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Peptidergic innervation irregularities in Hirschsprung's disease

Immunohistochemistry – Radioimmunoassay

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Summary. The distribution of vasoactive intestinal polypeptide (VIP)-containing nerves and the contents of both VIP and substance P (S-P) in the intestines from 12 children with Hirschsprung's disease were examined using immunohistochemical methods and radioimmunoassay. VIP-containing nerve fibers were markedly decreased in number in the true muscle coats of aganglionic segments, while extrinsic hypertrophic nerve bundles in these segments showed positive VIP-immunoreactivities. This finding suggests the existance of extrinsic origins of VIP-containing nerves in the human gut. The contents of VIP were 44.5 ± 8.2 in aganglionic segments and 130 ± 17.1 pg/mg wet tissue weight in normoganglionic segments. The contents of S-P were 0.42 ± 0.18 in aganglionic segments and 6.38 ± 2.3 pg/mg wet tissue weight in normoganglionic segments. Both VIP and S-P contents in aganglionic segments were significantly reduced as assessed by the use of radioimmunoassay ($p < 0.001$ and $p < 0.05$).

These abnormal peptidergic patterns of innervation might relate to the non-peristaltic state in Hirschsprung's disease.

Key words: Hirschsprung's disease – Vasoactive intestinal polypeptide – Substance P – Immunohistochemistry – Radioimmunoassay

The pathophysiology of the occurrence of the narrow segment in Hirschsprung's disease has not been fully defined. The abnormal autonomic nerve supply in the diseased bowel is, however, considered to be a causative factor (Whitehouse and Kernohan 1948; Kamijo et al. 1953; Ehrenpreis 1966; Meier-Ruge 1968).

Deficiencies in the non-adrenergic inhibitory system (Burnstock et al. 1963; Crema et al. 1968) have been demonstrated physiologically in the aganglionic segments (Frigo et al. 1973). The finding that impairment of

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the non-adrenergic pathway blocks peristaltic activity (Crema et al. 1970) suggests the significant role of this system in the pathogenesis of Hirschsprung's disease. The failure of the non-adrenergic-related relaxation may be one of the main factors related to the non-peristaltic state (Frigo et al. 1973).

Vasoactive intestinal polypeptide (VIP) is a putative neurotransmitter in the non-adrenergic inhibitory nerve system (Bryant et al. 1976; Fahrenkrug 1979; Goyal et al. 1980). This system is also referred to as the peptidergic nerve system (Sundler et al. 1980). Recently the content of VIP has been revealed to decrease in the aganglionic bowels by using radioimmunoassay (Freund et al. 1979; Dupont et al. 1980; Bishop et al. 1981). These authors estimated the content of VIP in aganglionic segments, compared with that in normoganglionic segments.

The distribution of VIP-containing nerves in the bowel of patients with Hirschsprung's disease should be determined morphologically, in order to elucidate the pathophysiology of the non-peristaltic state of aganglionic bowel. Two differently innervated forms of bowel are present in the aganglionic segments. One is the aganglionic segment with extrinsic hypertrophic nerve bundles and the other is that part without these bundles. These two forms of aganglionic segments are thought to have different peptidergic innervations.

Substance P (S-P), a regulatory peptide in both central and peripheral nerve system (Euler and Gaddum 1931), is reported to act as an excitatory neurotransmitter in peristaltic movement (Leander et al. 1981). Ehrenpreis and Pernow (1953) described bioassay evidence for a decrease of S-P activity in the aganglionic segments. The depletion of S-P innervation is also related to the non-propulsive effect of the aganglionic segments, therefore the content of S-P has to be estimated in detail with radioimmunoassay.

We examined immunohistochemically the detailed distribution of VIP-containing nerves. We measured the contents of both VIP and S-P, using radioimmunoassay, in resected intestines from 12 children with Hirschsprung's disease. We investigated the contents of VIP and S-P in 3 different parts of the resected bowel, the oligoganglionic as well as the aganglionic and normoganglionic segments.

