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Distribution of Substance P-Like Immunoreactivities in the Intramural Plexuses in the Mammalian Gastrointestinal Tract

(substance P/intramural plexuses/vagal ligation)

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A semi-quantitative study was performed to clarify the distribution of substance P in the myenteric and submucous plexuses of the rat gastrointestinal tract, using the unlabeled substance P antibody-peroxidase-antiperoxidase (PAP) method. In both plexuses, substance P-like immunoreactivities (SPLIs) were mainly found around the nerve cells. In addition, these SPLIs were localized in the structures identical to the terminal axons or varicosities; some of which were present in close proximity with nerve cell bodies, suggesting that a release of this peptide might exert some effect on the neurons. Counting of these SPLI-containing nerves in the plexuses, based on our tentative criteria, indicated a reasonable distribution; SPLI-containing nerve was highly condensed in the upper regions of the stomach and the ileum, and gradually decreased towards the duodenum. In the esophagus, SPLI-containing nerves were rarely detected. In cats, cervical ligation of the right vagus significantly decreased the number of SPLIs in the myenteric and submucous plexuses in the ileum, and SPLIs accumulated in the proximal ends of the ligation. The results suggest that a considerable number of SPLIs in the gut might occur in the central nervous system and flow centrifugally throughout the vagus.

Substance P was discovered in extracts of horse intestine and brain and was shown to induce peripheral vasodilatation and contraction of the isolated intestine (1). Later, it was identified as an undecapeptide (2) and bioactive substance P was synthesized (3). Radioimmunoassay techniques have been developed using specific antibodies against synthetic substance P (4). The distribution of this peptide has been investigated in several mammalian tissues such as brain (5), intestine (6) and other tissues (7). An immunohistochemical study has demonstrated the localization of substance P-like immunoreactive fluorescences in the central nervous system (8), intestine (9, 10) and genitourinary tract (11). More recently, electron microscopy showed that immunoreactive substance P was contained in some terminal axons; e. g. in dorsal horn of rat spinal cord (12) and the medial amygdaloid nucleus of the rat brain (13). Physiological studies have suggested that substance P may be an excitatory neurotransmitter in the primary sensory neuron (14, 15) or a

modulator of neuronal activity at the synaptic site of peripheral neurons (16, 17).

In the gastrointestinal tract, this peptide induces contractions of the intestine (1, 18) and the esophageal sphincter (19). It has been reported that immunofluorecent substance P is present in the nerves and the intramucosal endocrine-like cells (8, 9, 10). However, there is no systematic and quantitative evidence for the presence of this peptide in the nerves of the gastrointestinal tract. We report herein a semi-quantitative analysis on the nerves of the rat gastrointestinal tract using the unlabeled antibody enzyme method. The effect of vagal ligation was also evaluated by the estimation of the substance P-like immunoreactivities in the ileum of cats.

MATERIALS AND METHODS

Six adult male rats anesthetized with ether were perfused through the left ventricular cannula first with about 200 ml of Tyrode's saline, then with about 300 ml of Zamboni's fixatives (containing 2 % paraformaldehyde and 0.25 % picric acid in 320 m OsM phosphate buffer, pH 7.4). The dissected tissues were fixed with the same fixatives for 3 days at room temperature, washed with 320 m OsM phosphate buffer overnight, dehydrated, and embedded in soft paraffin. In other experiments, three adult cats, of which the right and cervical vagus had been ligated two days before sacrifice and two untreated cats were used. These animals were anesthetized with ketamine hydrochloride (20 mg/kg weight), and perfused through an arterial cannula with 1 liter of Tyrode's saline and 2 liters of Zamboni's fixatives. The dissected tissues were embedded by the same procedure as described above.

Four micron paraffin sections were processed for immunohistochemical detection of substance P by the unlabeled antibody peroxidase-antiperoxidase complex (PAP) method (20). The antiserum to substance P was raised in rabbits. Three mg of synthetic substance P (Peptide Institute) was dissolved in 1 ml of saline containing 25 % of polyvinylpyrrolidone (PVP, MW. 25000 −30000, Merck). Following homogenization with an equal volume of Freund's complete adjuvant, the mixture was injected subcutaneously at 40−50 sites in the dorsum in three rabbits so that each animal received approximately 1 mg of substance P at each immunization. The antiserum used for staining was collected from one rabbit after five immunizations at four week intervals.

