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Immunohistochemical study of neuron specific enolase and S-100 protein in Hirschsprung's disease

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Summary. The distribution of whole differentiated neurons in the intestines from 15 children with Hirschsprung's disease was investigated using neuron specific enolase (NSE) and the perineuronal elements were studied using S-100 protein immunostaining.

In aganglionic segments, NSE immunoreactive ganglion cells and S-100 positive satellite cells were absent, but the hypertrophic nerve trunks did show a markedly positive NSE and S-100 immunoreactivity.

Two different forms of aganglionic segment were present. One was the middle aganglionic segment of long segment aganglionosis which was almost completely dennervated. In the other type, there were several NSE positive nerve fibers in the muscularis propria of both the aganglionic segment of short segment aganglionosis and the distal aganglionic segment of long segment aganglionosis. These latter two aganglionic segments seemed to be innervated by extrinsic nerves.

Key words: Hirschsprung's disease – Neuron specific enolase – S-100 protein

Introduction

The pathophysiological mechanisms related to the narrow segment in children with Hirschsprung's disease has not been clearly defined. Hypotheses concerning the innervated form of aganglionic intestines include the denervation theory (Ehrenpreis 1966), the cholinergic nerve hyperplasia theory (Kamijo et al. 1953; Meier-Ruge 1968), the adrenergic nerve hyperactivity theory (Touloukian et al. 1973), deficiency in the nonadrenergic inhibitory system (Frigo et al. 1973), and deficiency of the peptidergic nerve system (Bishop et al. 1981; Taguchi et al. 1983).

Neuron specific enolase (NSE), a highly acidic soluble protein, was first discovered in brain extracts (Moore 1972). It is a dimetric protein consisting

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of two identical subunits (γ, γ) with a total molecular weight of 78,000 daltons (Marangos et al. 1975 and 1978). Recent immunohistochemical studies localized NSE in neurons, as well as in APUD (amine precursor uptake and decarboxylation) cells (Schmechel et al. 1978). Bishop et al. (1982) demonstrated that NSE was a common specific marker for all differentiated nerves in the alimentary tract.

S-100 protein was first described by Moore (1965) as a protein specific for nervous tissue. This protein has been detected in Schwann cells surrounding both myelinated and nonmyelinated nerve fibers, and in satellite cells around ganglion cells. It was also shown that neither axons nor ganglion cells contain this protein (Nakajima et al. 1982). It is now thought that S-100 protein is a marker of perineuronal elements of the nervous system.

In the present work, we have demonstrated the localization and the distribution of total neurons in the intestines in patients with Hirschsprung's disease, using NSE antiserum as a specific marker. Attention was also given to the distribution of S-100 protein in the same intestines.

Materials and methods

Twenty Japanese children aged 2 months to 17 years were diagnosed as having Hirschsprung's disease, following barium enema, manometric study, and histochemical study using acetylcholinesterase staining of suction biopsied materials (Karnovsky and Roots 1964; Meier-Ruge et al. 1972). The intestines were resected using the Z-shaped anastomosis procedure (Ikeda 1967).

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

The *material* was fixed in Zamboni's solution (1967) for 24 h at 4° C. Fixation was followed by thorough rinsing in 75% ethyl alcohol for 24 h, then dehydration in alcohol. The specimens were embedded in paraffin and 4 μm thick sections were prepared for immunohistochemistry and routine haematoxylin and eosin staining.

For the *immunohistochemical study*, an enzyme-labeled antibody techniques; the ABC (Avidin Biotin Peroxidase Complex) method was used (Guesdon et al. 1979; Hsu et al. 1981).

