immunoreactivity index was calculated by multiplying each intensity value by the number of pixels exhibiting this intensity value. The numbers of substance P-immunoreactive nerve fibers in the lamina propria were markedly increased, and their fluorescence intensity was enhanced in ulcerative colitis. The longitudinal muscle layer contained substance P-immunoreactive nerve fibers in ulcerative colitis, but not in the controls. The substance P-immunoreactive index (= number × intensity of nerve fibers) was 3.42 ± 1.49 in controls, 21.19 ± 7.79 in mild ulcerative colitis regions ($P < 0.05$), and 29.68 ± 9.81 in severe ulcerative colitis regions ($P < 0.01$). Increase in the number of substance P nerve fibers is in accordance with the hypothesis that substance P contributes to neurogenic mediation of inflammation in ulcerative colitis.

KEY WORDS: inflammatory bowel disease; neuropeptide; immunohistochemistry; morphometry.

Ulcerative colitis (UC) is an inflammatory, ulcerating process of the colon with many remissions and exacerbations. Its etiology remains unknown (1, 2). Microscopically, ulcerative colitis is primarily a mucosal and submucosal disease. Histological changes of the severe form of UC include an increased number of inflammatory cells in the lamina propria, crypt abscesses, destruction of the glands, and ulcerations. In the quiescent stage of the disease, the mucosa may appear to be almost normal, although subtle microscopic abnormalities are often detected.

Substance P is associated with both sensory and motor functions of the intestine. The gastrointestinal tract is innervated by sensory fibers that contain and release neuropeptides. It has been suggested that neuropeptides are involved in the regulation of inflammatory and immune responses of inflammatory diseases of humans (3–5). Generally, the amount of substance P in the colon is higher in UC patients (6, 7). Likewise, the number of substance P receptors is increased in UC colon (4, 8). Since nerves containing

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substance P contribute to the innervation of normal human colon (9, 10), the changes in the levels of substance P and its receptors suggest the possibility that this peptide functions as a mediator of neurogenic inflammation in UC. Substance P also controls intestinal peristalsis by contracting intestinal smooth muscle and by stimulating acetylcholine release from other nerves (11). The present study was undertaken to localize the structures responsible for elevated levels of substance P in the colon of UC patients and to determine whether the changes in substance P correlate with altered innervation. In addition, since ileitis is found in about 10% of specimens taken from UC patients subjected to colectomy (12), ileum specimens were included in this study.

MATERIALS AND METHODS

Specimens of normal and diseased gut were obtained from intestine immediately after resection. Permission for human studies was granted by the Ethical Committee of the Helsinki University Central Hospital.

Specimens of Normal Colon. Specimens were taken from resected colon of nine patients (five women and four men) with neoplasia of colon or rectum undergoing colectomy. Their mean age was 65 years (range 45–81). No patient had bowel obstruction or other additional colonic disease. Several whole-wall specimens were taken from a region at the farthest possible distance from the tumor. The specimens were stained for routine histological examination.

Specimens of ileum were obtained from the above patients undergoing right hemicolectomy. This group consisted of seven patients (two women and five men). Their mean age was 66 years (range 45–81 years).

Specimens of Ulcerative Colitis. The diagnosis of UC was based on history and clinical examination, endoscopic examination, and histopathological findings of inflammatory cellular infiltrates in the lamina propria, paucity of goblet cells, and crypt abscess. Specimens of ileum and colon were taken from the resected bowel of patients with UC undergoing proctocolectomy. UC patients were operated on either for failed conservative treatment or for severe side effects of corticosteroids. None of the patients were operated on for dysplasia or fulminant colitis.

Specimens were taken from ileum, the least affected region of colon, and the most affected region of colon, but not from the ulcerated region with destroyed epithelium. The specimens were stained for routine histological examination. Specimens of ileum and colon were taken from 10 patients (one woman and nine men, mean age 44 years, range 24–65 years). The mean duration of the disease was 5.8 years, range 0.5–14 years.