The characterizations of the anti-substance P antiserum were determined by radioimmunoassay according to the procedures described by Yanaihara *et al.* (21) using the Tyr^8-substance P (Peptide Institute) after iodination by chloramine T methods. By radioimmunoassay, the antiserum diluted to 1 : 50000 permits the detection of 100 pg of substance P in 0.7 ml of incubation mixture. Under these circumstances, the antiserum did not cross-react with 20 ng of synthetic somatostatin, eledoisin, enkephalin, angiotensin II, Neurotensin, LHRH and thyrotropin releasing hormone. Physalaemin, known to have the structure identical to that of substance P at their carboxy-terminal

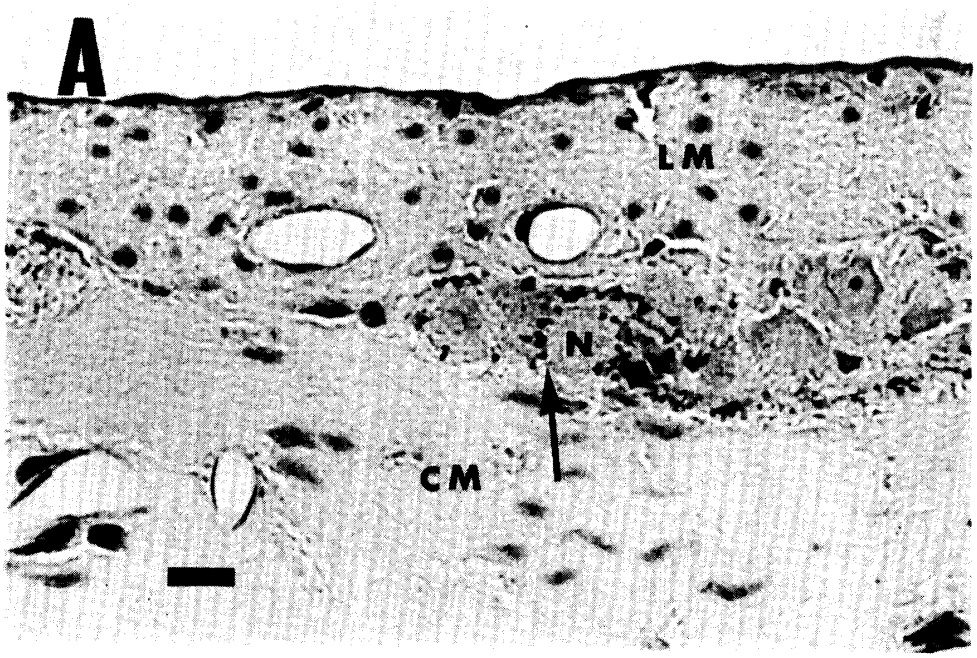
end, showed very weak cross-reactivity with the antiserum to substance P. This antiserum was used in a dilution of 1 : 40 for histochemical staining of substance P. Another anti-substance P antiserum, generously provided by Prof. Yanaihara (Shizuoka College of Pharmacy, Shizuoka, Japan) was used in a dilution of 1 : 100 for the staining. The anti-rabbit IgG goat serum (Research Institute for Microbial Diseases, Osaka) was used in a dilution of 1 : 30. The PAP prepared according to the methods by Sternberger *et al.* (20) was used in a dilution of 1 : 20. A part of the used PAP was generously provided by Prof. Daikoku (Tokushima University, Tokushima, Japan).

The substance P-like immunoreactivity (SPLI) was visualized as the reaction products between PAP and DAB about 40 min after the immersion in Tris-HCl buffer (pH 7.6) containing 0.003 % of DAB and 0.001 % of hydrogen peroxide at room temperature. Normal rabbit serum or the anti-substance P antiserum treated with an excess of antigen were used for the controls. Specific SPLI deposits were not detected with either control serum. The observations and calculations of SPLI deposits were performed on 4 to 9 sections for each tissue.

RESULTS AND DISCUSSION

The substance P-like immunoreactivities (SPLIs) were detected mainly in nerve fibers in the myenteric and submucous plexuses and in the muscle layers and were also detected weakly, presumably in endocrine-like cells existing in the pyloric and intestinal glands. This report concerns a semi-quantitative study on the distribution of SPLIs in the myenteric and submucous plexuses in the gastrointestinal tract.