Antisera used were anti-native bovine brain NSE rabbit serum and anti-cow S-100 protein rabbit serum (Dakopatts, Denmark). The deparaffinized sections were immersed in methanol for 30 min and then in 0.03% (v/v) hydrogen peroxide in 0.01 M PBS (phosphate buffered saline, pH 7.4) for 30 min at room temperature. NSE antisera were applied to the sections at 1:500 dilution and the preparations incubated at room temperature in a moist chamber for about 18 h. S-100 antisera were used at a dilution of 1:200. The diluted biotinylated antirabbit IgG goat sera and the diluted avidin-biotinylated peroxidase were each incubated for 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 sera were used instead of NSE or S-100 antisera for the negative control study.

Results

Histology

Aganglionosis in the resected portion of the colon was confirmed histologically. All but two cases included aganglionic segments, because of the opera-

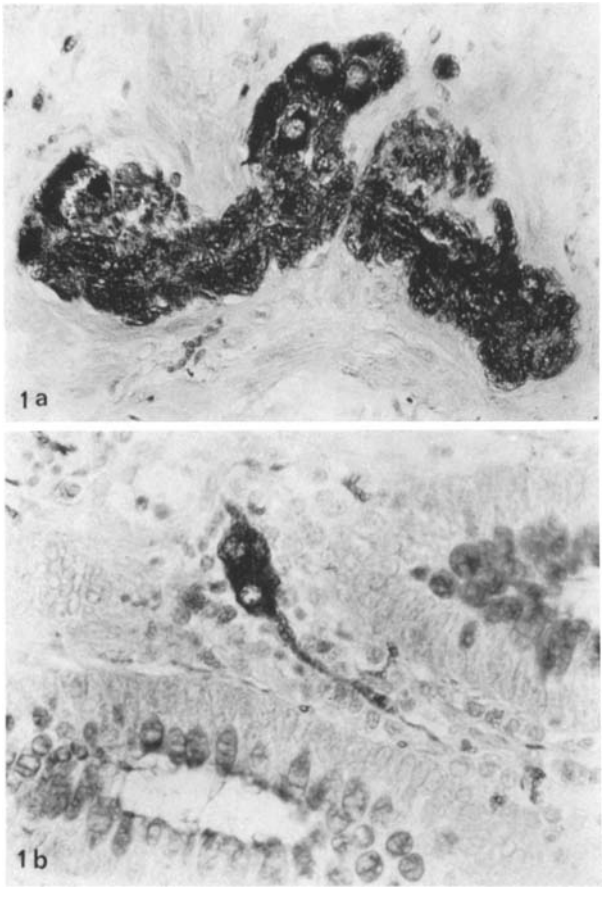


Fig. 1a, b. NSE immunostaining of ganglion cells in Normoganglionic segment. **a** Myenteric plexus (×320): Several immunoreactive ganglion cells are present without nuclear staining. Nerve endings around ganglion cells and nerve fibers also show positive immunoreactivity. **b** NSE immunoreactive ganglion cells in the lamina propria mucosae (×380): Two immunoreactive ganglion cells with nerve fibers are present around the crypt of the lamina propria mucosae

tion procedure (Ikeda 1967). Oligoganglionic segments were included in the distal site of the resected colons in these two cases.

Histological findings revealed that 10 cases were short segment aganglionosis (SSA) and 5 were long segment aganglionosis (LSA).