Materials and methods

Twelve Japanese children ranging in age from 2 months to 17 years were clinically diagnosed as Hirschsprung's disease, following barium enema, manometric study and histochemical study using acetylcholine-esterase staining in suction biopsy material (Karnovsky and Roots 1964; Meier-Ruge et al. 1972). The intestine was resected using the Z-shaped anastomosis method (Ikeda 1967).

As a control study, transverse and sigmoid colons, and anorectal portions obtained at the time of autopsy on patients without gastrointestinal disease were studied. Here, the ages ranged from newborn to 16 years. All materials were obtained within 5 h after death.

Histology and immunohistochemistry

The materials were fixed with Zamboni's solution (1967) for 1 h at 4° C, then were cut 2 mm thick and fixed in the same solution for an additional 24 h at 4° C. Fixation was followed

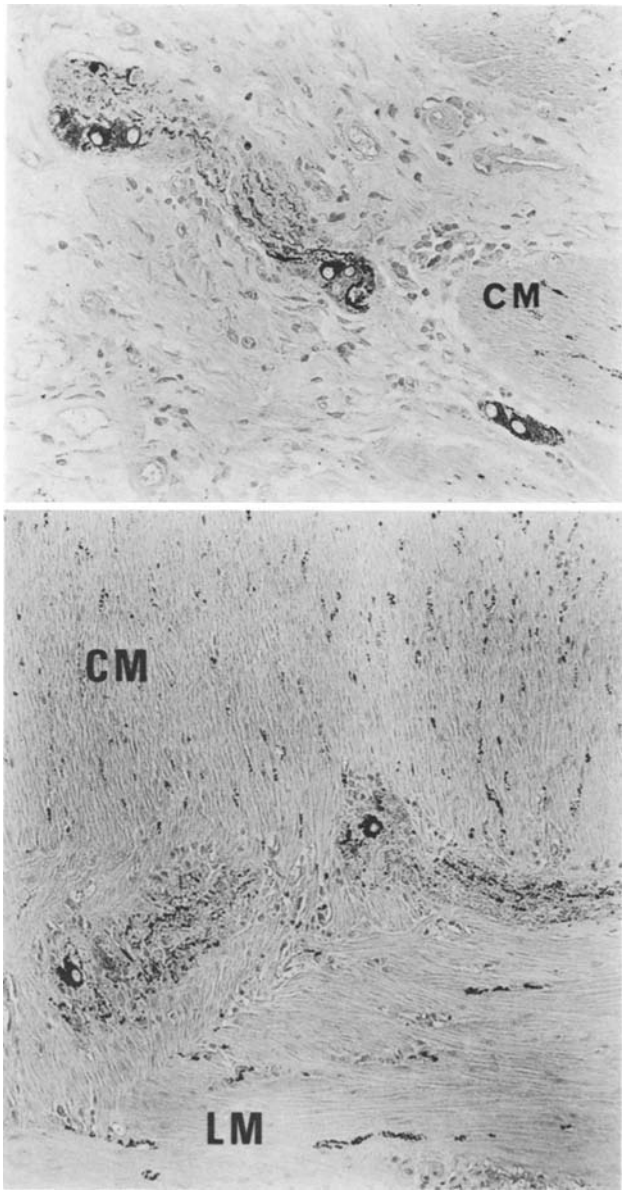
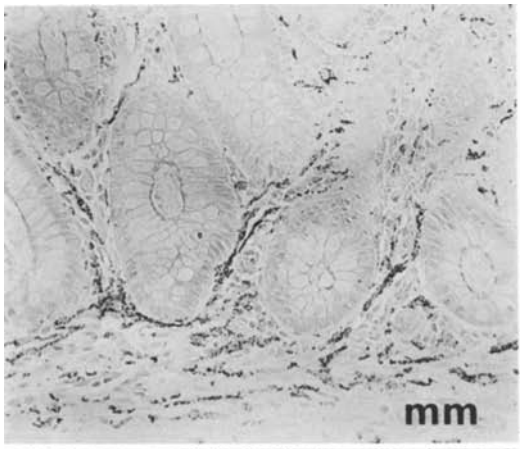