The myenteric plexus was observed throughout the gastrointestinal tract with variable size and distribution among the tissues, but the submucous plexus



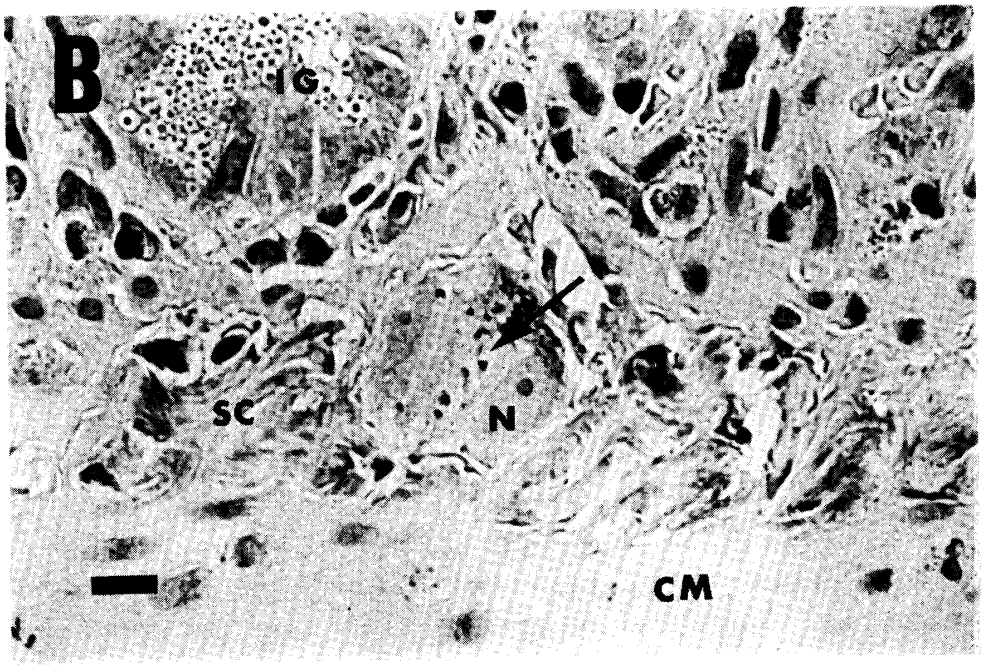


Fig. 1. Myenteric plexus (A) (in the colon) and submucous plexus (B) (in the ileum) of rat stained by the PAP method. Nuclei are counterstained with hematoxylin. Bar represents 10 μ in both (A) and (B). One of the SPLI deposits is indicated by the arrow. CM : circular muscle layer, LM : longitudinal muscle layer, N : nerve cell, SC : submucous connective tissue, IG : intestinal gland.

appeared invariably in the intestine. As shown in Fig. 1, the reaction products ranging from 0.5 to 1 μ in diameter, were recognized as the SPLI deposits around the nerve cells and in the running fibers in both plexuses, and were more numerous in the myenteric plexus than in the submucous plexus. Identification of the cells producing substance P is most important with respect to considerations of the function of this peptide. Using an immunofluorescence technique, Pearse & Polak (10) observed substance P-containig cells in both plexuses but others (8, 9) stated that the SPLIs were localized only in the nerve fibers. We are now attempting to determine what type of nerve cell in the intramural plexuses produces substance P.

To compare the frequency or density of SPLIs in the intramural plexus of several tissues, some criteria were devised. Although the plexus consists of a three dimensional meshwork of nerve cells and fibers, it appears in the sections as several groups of nerve cells and fibers. In the present study, a group which contained one or more nerve cells in the plexus was refered to as one ganglion. To standardize the comparison, only the SPLI deposits attached to the nerve cells in the ganglion were counted, and the nerve cell associated with two or more SPLI deposits was termed "positive cell". Fig. 2 shows the relative frequency of the ganglia containing at least one positive cell (positive ganglia) in the plexus observed. As shown in Fig. 2 (A), more than 50 % of myenteric plexus-ganglia in the stomach and intestine contained the positive cells. In the esophagus, only 2 of 15 observed ganglia (13 %) contained the positive cells. Except for the esophagus, the duodenum showed the lowest