Immunohistochemistry. Immediately after resection, the specimens were immersed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.2, for 24 hr and transferred to 20% sucrose in PBS. Then 10-μm cryostat sections were cut on chrome–alum–gelatin-coated glass slides. The sections were incubated with the primary antiserum (Incstar, Stillwater, Minnesota; diluted 1:500) overnight at +4°C, rinsed, and incubated with fluorescein-conjugated swine anti-rabbit IgG (1:100) for 1 hr at room temperature and mounted in Na–veronal–glycerol mixture. The specimens were examined immediately with a Leitz Aristoplan fluorescence microscope and photographed.

For specificity controls, the primary antiserum was omitted from some sections, which resulted in disappearance of the staining. Substance P immunoreactivity of lamina propria nerve fibers was largely abolished after preincubation of the antiserum with 0.1 μM substance P (Sigma) and totally disappeared after preincubation with 1 μM substance P. Immunoreactivity of the myenteric plexus disappeared after preabsorption with 10 μM substance P. Cellular fluorescence in the lamina propria was unaffected by preabsorption with substance P.

Morphometry. From the sections containing longitudinal profiles of the villi, three sections were randomly selected, photographed, digitized through a video camera and digitizing board (PCVision Plus, Image Technology), and stored in the computer. The area measured was determined by the epithelial basement membrane of the villus and the muscularis mucosae. The area measured frequently contained fluorescent cellular profiles, which were considered nonspecific based on the yellow–brownish, instead of green, color of the fluorescence and on the persistence of staining after preabsorption with substance P. These profiles were identified in the original photographs and manually removed from the electronic image. The overall average fluorescence intensity of the villus was measured as the average degree of grayness over the measuring area. Intensity histograms indicating the number of pixels in each intensity class were obtained. The threshold of specific substance P immunofluorescence intensity was empirically determined by comparing photomicrographs of immunoreactive nerve fibers with their digitized images. Pixels representing values below the threshold were omitted. A value for specific substance P immunoreactivity was obtained by summing the total number of pixels exceeding the threshold.

TABLE 1. VISUAL ESTIMATION OF DENSITY OF SUBSTANCE P-IMMUNOREACTIVE NERVE FIBERS IN VARIOUS LAYERS OF NORMAL AND UC GUT*

	Ileum		*Colon*		
Layer	*Normal*	*UC*	*Normal*	*UC†*	*UC‡*
Epithelium	–	–	–	–	–
Lamina propria	++	+++	+	+++	++++
Muscularis mucosae	+	+	+	+	+
Submucosa					
Nonvascular	++	++	++	++	++
Vascular	–	±	±	±	+
Circular muscle	+	++	+	+	++
Myenteric plexus	++	+++	++	+++	++++
Longitudinal muscle	±	+	–	±	+

*–, none; ±, occasional; +, sparse; ++, moderate; +++, dense; ++++, very dense.
† Least affected region.
‡ Most affected region.

The substance P immunoreactivity index was calculated by multiplying each intensity value by the number of pixels exhibiting this intensity value.

The values obtained from the three sections of each specimen were averaged. The average for each patient group was calculated and statistically analyzed using the Student's *t* test.

RESULTS

Histochemical Observations (Table 1)

Normal Colon. No substance P-immunoreactive cells or nerve fibers were observed in the epithelium. The lamina propria consistently contained some thin, varicose substance P-immunoreactive fibers (Figure 1A). These fibers extended throughout the core of the villus, sometimes following closely the basement membrane. Some substance P-immunoreactive fibers were also seen in the muscularis mucosae. Solitary nerve fibers in the submucosa showed substance P immunoreactivity. Substance P-immunoreactive fibers were occasionally observed in the submucous vascular walls. Several small ganglia consisting of substance P-immunoreactive neurons were also seen in the submucosa. The circular muscle layer contained some substance P-immunoreactive fibers, while the longitudinal muscle layer lacked such fibers. The myenteric plexus contained both substance P-immunoreactive fibers and ganglion neurons. There was no difference in these findings between young and old patients.

Least Affected Region of UC Colon. The immunohistochemical technique revealed no substance P staining in the epithelial cells. The lamina propria contained many fibers showing bright immunofluorescence for substance P (Figure 1B). By visual estimation, their number was greater than that in normal colon. The muscularis mucosae and the submucosa appeared similar to those in the controls, ie, they consistently contained some substance P-immunoreactive fibers. Notably, the number of vascular fibers and the fibers near lymph nodules appeared identical to that in the normal colon. Both the circular and longitudinal muscle layers contained some substance P-immunoreactive fibers. Substance P immunoreactivity in the myenteric plexus was more intense than in the normal colon. Routine histological examination indicated no changes in some of these colon specimens, while in others epithelial proliferation suggestive of regeneration, as well as signs of chronic inflammation, were observed.