Immunohistochemistry

A. Normoganglionic segment (NGS) and control colon

1. Neuron specific enolase (NSE). The distribution of NSE immunoreactivities in NGS was same as in control colons.

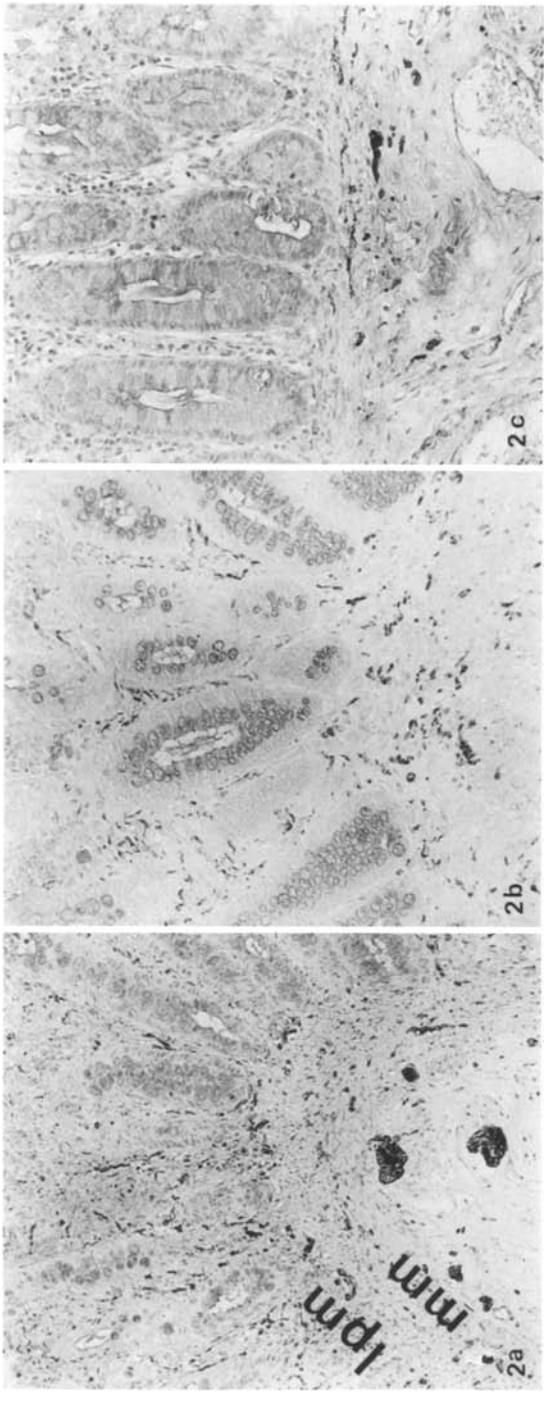


Fig. 2a–c. NSE immunostaining of the mucosa and the submucosa. (*mm*: muscularis mucosae, *lpm*: lamina propria mucosae) **a** Normoganglionic segment (×116): The submucosal plexuses show markedly positive immunoreactivity. NSE positive nerve fibers run parallel to the smooth muscle cells in the muscularis mucosae, and in the lamina propria they are present around the crypts and some extend from the muscularis mucosae. **b** Aganglionic segment of short segment aganglionosis (×150): No immunoreactive ganglion cells are seen in the submucosa. The distribution of mucosal immunoreactive nerve fibers is similar to that in NGS. **c** Middle aganglionic segment of long segment aganglionosis (×116): A few immunoreactive nerve fibers are seen in the muscularis mucosae. NSE fibers in the lamina propria are absent