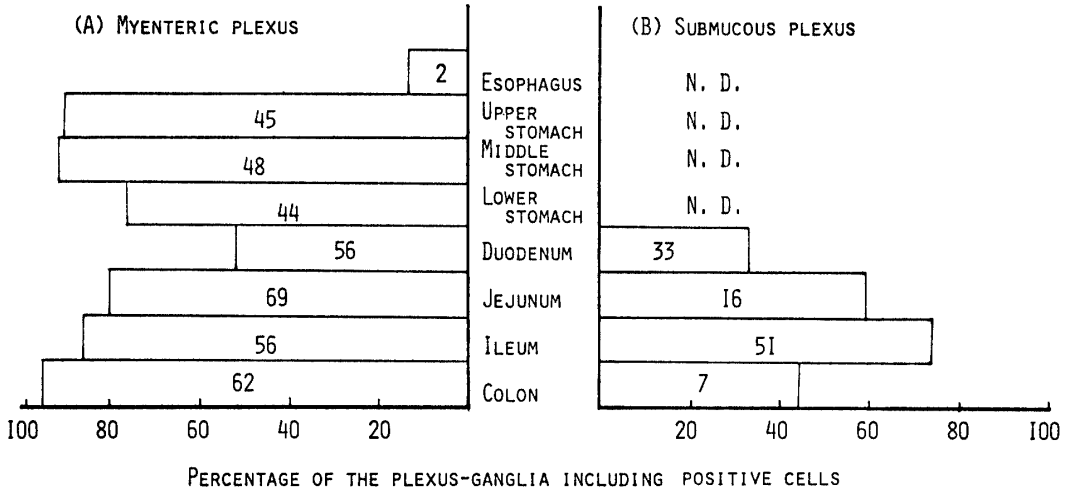


Fig. 2. The relative frequency of the myenteric and submucous plexus-ganglia including the nerve cell associated with SPLI deposits (positive cell) in rat gut. The number in the column shows the actual number of the plexus-ganglia observed and which includes the positive cells. N. D. : not detected.

frequency of positive ganglia and this frequency increased gradually towards the upper stomach and colon. Since the submucous plexus was hardly detected in the rat esophagus and stomach, the comparison was restricted only to the intestine, as shown in Fig. 2 (B). The frequency of the positive ganglia in the submucous plexus was lower than that of the myenteric plexus in each tissue. This frequency increased from duodenum towards ileum and decreased in the colon. Fig. 3 shows the relative frequency of the nerve cells associated with SPLI deposits (positive cells) in all the nerve cells observed in the myenteric and submucous plexuses. These calculations were performed on about 50 to 100 myenteric plexus-ganglia, except for 15 in the esophagus and on 30 to 130 submucous plexus-ganglia on the same sections as in Fig. 2. The results shown in Fig. 3 coincide with those in Fig. 2.

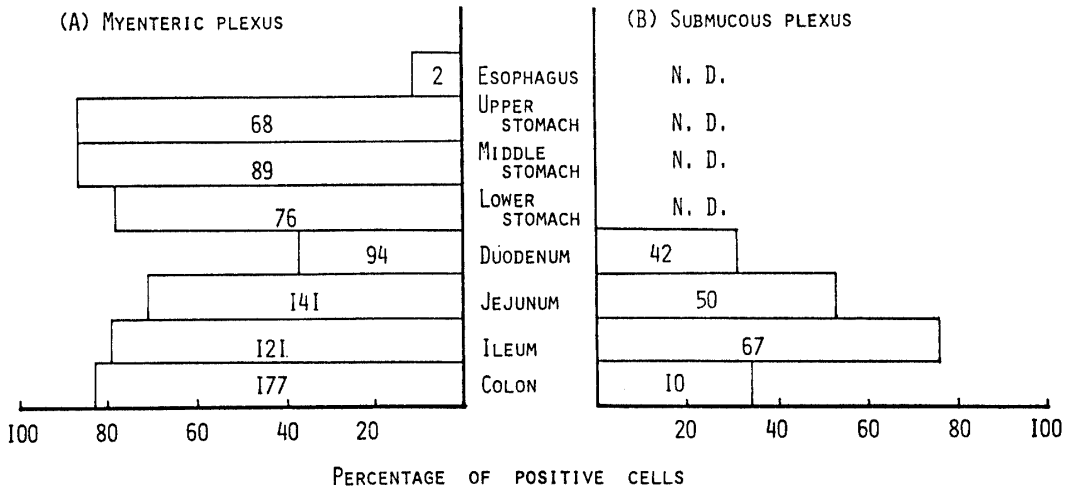


Fig. 3. The relative frequencies of the nerve cells associated with SPLI deposits (positive cell) in the myenteric and submucous plexuses of rat gut. The number in the column shows the actual number of the positive cells observed. N. D. : not detected.