Fig. 1 a, b. Control colon (Plexus and Proper muscle). **a** VIP-containing ganglion cells and VIP-fibers in Meissner's plexuses. *CM*, circular muscle layer. ×190. **b** Several VIP-containing cells and numerous VIP-fibers were present in Auerbach's plexuses. In the proper muscle, VIP-fibers ran in the same direction of smooth muscle fibers and distributed uniformly and densely within the smooth muscle bundles. *LM*, longitudinal muscle layer. ×180



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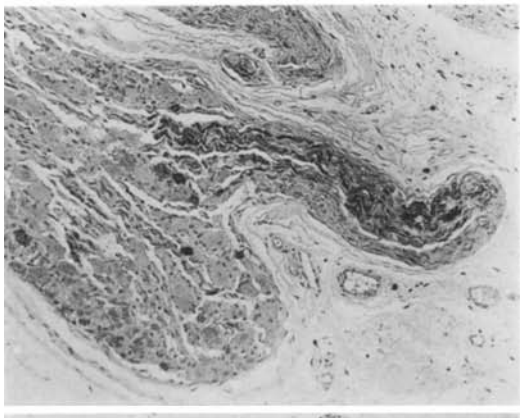

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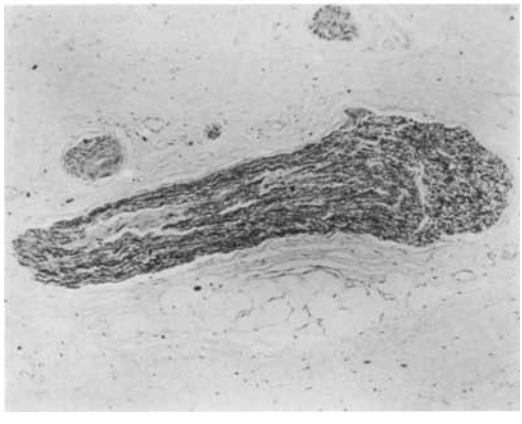


3b

Fig. 2. Control colon (Mucosa). VIP-fibers were seen as single strands or varicose forms, partly forming networks, in the lamina propria mucosae, and ran in the same direction of smooth muscle fibers in the muscularis mucosae (*mm*). ×200

Fig. 3a, b. Controls (Mesenterium and Anorectal portion). **a** VIP-containing nerve cells, forming a plexus, and VIP-containing nerve bundles which seemed to originate from these cells in the mesenterium. ×100. **b** The nerve bundles in the adjacent soft tissue of the rectum showed positive VIP immunoreactivities. ×110

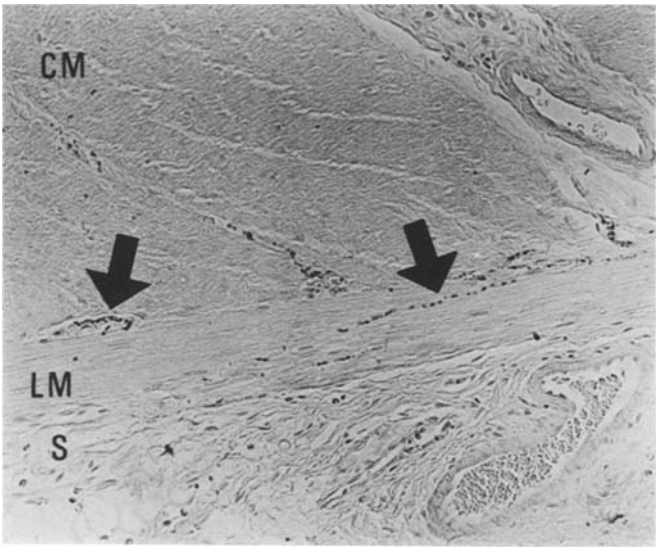


Fig. 4. AGS-1 (Proper muscle). Few VIP-containing slender fibers (*arrow*) were present in the intermuscular space and in the proper muscle. *S*, serosa. ×180

by thorough rinsing in 75% ethyl alcohol for 24 h. The specimens were embedded in paraffin wax and 3 μm thick sections were prepared for histological examination and immunohistochemical study. For histological study, sections were stained with Haematoxilin-Eosin and Cresyl violet.