Most Affected Region of UC Colon. Immunostaining for substance P revealed no reactivity in the epithelial cells. The number of substance P-immunoreactive nerve fibers in the lamina propria was markedly increased as compared to the two other groups. Their fluorescence intensity also was greater than in the controls (Figure 1C). Substance P-immunoreactive nerves in the muscularis mucosae appeared as fragmented fibers, rather than as a uniform fiber plexus, as in the other groups. Distribution of substance P immunoreactivity in the submucous layer was identical to that in the two previous groups. Both the circular and longitudinal muscle layers contained substance P-immunoreactive fibers. The myenteric plexus showed very intense immunoreactivity for substance P. Routine histological examination showed ulceration of mucosa in all specimens, indicating a severe stage of UC. The mucosal thickness varied and the total number of glands and goblet cells was clearly reduced. In the lamina propria there were large numbers of lymphocytes and plasma cells and occasionally neutrophils and crypt abscesses. The epithelium showed signs of regenerative atypia. No inflammatory changes were observed in the muscular layer. No signs of malignancy were seen in any specimens.

Normal Ileum. No epithelial cells were stained for substance P. The lamina propria consistently contained substance P-immunoreactive fibers (Figure 2A). The density of these fibers was essentially similar to that in colon. Some substance P fibers were also seen in the muscularis mucosae. Several small ganglia consisting substance P-immunoreactive neurons were observed in the submucosa, but no fibers were observed in the vascular walls. The circular and longitudinal muscle layers contained some substance P-immunoreactive fibers. The myenteric plexus contained substance P-immunoreactive fibers and ganglion neurons.

Ileum of UC Patients. No epithelial cells showed substance P immunoreactivity. The lamina propria contained many fibers showing bright immunofluorescence for substance P (Figure 2B). The muscularis mucosae appeared similar to that in controls. The submucosa contained some substance P-immunoreactive fibers and small ganglia, and substance P-immunoreactive fibers were occasionally observed in vascular walls. The circular and longitudinal muscle layers contained some substance P-immunoreactive fibers. The myenteric plexus showed very intense immunoreactivity for substance P. Histological examination revealed no ileitis or other abnormalities in these specimens.