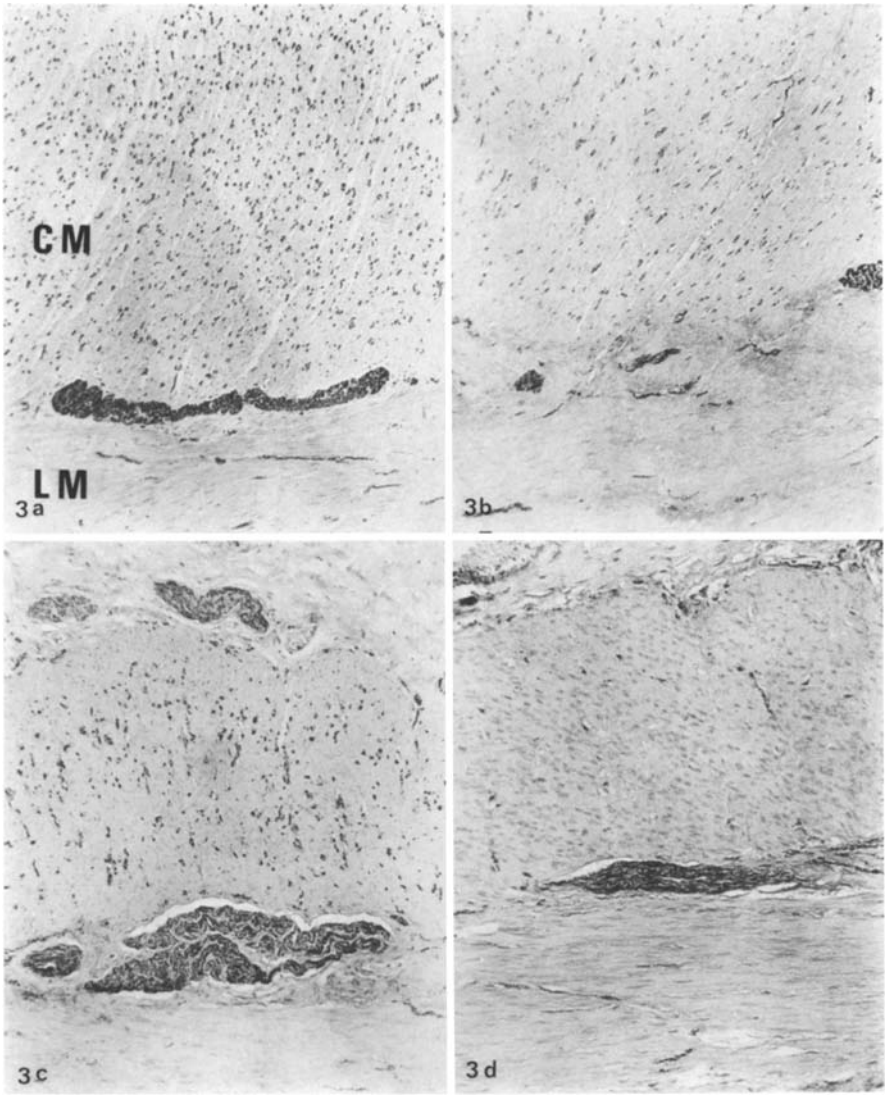


Fig. 3a–d. NSE immunostaining in the muscularis propria (*CM* circular muscle layer; *LM* longitudinal muscle layer). **a** Normoganglionic segment (×90): Fine NSE-positive fibers are densely and uniformly distributed in *CM*. In LM their number is fewer than that in *CM*. **b** Oligoganglionic segment (×90): Immunoreactive nerve fibers are present in uniform distribution. Compared with normoganglionic segment, they are decreased in number. **c** Distal aganglionic segment of long segment aganglionosis (×130): Hypertrophic nerve trunks in the intermuscular zone show positive immunoreactivity. Several NSE fibers are present between and in the smooth muscle bundles. **d** Middle aganglionic segment of long segment aganglionosis (×130): Hypertrophic trunks show NSE immunoreactivity. Very few NSE fibers are noted in the muscularis propria and are mostly found between the smooth muscle bundles