For the immunohistochemical studies, the enzyme-labeled antibody method was used; PAP method (Immunoperoxidase staining, Sternberger 1974) and ABC method (Avidin-biotin-peroxidase complex method, Guesdon et al. 1979, Hsu et al. 1981). VIP antisera were anti synthetic porcine VIP rabbit sera (R-502; Yanaihara N et al. 1977). The deparaffinized sections were immersed in 0.03% (v/v) hydrogen peroxide in 0.01 M PBS (phosphate buffered saline) for 30 min at room temperature to eliminate the endogenous peroxidase activity. Possible background staining was also removed by the application of normal goat serum, diluted 1:10, for 30 min at room temperature. VIP antisera were applied to the sections at 1:1,000 dilution and the preparations incubated at room temperature in a moist chamber for 24 h. Both the second and the third layers were incubated for every one hour at room temperature. Visualization of the peroxidase was achieved by the diaminobenzidine method. The sections were then stained with Methyl green and examined under a transmitted light microscope. Non-immune rabbit serum was used, as controls instead of VIP antisera.

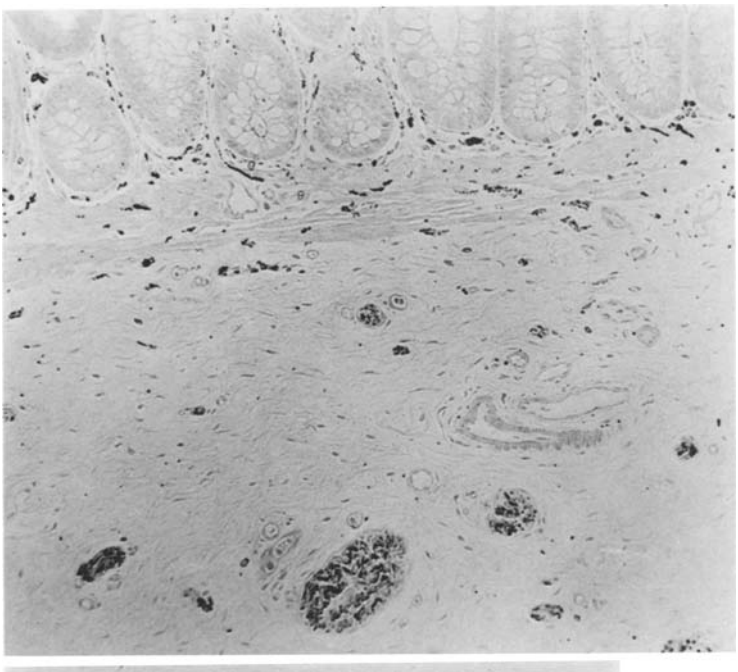
Radioimmunoassay

The tissues were rapidly frozen in n-hexane cooled by acetone-dry ice and were kept frozen at −70° C. Samples were extracted by the method of boiling water-0.1 M acetic acid (Yanaihara C et al. 1976). VIP-like immunoreactivities were assayed by the radioimmunoassay using antisera R-502 (Yanaihara N et al. 1977). S-P like immunoreactivities were assayed by the specific radioimmunoassay using antisera R-400 (Yanaihara C et al. 1976).

Results

1. Histological findings

In cases of Hirschsprung's disease the aganglionic segments of the bowel were most apparent. In one case (case No. 4) an aganglionic segment was