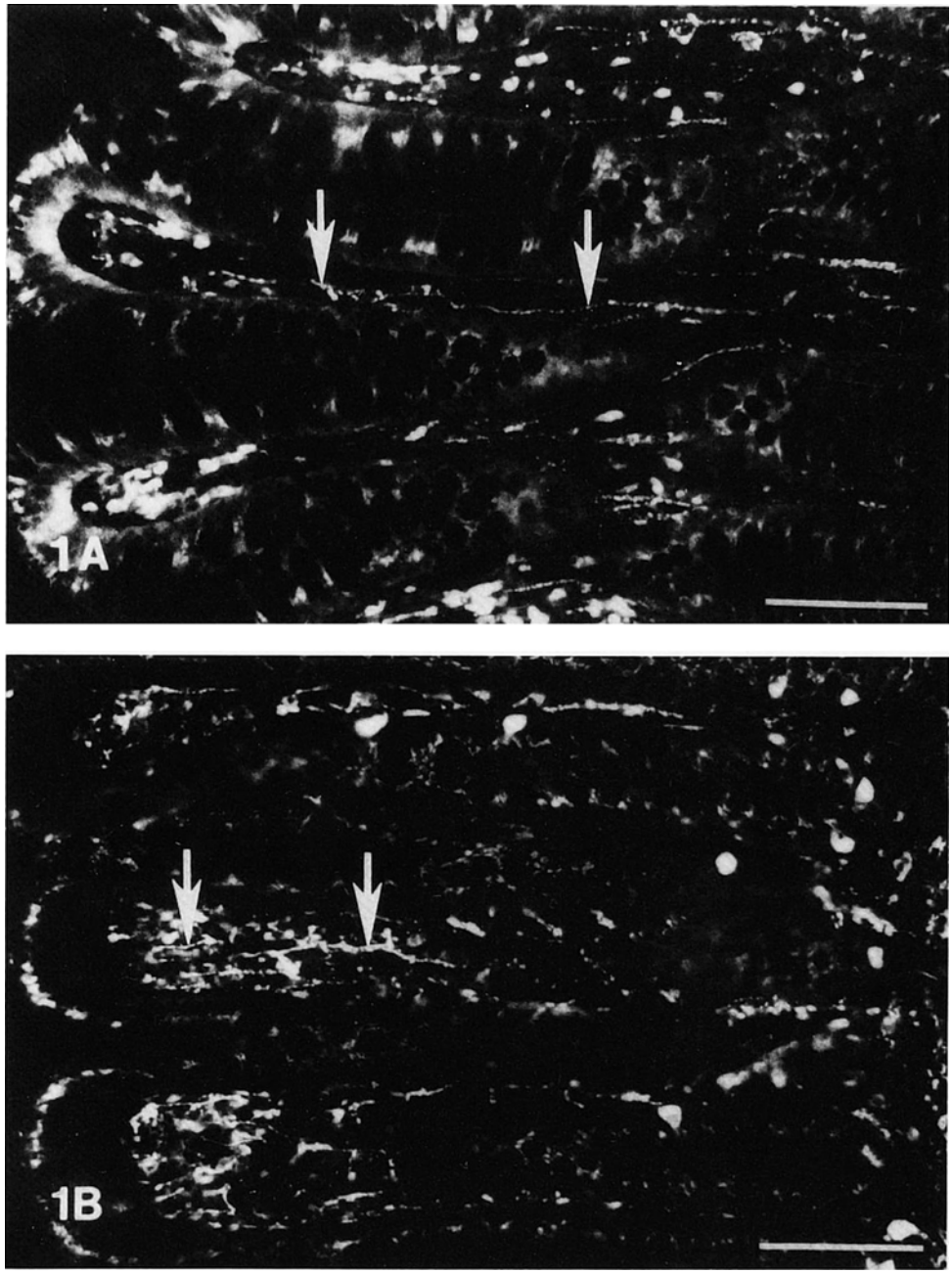

Fig 1. Fluorescence micrographs of substance P immunoreactivity in normal colon (A), the least affected region of UC colon (B), and the most affected region of UC colon (C). Nerve fibers show specific immunoreactivity, while the staining in the cellular profiles in the lamina propria and that in the epithelium is nonspecific (see Materials and Methods). Arrows indicate substance P-immunoreactive nerve fibers. Bar = 100 μm.

Morphometric Measurements

The substance P immunoreactivity index as defined in this study (see Materials and Methods) takes into account both the number of nerve fibers and their intensity. The index for the most affected UC colon (29.68 ± 9.81) was 8.7 times higher than that of normal colon (3.42 ± 1.49, $P < 0.01$), and that of the least affected UC colon (21.19 ± 7.79) was 6.2 times higher than that of normal colon ($P < 0.05$) (Figure 3). The indices obtained for normal ileum (1.74 ±