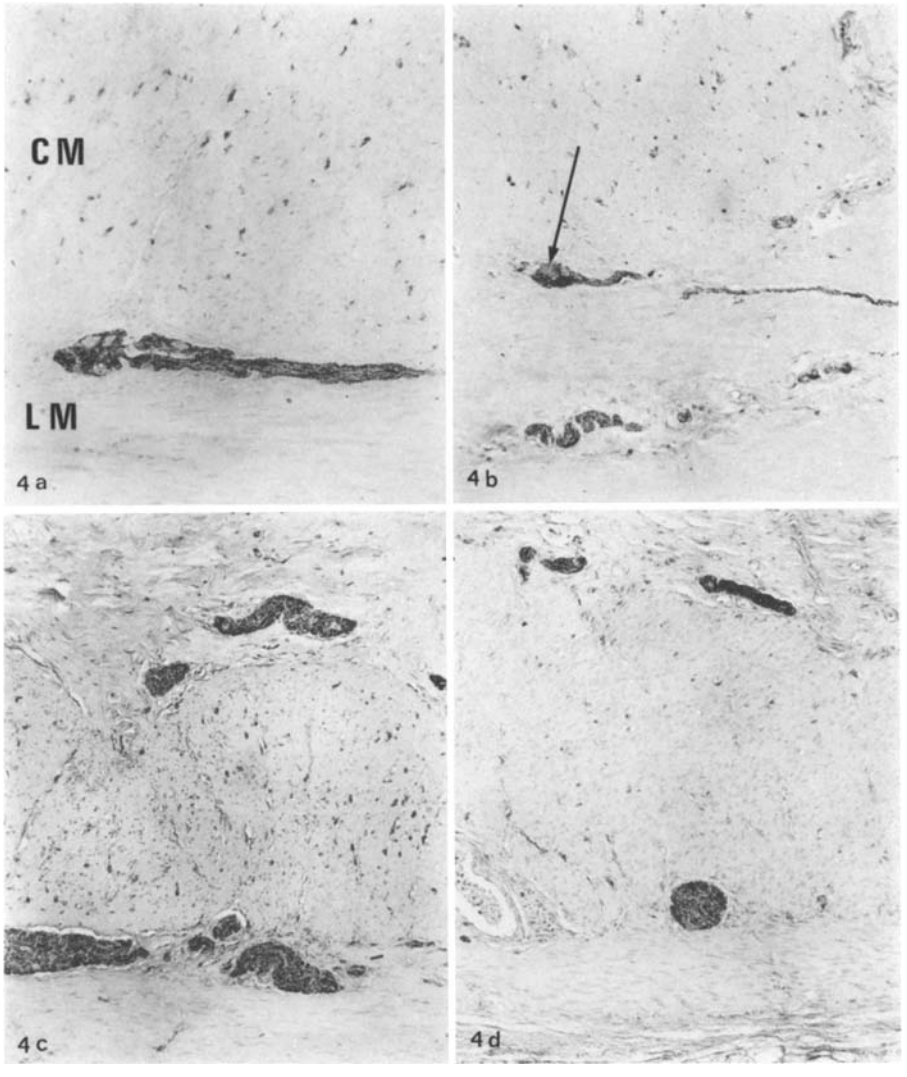
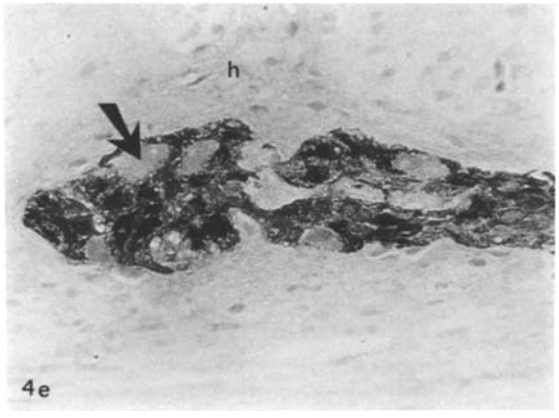


Fig. 4a–e. S-100 protein in the muscularis propria. (*CM* circular muscle layer; *LM* longitudinal muscle layer) **a** Normoganglionic segment (×120): Immunoreactive nerve sheaths and Schwann cells are distributed uniformly in the muscularis propria. S-100 immunoreactivities are more prominent in CM than LM. In Auerbach's plexus, S-100 immunoreactivities are demonstrated around S-100 negative ganglion cells (Fig. 4e). **b** Oligoganglionic segment (×120): The number of S-100 negative ganglion cells (*arrow*) is smaller than in normoganglionic segment. S-100 immunoreactivities in the muscularis propria are slightly decreased in number. **c** Distal aganglionic segment of Long segment aganglionosis (×120): Hypertrophic nerve trunks in both the submucosa and the intermuscular zone show marked positive immunoreactivities. S-100 immunoreactivities are also seen in the muscularis propria. **d** Middle aganglionic segment of long segment aganglionosis (×116): There are a few hypertrophic nerve trunks which show S-100 immunoreactivities. None or very little S-100 immunoreactivity is seen in the muscularis propria. **e** Auerbach's plexus in normoganglionic segment (×390): High magnification of Fig. 4a. S-100 immunoreactivities are observed around S-100 negative ganglion cells (*arrow*)