a

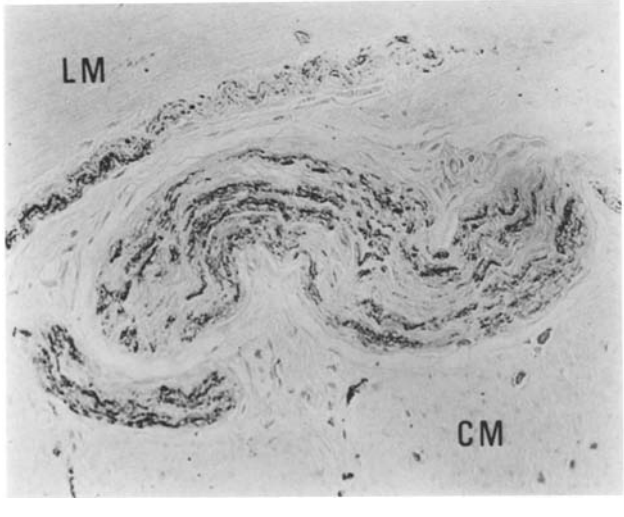


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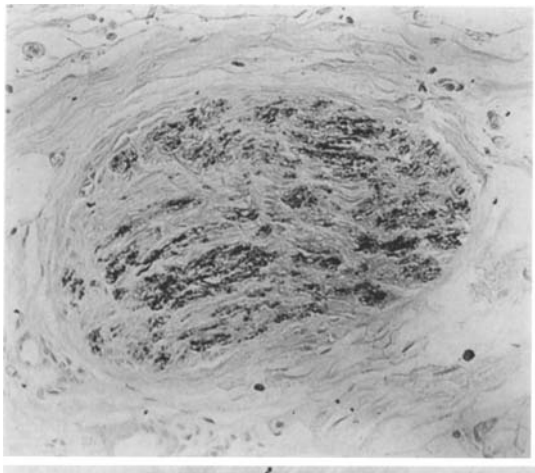
Fig. 5a–d. AGS-2. **a** *Mucosa and submucosa.* Several VIP-containing hypertrophic nerve bundles were noted in the submucosa. The distribution of VIP-fibers in the mucosa were same as that of controls. ×160. **b** *Intermuscular space and* **c** *Serosa;* Nonmyelinated hypertrophic nerve bundles showed positive VIP immunoreactivities. ×220, ×220. **d** *Proper muscle.* A few VIP-containing fibers were present between but not within the smooth muscle bundles (*arrow*). VIP-containing hypertrophic nerve bundles were also noted in the intermuscular space. ×220



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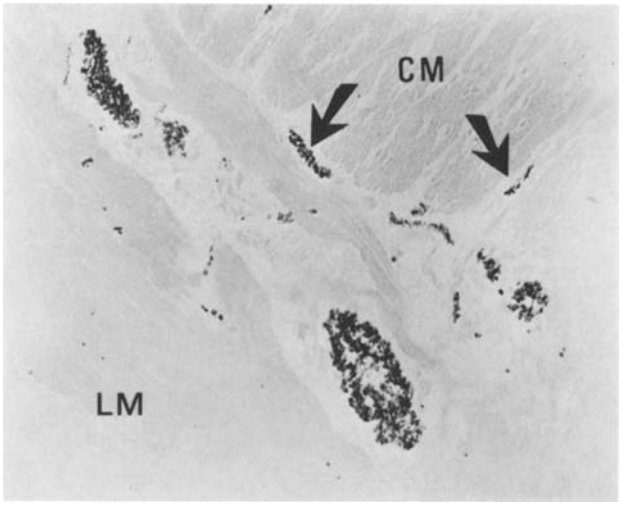

d

not included in the resected specimen and an oligoganglionic segment was observed in the distal site. In cases No. 8, 9, 10 and 12, oligoganglionic segments were very short. All specimens included normoganglionic segments (NGS).

Hypertrophic nerve bundles composed of nonmyelinated fibers were present in the aganglionic segments. The bundles ran in the subserosa and penetrated to both the intermuscular space and the submucosa through the proper muscle. These findings support the suggestion that these bundles have extrinsic origins. We devided aganglionic segments into two types; one included aganglionic segments without hypertrophic nerve bundles (AGS-1) and the other aganglionic segments with hypertrophic nerve bundles (AGS-2).

2. Immunohistochemical findings

a) Controls. VIP-immunoreactive ganglion cells were present in both Auerbach's and Meissner's plexuses. The number of VIP-containing cells were more extensive in the latter (Fig. 1a) than the former (Fig. 1b).