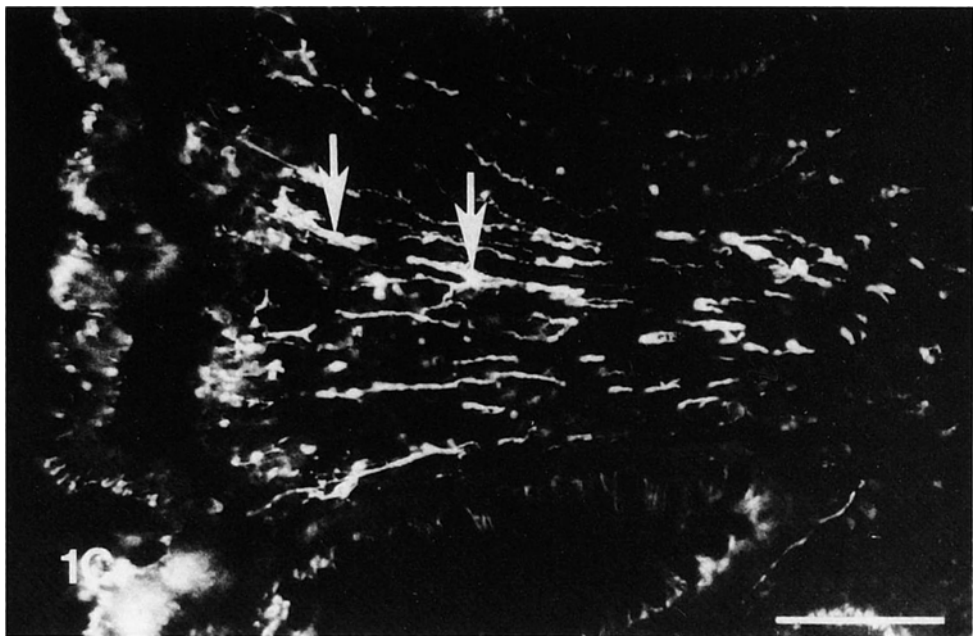

Fig 1. Continued.

0.56) and ileum of UC patients (11.17 ± 3.31) showed a significant difference ($P < 0.05$).

DISCUSSION

The distribution of substance P in normal human ileum and colon described in the present study is similar to that reported in previous histochemical studies (9–11, 13–15). The density of substance P-immunoreactive fibers, as estimated visually, correlates with the chemically measured substance P levels in the various layers of ileum and colon. Thus, the density of substance P-immunoreactive fibers was greatest in the myenteric and submucous plexuses, while abundant immunoreactive fibers were consistently present also in the lamina propria, muscularis mucosae, submucosa, and the circular (ileum and colon) and longitudinal (ileum) muscle layers. Radioimmunological determination of substance P has indicated that of the total substance P content of the gut, about 40% originates from the lamina propria, 40% from the external muscle layer, and 20% from the submucosa (10). In light of our observation, the values for the muscle layer and submucosa include substance P-immunoreactive nerves and neurons in the myenteric and submucous nerve plexuses, respectively, while the value for lamina propria represents predominantly the individual fibers distributed throughout this layer. Rich substance P-immunoreactive innervation of lamina propria was also demonstrated by Milner et al (16), who showed that the amount of substance P per unit mass is several-fold higher in lamina propria than in the rest of the colonic wall.

We did not observe substance P in the intestinal epithelium. Our observations corroborate previous results that isolated epithelium of human ileum and colon lacks chemically detectable substance P (10). Likewise, previous immunohistochemical studies (10, 16) reported the absence of substance P immunoreactivity in the gut epithelium, although substance P-immunoreactive endocrine cells have been described in the gut in other mammalian species (17, 18). We conclude therefore that substance P in human intestine is located exclusively in neuronal structures. The distribution of substance P-immunoreactive nerve fibers correlates with that of substance P binding sites (19), suggesting that these nerves are functional in normal human colon.

The present study reports an increased immunoreactivity for substance P in the colonic mucosa of patients suffering from severe ulcerative colitis. Quantitative histochemical analysis revealed that this change is due to an increased number of positively stained nerve fibers in the lamina propria. In addition, the external longitudinal muscle layer of UC patients showed substance P-immunoreactive fibers that were not seen in normal colon. These findings were not seen in normal or UC patient ileum. Because of their