Each ganglion cell showed a cytoplasmic NSE-positive reaction product in both the submucosal and myenteric plexuses (Fig. 1a). Immunoreactive ganglion cells were also demonstrated in the lamina propria mucosae (Fig. 1b). Some variation in the staining intensity was noted among the immunoreactive ganglion cells. NSE positive nerve endings and fibers were present around ganglion cells (Fig. 1a).

The immunoreactive nerve fibers present in all layers of the colon were slender or varicose, and formed a fine network. In the lamina propria mucosae, they were seen mostly around the crypts and seemed to extend from the muscularis mucosae (Fig. 2a). In the muscularis mucosae, they were in parallel with the smooth muscle fibers (Fig. 2a). In the submucosa, some slender fibers were in a single form, and a few nerve trunks were also present. Fine NSE-positive fibers were densely and uniformly distributed in the circular muscle layer and ran in parallel with the smooth muscle cells. They seemed to be distributed between the individual smooth muscle cells (Fig. 3a). In the longitudinal muscle layer, the number was less than in the circular muscle layer (Fig. 3a). Some slender fibers were also noted around blood vessels and were found mainly in the medioadventitial border of arteries in the submucosa and the subserosa.

2. S-100 protein (S-100). S-100 containing nerve sheaths and Schwann cells were present in the muscularis propria (Fig. 4a). Their distribution was similar to that of NSE. However, they were fewer than those in the NSE positive nerve fibers. S-100 immunoreactivity was not seen in the mucosa. S-100 immunoreactive satellite cells were demonstrated around the S-100 negative ganglion cells, in both Meissner's and Auerbach's plexuses (Fig. 4e).

B. Oligoganglionic segment (OGS)

1. NSE. Immunoreactive ganglion cells were decreased in number. NSE containing hypertrophic nerve trunks were present in the submucosa, the intermuscular zone, and the subserosa. Fine immunoreactive fibers were

also present in the muscularis propria, in a uniform distribution (Fig. 3b). However, their number was fewer than that in NGS. The distribution of NSE containing nerves in the mucosa was similar to that in NGS.

2. S-100. S-100 immunoreactivities were slightly decreased in number in the muscularis propria, compared with those in NGS. The number of S-100 negative ganglion cells was smaller than in NGS (Fig. 4b).

C. Aganglionic segment (AGS)

1. NSE. NSE positive ganglion cells were absent. The distribution of immunoreactive nerve fibers and trunks differed between the various forms of aganglionosis.

a. Short segment aganglionosis (SSA). Several immunoreactive hypertrophic nerve trunks and a few slender nerve fibers were present in the submucosa, the intermuscular zone, and the subserosa. Some of the nerve trunks seemed to penetrate into the muscularis propria, where various sized NSE positive fibers were present, mainly between the bundles of smooth muscle cells. A few immunoreactive fibers were also present in the smooth muscle cell bundles. In the mucosa, the localization of NSE positive nerve fibers was similar to that seen in the NGS (Fig. 2b).

b. Long segment aganglionosis (LSA).
I. Distal portion. The distribution of NSE containing nerve trunks and fibers was the same as that of SSA (Fig. 3c).

II. Middle portion. A few immunoreactive hypertrophic nerve trunks and varicose nerve fibers were present in the intermuscular zone and in the submucosa (Fig. 3d), however their number was fewer than in the distal AGS.