VIP-containing nerve fibers were observed throughout the whole thickness of the bowel and most fibers were thin and varicose. In the mucosa the fibers were found in the lamina propria, especially around the crypts, sometimes formed fine networks (Fig. 2). In the muscularis mucosae, VIP-fibers ran in the same direction of smooth muscle fibers. In the submucosa, most of VIP-fibers were present within Meissner's plexuses and a few fibers in the form of single strands. In the proper muscle, VIP-fibers ran in the same direction of smooth muscle fibers and the distribution was uniform (Fig. 1b). The density of VIP-fibers was more extensive in the circular muscle layer than in the longitudinal layer. Abundant VIP-fibers were seen in Auerbach's plexuses, and formed dense networks (Fig. 1b).

In the mesenterium near the colon, VIP-containing nerve cells, forming a plexus together with VIP-containing nerve bundles were present in some cases (Fig. 3a). VIP-bundles were also noted in the subserosa in several cases.

In anorectal portions VIP-nerve bundles were prominent in the adjacent soft tissue around the rectum (Fig. 3b).

b) Normoganglionic segments in Hirschsprung's disease (NGS). The distribution of VIP-containing nerves was similar to that seen in controls.

c) Aganglionic segments without hypertrophic nerve bundles (AGS-1). Few VIP-fibers were present in the intermuscular space, the proper muscle and the mucosa. VIP-containing ganglion cells were absent in these segments (Fig. 4).

d) Aganglionic segments with hypertrophic nerve bundles (AGS-2). Both in the lamina propria and in the muscularis mucosae, the VIP-fibers were similar in distribution and density to those seen in cases of NGS. However, few VIP-fibers were present in the proper muscle. Hypertrophic nerve bundles, which represented to the extrinsic fibers mentioned above, showed markedly positive VIP-immunoreactivities in the submucosa (Fig. 5a), the intermuscular space (Fig. 5b) and the serosa (Fig. 5c). Some VIP-fibers were also present between but not within the smooth muscle bundles in the muscle coats (Fig. 5d). Thus the extrinsic VIP nerve bundles were not seen to innervate smooth muscle cells in the muscle coats.

The distribution of VIP-immunoreactive nerves of Hirschsprung's disease is summarized in Fig. 6.

3. Radioimmunoassay (Tables 1 and 2)

Immunoreactive VIP content in the bowel of Hirschsprung's disease was 44.5 ± 8.2 in aganglionic segments, 66.6 ± 11.1 in oligoganglionic segments