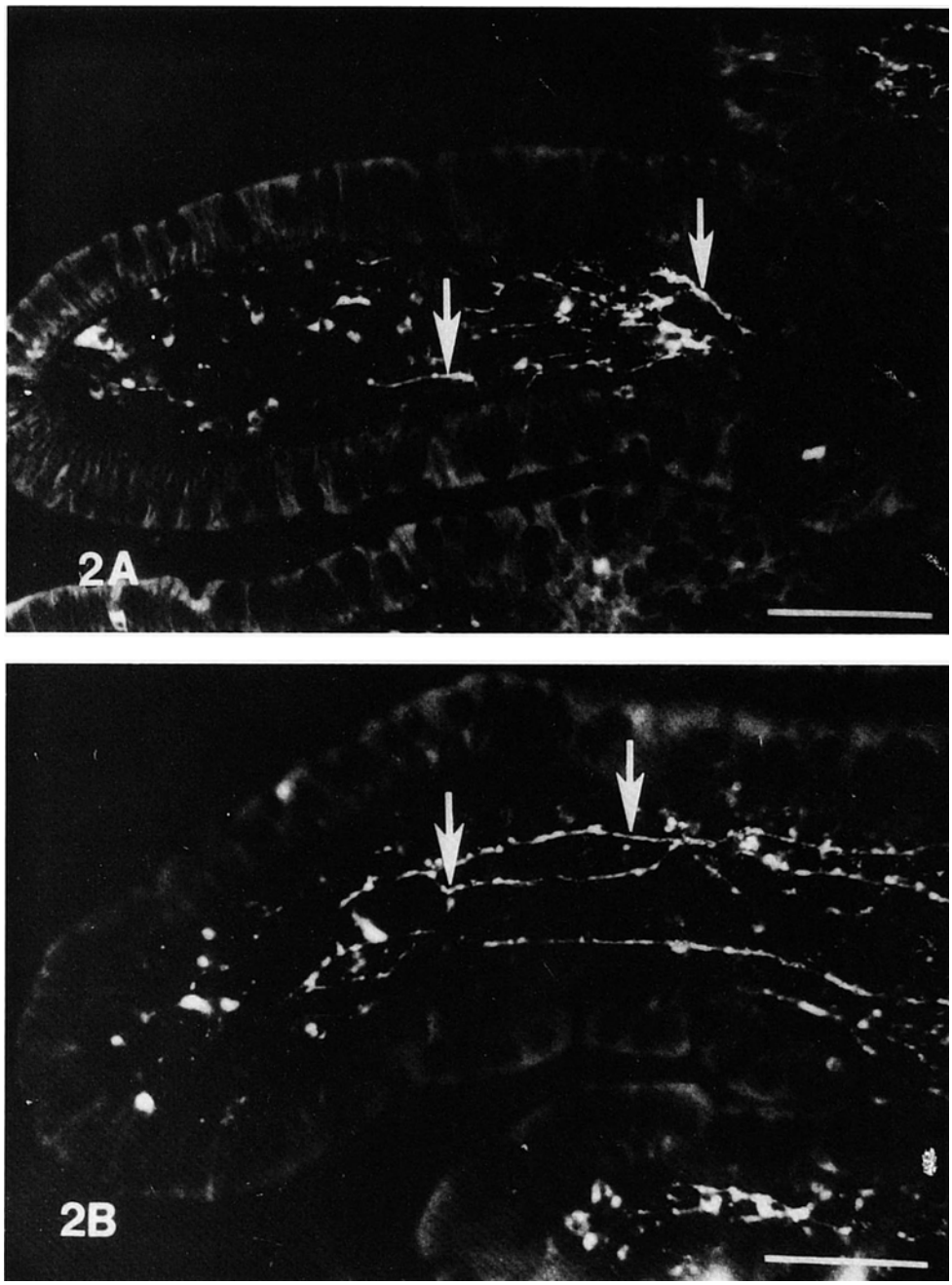

Fig 2. Substance P immunoreactivity in normal ileum (A) and in ileum from UC patient (B). Nerve fibers show specific immunoreactivity, while the staining in the cellular profiles in the lamina propria and that in the epithelium is nonspecific (see Materials and Methods). Arrows indicate substance P-immunoreactive nerve fibers. Bar = 100 μm.

disease, the control patients were, in general, older than UC patients, which potentially might contribute to the different findings between these groups. This is, however, unlikely, since no difference in the occurrence of SP was observed between young and old patients of the control group. Our results provide a morphological correlate to previous studies reporting changes in chemically determined substance P in UC. Goldin et al (7) reported on 2.5-fold increase in the average substance P content of colonic mucosa in UC patients, and a 76% increase in substance P content when the inflamed and uninflamed colonic regions of