None or very few NSE containing fibers were noted in the muscularis propria, and when present, were distributed sparsely between the muscle bundles (Fig. 3d). In the muscularis mucosae, NSE fibers were decreased. No immunoreactive nerve fibers were seen in the lamina propria mucosae (Fig. 2c).

III. Proximal portion. In one out of five cases, the localization of NSE fibers and trunks was similar to that in the distal portion, while, in the other four cases, it was the same as that in the middle portion.

2. S-100. Hypertrophic nerve trunks showed a marked positive S-100 protein immunoreactivity (Fig. 4c and d). Their number was fewer in the middle AGS than in the proximal AGS of LGS and the whole AGS of SSA.

S-100 positive nerve sheaths and Schwann cells were noted in the muscularis propria of AGS of SSA and the distal AGS of LSA (Fig. 4c). None or very little S-100 immunoreactivity was noted in the muscularis propria of the middle AGS of LSA (Fig. 4d).

Discussion

Ehrenpreis (1966) proposed that a state of denervation induced spasticity of smooth muscles in AGS. Meier-Ruge (1968) found that the hypertrophic nerve trunks showed a highly positive acetylcholinesterase activity in the intermuscular zone and a marked proliferation of thick acetylcholinesterase positive fibers in the muscularis propria in AGS. Garret et al. (1969) reported the proliferation of catecholamine fluorescent nerves in the muscularis propria of aganglionic bowels in 9 of 13 cases. We have previously shown that few VIP containing nerve fibers were distributed among the smooth muscle bundles in the muscularis propria, while the extrinsic hypertrophic nerve trunks showed highly positive VIP immunoreactivity, in aganglionic bowels (Taguchi et al. 1983). Thus, we proposed that peptidergic innervation was deficient in intestines of aganglionosis.

Until recently, acetylcholinesterase staining has been used to demonstrate cholinergic nerves. Bishop et al. (1982) showed that this is not a specific marker for cholinergic nerves, nor it is a common marker for whole neurons. They showed that NSE immunoreactivity was present in a larger proportion of nerve fibers and ganglion cells when compared with acetylcholinesterase. They proposed that NSE is a better common marker of the enteric nervous system.

This study, using NSE immunostaining, showed that the distribution of enteric nerves is different in the two forms of aganglionic segments. One form is seen in the middle AGS in LSA. In this segment, none or very few nerve fibers were present in the muscularis propria. This finding supports the denervation theory (Ehrenpreis 1966) only in LSA, as seen morphologically. In the other form of AGS, several NSE containing nerve fibers were present in the muscularis propria in the distal AGS of LSA and the whole AGS of SSA. Although fewer in number than those of NGS, these AGS seemed to be innervated by extrinsic nerves. The denervation theory seems to be inappropriate in these aganglionic intestines. These findings are consistent with the electro-physiological studies by our colleagues (Kubota et al. 1983).

A uniform and dense distribution of fine NSE positive nerve fibers was observed in the muscularis propria of NGS, but it was not seen in AGS. These uniformly distributed nerve fibers were probably of an intrinsic origin and were therefore derived from intramural ganglion cells. However, both forms of AGS showed a lack of regular and dense innervation in the muscularis propria, and this fact seems to be related to the nonperistaltic state.

The distribution of S-100 protein in aganglionosis was also investigated in this study. Hypertrophic nerve trunks showed marked positive S-100

immunoreactivity and thus we think that this protein can be used as one of the markers of hypertrophic nerve trunks in Hirschsprung's disease.

It is interesting that no S-100 immunoreactivity was observed in the mucosa of normal colons. This probably means that the nerve fibers have neither Schwann cells nor sheaths in the mucosa of intestines.

The distribution of NSE and S-100 was different in that NSE was present diffusely in nerve cell bodies and nerve fibers; while S-100 was contained in perineural elements, for example, satellite cells, Schwann cells, and nerve sheaths.

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