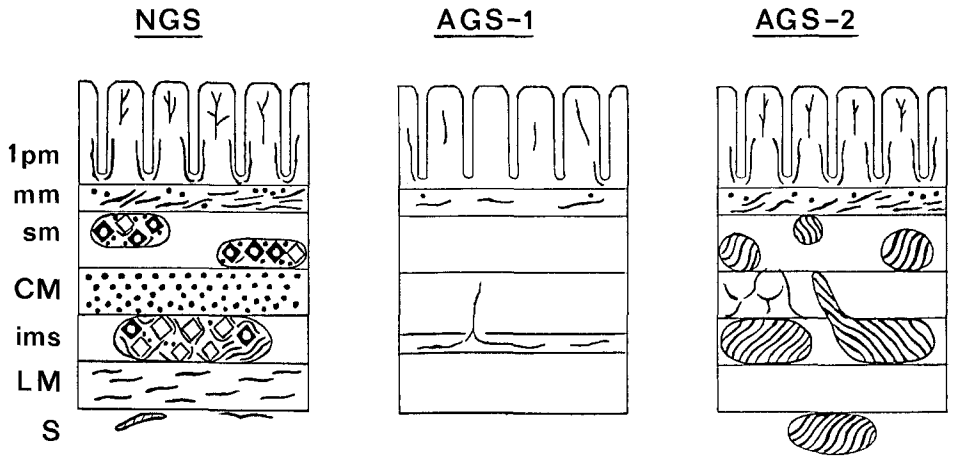

Fig. 6. The distribution of VIPergic nerves in Hirschsprung's disease. (◆ immunoreactive ganglion cell; ◇ non-immunoreactive ganglion cell; ∿ immunoreactive nerve fibers; ▭ immunoreactive nerve bundles.) *lpm;* lamina propria mucosae. *mm;* muscularis mucosae. *sm;* submucosa. *CM*, circular muscle layer. *ims;* intermuscular space. *LM;* longitudinal muscle layer. *S;* serosa. *NGS; VIP*-containing ganglion cells were present in both plexuses and VIP-fibers distributed in the proper muscle densely and uniformly. *AGS-1;* Few VIP-fibers were noted throughout all layer of the bowels. *AGS-2;* VIP-containing extrinsic nerve bundles penetrated to the intermuscular space and the submucosa through the proper muscle, and distributed in the mucosa and the muscularis mucosae. In the proper muscle, a few VIP-fibers were seen between but not within the smooth muscle bundles. The proper muscle was not seemed to be innervated by VIP-containing nerves. *NGS* = Normoganglionic segment; *AGS-1* = Aganglionic segment without hypertrophic nerve bundles; *AGS-2* = Aganglionic segment with hypertrophic nerve bundles

Table 1. VIP-content of the intestines was examined by the use of radioimmunoassay with R-502. VIP-content in aganglionic segments was significantly reduced, compared with those in oligoganglionic segments ($p < 0.05$) and normoganglionic segments ($p < 0.001$) (N.E. = not examined; S.E. = standard error; Unit = pg/mg wet tissue weight)

Case	Patient	Age	Sex	Type	Aganglia	Oligoganglia	Normoganglia
1.	I.K.	2 mo.	f	short	44.0	70.0	80.5
2.	A.K.	4 mo.	m	short	24.7	41.8	58.4
3.	Y.H.	4 mo.	m	short	65.7	79.2	148.5
4.	H.Y.	8 mo.	m	short	N.E.	94.6	118.0
5.	I.S.	1yr.	m	short	39.6	42.8	116.1
6.	U.K.	12 yr.	m	short	88.0	100.7	217.9
7.	I.T.	17 yr.	m	short	82.2	91.7	192.8
8.	Y.Y.	4 mo.	m	long	51.1	N.E.	170.8
9.	N.N.	9 mo.	f	long	43.3	N.E.	172.0
10.	I.H.	10 mo.	m	long	42.3	N.E.	180.0
11.	A.Y.	10 mo.	m	long	1.7	11.8	76.6
12.	S.T.	1 yr.	m	entire	7.2	N.E.	31.0
Mean ± S.E.					44.5 ± 8.2	66.6 ± 11.1	130 ± 17.1
					$p < 0.05$		$p < 0.02$
						$p < 0.001$	

Table 2. Substance P-content of the intestines was examined by the use of radioimmunoassay with R-400. S-P content in aganglionic segments was significantly decreased, compared with that in normoganglionic segments ($p<0.05$) (N.E. = not examined; N.S. = not significant; S.E. = standard error)

Case	Patient	Age	Sex	Type	Aganglia	Oligoganglia	Normoganglia
1.	I.K.	2 mo.	f	short	0.2	0.2	0.3
2.	A.K.	4 mo.	m	short	0.3	0.3	1.3
3.	Y.H.	4 mo.	m	short	0.2	0.2	0.9
4.	H.Y.	8 mo.	m	short	N.E.	12.1	25.7
5.	I.S.	1 yr.	m	short	0.0	0.1	1.4
6.	U.K.	12 yr.	m	short	0.1	1.2	15.8
7.	I.T.	17 yr.	m	short	0.5	0.5	9.4
8.	Y.Y.	4 mo.	m	long	0.8	N.E.	5.2
9.	N.N.	9 mo.	f	long	0.0	N.E.	2.3
10.	I.H.	10 mo.	m	long	2.2	N.E.	12.3
11.	A.Y.	10 mo.	m	long	0.3	0.7	1.5
12.	S.T.	1 yr.	m	entire	0.0	N.E.	0.5
Mean ± S.E.					0.42 ± 0.18	1.93 ± 1.47	6.38 ± 2.31
					N.S.	N.S.	
					$p<0.05$		

and 130 ± 17.1 pg/mg wet tissue weight in normoganglionic segments. The VIP content was significantly reduced in aganglionic segments compared with that in oligoganglionic segments ($p<0.05$) and that in normoganglionic segments ($p<0.001$).