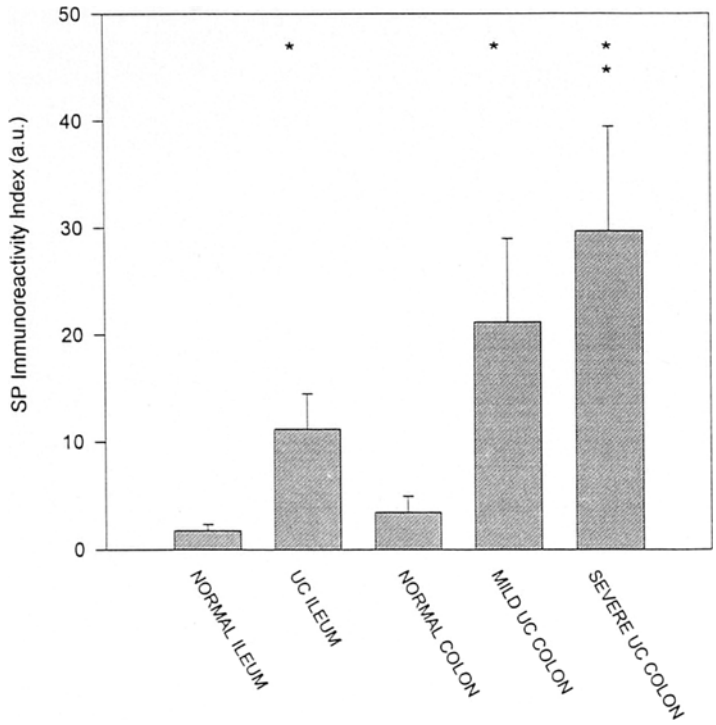

Fig 3. Intensity index representing substance P immunoreactivity in nerves of the lamina propria of ileum and colon. Asterisks indicate statistically significant differences from control (normal ileum or colon) ($^*P < 0.05$, $^{**}P < 0.01$, Student's t test).

same patients were compared. Koch et al (6) discovered a threefold increase in substance P concentration in the mucosa–submucosa of descending colon of UC patients. Using autoradiographic techniques, Mantyh et al (4, 8, 19) demonstrated an increased number of substance P receptors in blood vessels and lymph nodules of the submucosa, as well as the longitudinal and circular muscle layer of UC colon. Taken together, these observations support the idea that substance P is involved in the pathogenesis of UC through a neurogenic mechanism.

Substance P in the sensory nerve endings of the gastrointestinal tract is released upon sensory stimulation (20). In addition, increasing evidence supports the view that substance P contributes to neurogenic mediation of inflammation. This effect consists of several distinct components: First, substance P is a potent vasodilator and is thus responsible in part for mucosal hyperemia and extravasation (4, 20). A low density of substance P receptors has been demonstrated autoradiographically in blood vessel walls of normal human colonic submucosa. In corresponding UC specimens, substance P receptors are present in vascular walls from muscularis mucosae to the longitudinal external muscle layer and their density is greatly increased (4). Second, substance P induces chemotaxis by enhancing leukocyte attachment (21). This mechanism may be mediated by endothelial leukocyte adhesion molecule 1 (ELAM-1), since immunoreactivity for ELAM-1, which is absent in normal colon, appears in UC colon (22). In the skin, substance P induces synthesis of ELAM-1 (23). Third, substance P receptors appear in the germinal centers of submucosal lymph nodules in UC patients, while control patients lack such receptors (4), suggesting that the peptide regulates the immune response. In fact, several cell types that are involved in immune and inflammation reactions are known to possess substance P receptors (23). Under experimental conditions, substance P suppresses activation of lamina propria leukocytes (24). Sensory innervation by substance P terminals may also have a role in the healing of the epithelial ulcerations characteristic of UC. This is suggested by analogy with other tissue models: experimental destruction of substance P innervation leads to delayed wound healing in the skin (25) and cornea (26).

The increase of substance P-immunoreactive nerve

fibers in the lamina propria could occur through several mechanisms: (1) Inflammation might stimulate substance P synthesis so that intracellular peptide levels, which normally fall below the detection limit, become visible. An increase in the level of substance P in sensory nerves has been demonstrated in other inflammatory conditions, such as experimental arthritis (27, 28) and psoriasis (29, 30). (2) Inflammation might trigger nerve sprouting, which leads to an increased number of detectable fibers in tissue sections, as has been demonstrated in inflamed dental pulp (31). (3) The number of neurons innervating the lamina propria might increase, although it is unlikely that the neuron population proliferates, since mature neurons are thought to be postmitotic cells.

CONCLUDING REMARKS

The present observations support the idea that substance P mediates several inflammatory processes in the human colon (32). Regardless of the causal relationship between UC and substance P, elucidation of substance P-related processes may offer possibilities for new therapeutic interference of inflammatory bowel diseases.

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