To determine the density of SPLIs in the plexus, the number of SPLI deposits detected only around the positive cells were counted. Fig. 4 shows the average number of SPLI deposits per one positive cell in the myenteric and submucous plexuses of each tissue. The spectrum of the densities also correlated with that of frequencies in Figs. 2 and 3. Thus, in the upper part of the stomach and ileum about 90 % of the myenteric plexus-ganglia included the nerve cells associated with 4 to 5 SPLI deposits and these cells comprise about 80 % of the cells in the plexus. But in the duodenum, about half of the myenteric plexus-ganglia included the nerve cells associated with 3 SPLI deposits and these cells were numbered only 40 % in the plexus. The submucous plexus can be described in almost the same manner as the myenteric plexus except for the low frequency and density.

Nilsson and Brodin (7) reported the tissue concentrations of SPLI in extracts from rat gastrointestinal tract and the results are as follows: The highest concentration (7.0 ng/g) in colon; the jejunum (5.5 ng/g) and the extra antral portion of stomach (5.0 ng/g) are relatively higher; low concentrations in esophagus (1.4 ng/g) or distal duodenum (2.5 ng/g). These distributions of SPLI essentially explain the distribution obtained immunohistochemically in our work, except for the jejunum. Since the endocrine-like cells also contain the SPLIs, the concentrations determined by radioimmunoassay may include

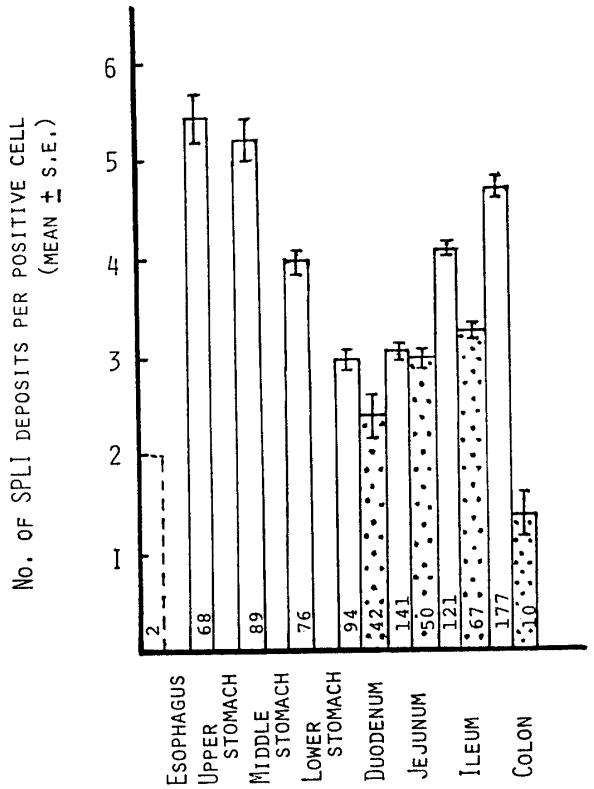


Fig. 4. The relative density of SPLI in the myenteric and submucous plexuses in rat gut indicated by the average number of SPLI deposits associated with the positive nerve cell. The number in the column shows the actual number of the positive cells observed. The blank column shows the myenteric plexus and the dotted column the submucous plexus. In the esophagus such is shown by the perforated line, for the number of observed cells were too little.

the SPLIs in these cells. In conclusion, the indexes adopted for our work are useful for quantitative immunohistochemical studies done to evaluate the SPLIs in the gastrointestinal tract.

In cats of which the right vagus was ligated in the cervix, the SPLIs were accumulated in the nerve fibers immediately above the ligation (data have been presented separately). Changes in the quantities of SPLI deposits in both myenteric and submucous plexuses following the vagal ligation were evaluated. The comparisons were performed on 5 sections for each group. As shown in Table 1, the SPLI deposits around the nerve cell in both plexuses apparently

TABLE I. *Effects of Vagal Ligation on the Frequency of Positive Nerve Cells in the Plexuses of the Cat Ileum*

Cats	Myenteric plexus			Submucous plexus		
	Observed nerve cells	Positive cells	Frequency of positive cells	Observed nerve cells	Positive cells	Frequency of positive cells
Non-treated	84	57	67.9%	91	39	42.9%
Treated	91	40	44.0%	175	40	22.9%

decreased in the ileum of the treated animal. The decrease of the frequency was statistically significant at the 1 % level of probability by x^2 test of 2×2 table (myenteric plexus : $x^2 = 10.10$, submucous plexus : $x^2 = 11.47$).

The result suggests that a considerable amount of substance P flows centrifugally with the axonal transport in the vagus, and that the related fibers probably connect with both intrinsic and postganglionic neurons. Thus, a release of substance P from the nerve terminals of the substance P-containing fibers may exert effects on the neurons.

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