Immunoreactive S-P content was 0.42 ± 0.18 in aganglionic segments, 1.93 ± 1.47 in oligoganglionic segments and 6.38 ± 2.31 pg/mg wet tissue weight in normoganglionic segments. The S-P content was also decreased in aganglionic segments, as compared with that in normoganglionic segments ($p<0.05$).

Discussion

The presence of non-adrenergic inhibitory neurons has been postulated in order to explain many features of gut physiology (Crema et al. 1968). In the aganglionic segment in patients with Hirschsprung's disease, there is a lack or failure of nerve mediated non-adrenergic relaxation (Frigo et al. 1973). Recently Bryant et al. (1982) reported that VIP only was initially present in the myenteric plexus at the 12 week of gestation having looked for 8 kinds of gut peptides. VIP is considered to be a neurotransmitter belonging to the non-adrenergic inhibitory system, so-called VIPergic system (Polak and Bloom 1980), and which contributes to the inhibitory components of peristaltic activity (Furness et al. 1980).

Bishop et al. (1981) and Tsuto et al. (1982) noted a decrease of VIP-containing fibers in aganglionic segments in Hirschsprung's disease. Bishop et al. (1981) also suggested that depletion of VIP-containing nerve fibers would be caused by the absence of ganglion cells, and therefore, by immuno-

histochemistry supported the suggestion that VIP-containing nerves had an intrinsic origin (Schultzberg et al. 1978; Jessen et al. 1980; Malmfors et al. 1980).

The distribution of VIP-immunoreactive fibers in AGS-1 was similar to that in the previous study reported by Bishop et al. (1981), at that time we agreed that intramural VIP-fibers were of intrinsic origin. However, we demonstrated that the hypertrophic nerve bundles in AGS-2 show markedly positive VIP-immunoreactivities, in spite of the absence of intramural ganglion cells. These findings suggest that the VIP-containing nerve bundles have an extrinsic origin, and might originate from the mesenteric ganglion cells or the sacral plexuses, then penetrating the muscle coats and distribute in the mucosa in AGS-2.

The vagal trunks possess peptide-containing nerve fibers (Lundberg et al. 1978). In our study, VIP-containing nerve bundles were noted in the soft tissue in the anorectal area of tissues from the controls, and we also demonstrated VIP-containing nerve cells and bundles in the mesentery near the colon in controls. These bundles are considered to be the putative origins of intramural VIP-containing fibers. Thus the VIP-containing fibers in the intestines might be of extrinsic and intrinsic origin.

In AGS-2 the extrinsic VIP-containing fibers ran in the subserosa and penetrated the muscle coats or were dispersed between the smooth muscle bundles, but did not reach the individual smooth muscle cells. In AGS-1, VIP-containing fibers were certainly decreased in number throughout the entire thickness of the gut wall. Thus very few VIPergic neurons are considered to innervate the smooth muscle cells in the proper muscle of both aganglionic segments.

The VIP content in the aganglionic segment was decreased, as recently reported by other workers (Freund et al. 1979; Dupont et al. 1980; Bishop et al. 1981). We found that the content of VIP was gradually decreased in proportion to the number of ganglion cells. The findings obtained by radioimmunoassay were consistent with those of the immunohistochemical examination of VIP neurons.

S-P acts as a regulatory peptide in the gastrointestinal tract in that it functions as an excitatory neurotransmitter in the peptidergic nerve system related to peristaltic movements. The decrease of S-P content in the aganglionic segments, clearly demonstrated in our study using radioimmunoassay, may relate to the non-propulsive state in the aganglionic segment.

The failure of this peptidergic innervation seems to be a significant factor in the non-peristaltic state in Hirschsprung's disease.

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