

An Immunohistochemical Study of Serotonergic Nerves in the Colon and Rectum of Children with Hirschsprung's Disease

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Summary. Serotonin (5-HT)-like immunoreactive nerve fibers were investigated in gut tissue obtained from seven Japanese children with Hirschsprung's disease. In the control untreated tissues, 5-HT-like immunoreactive fibers were observed neither in the normoganglionic nor in the aganglionic regions. After pargyline treatment, 5-HT-positive neuropils were consistently detected in association with the myenteric plexus in the normoganglionic segment, while in the aganglionic segment immunoreactive fibers could not be demonstrated through the entire layer of the bowel tissue. The occurrence of 5-HT-like immunoreactive neuropils by pargyline treatment strongly suggests that the infant bowel is innervated with serotonergic elements. After treatment with 5-hydroxy-L-tryptophan (5-HTP), the immunoreactive neuropils were localized in the myenteric and submucous plexuses of the normoganglionic segment. On the other hand, another type of 5-HT-positive fibers was characteristically demonstrated in aganglionic segments following the drug treatment; moderate numbers of 5-HT-like immunoreactive fibers appeared in the intermuscular zone and within the circular and longitudinal muscle layers. Several 5-HT-positive fibers were present in the hypertrophic nerve bundles seen in a diseased bowel. A possible origin of this type of 5-HT-positive nerve fibers was discussed.

Key words: Serotonin – Immunohistochemistry – Hirschsprung's disease

Introduction

Hirschsprung's disease is characterized by a maintained contraction of the aganglionic segment.

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Normal peristaltic movement is lacking in the constricted aganglionic segment. It has been reported that the absence of non-cholinergic, non-adrenergic inhibitory nerves leads to a contraction of the gut segment in humans (Frigo et al. 1973) and murine models (Richardson 1975) of Hirschsprung's disease. Gonella (1981) proposed that the enteric serotonergic neurons may be intramural interneurons, acting on non-cholinergic, non-adrenergic inhibitory nerves. Immunohistochemical studies suggested that neurons containing 5-HT are located in the enteric nervous system of mammals, including man (Gershon et al. 1977; Costa et al. 1982; Griffith and Burnstock 1983; Kurian et al. 1983). Most histochemical studies, however, have failed to reveal 5-HT-containing nerves in the normal enteric nervous system. Pretreatment with precursors or analogues of 5-HT was considered necessary to visualize the enteric 5-HT-containing nerves. We used highly sensitive immunohistochemical procedures involving avidin-biotin interaction for localizing serotonin antigen in cryostat sections. We now report the distribution of serotonin-like immunoreactive nerves in the normoganglionic and aganglionic segments of the gut resected from infants with Hirschsprung's disease.

Materials and Methods

The specimens were obtained from the resected bowels of seven Japanese children with Hirschsprung's disease aged 3 months to 9 years. All seven were cases of recto-sigmoid aganglionosis. The patients underwent recto-sigmoidectomy according to a modification of Duhamel's method (Ikeda 1967). The bowels were excised within 60–150 min after ligation of the branches from the inferior mesenteric artery. Immediately after resection, the intestine was cut open lengthwise along the mesenteric border and cut into small pieces (about 0.5 × 0.5 cm). The tissues were incubated in non-oxygenated phosphate-buffered saline (0.9% NaCl in 0.01 M phosphate buffer, pH 7.3) containing pargyline, an inhibitor of monoamine oxidase, or 5-hydroxy-L-tryptophan (5-HTP), a precursor of 5-HT, for 60 min at room temperature.

Pargyline or 5-HTP was added to the incubation medium to make a final concentration of 10^{-3} M and 10^{-4} M or 5×10^{-5} M, respectively. The drug-treated and untreated tissues were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) for 1–2 days at 4°C. After three brief washes in 80% ethanol, the tissue blocks were rinsed with the same phosphate buffer containing 7% sucrose overnight, then frozen in melting isopentane cooled with solid carbon dioxide. Sections were cut on a cryostat at 10 μ m, collected on glass slides coated with chrome-alum gelatin. After being left to dry for 60 min at room temperature, these sections were processed using the immunoperoxidase technique of avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA) (Hsu et al. 1981). The immunogen for serotonin was prepared by coupling 5-hydroxy-tryptamine creatinine sulfate to rabbit serum albumin, as described by Grotta and Brown (1974). The anti-serotonin antiserum was produced in rabbits. We have already reported the cross-reactivity of the antiserum and control experiments (Nada et al. 1984). The antiserum was used at a dilution of 1:4,000 in the phosphate-buffered saline (PBS), pH 7.3.

Results

In the untreated gut tissue, 5-HT-like immunoreactive nerves were not to be evidenced in either the normoganglionic or the aganglionic segments.

Pargyline-treated Bowels

The simplified incubation procedure made feasible visualization of the 5-HT-like nerve fibers. The 5-HT-like immunoreaction was found in preparation from normoganglionic regions, when small tissue blocks of bowels were preincubated with 10^{-4} M pargyline. The immunoreactive nerve fibers were consistently observed in the myenteric plexus, although the perikarya were not detected. The immunoreactive fibers were mostly short, varicose, and localized around the ganglion cells which were not immunostained. In addition, the 5-HT fibers were found only infrequently in the submucous plexus. In the submucosa, weakly immunostained nerve fibers were observed in the walls of blood vessels. These fibers were present in relatively large, but not in small vessels. In contrast, 5-HT-like immunoreactive fibers were rare in preparations from the aganglionic region. Only a few fibers with weak 5-HT-like immunoreactivity were encountered in association with submucous blood vessels. 5-HT fibers were never detected in the circular and longitudinal muscle layers of normoganglionic and aganglionic segments (Fig. 1).

5-HTP-treated Bowels

The treatment with 5-HTP intensified the immunoreactivity of nerve fibers, although the concomitant background staining was enhanced. In normoganglio-

nic segments, the immunoreactive fibers were localized in the myenteric and submucous plexus and in the submucous vessel walls. The distribution of 5-HT-positive nerve fibers showed a pattern similar to that in the pargyline-treated bowels. The short varicose fibers formed basket-shaped networks around the neuronal cell bodies of the myenteric plexuses (Fig. 2), while the ganglion cells were not stained.

In aganglionic bowels, 5-HTP treatment made feasible visualization of additional 5-HT-positive fibers not demonstrable by pargyline. The hypertrophic nerve bundles commonly occurred in the intermuscular zone and longitudinal muscle layer of the aganglionic bowels. These bundles contained several 5-HT-like immunoreactive fibers (Fig. 3), whereas the nerve bundles seen in the submucosa had no immunoreactive fibers. There were moderate numbers of 5-HT-like immunoreactive fibers within the circular and longitudinal muscle layers (Fig. 4). The 5-HT-positive fibers lay along the muscle bundle and usually followed a long, tortuous course and gave off a few branches.

The 5-HT-like immunoreaction could not be demonstrated in the three resected bowels exposed to poor circulation for more than 2 h during the operation, even when the tissues were incubated with pargyline or 5-HTP.

Discussion

It is difficult to demonstrate serotonergic nerves in the normal gut, since serotonin contents are very low in the neurons (Robinson and Gershon 1971). For this reason, the precursors and analogues of 5-HT or tritiated 5-HT have been used for histochemical visualization of serotonergic nerves in bowels from human (Rogawski et al. 1978; Kurian et al. 1983) and rodents (Costa et al. 1982). It has been suggested that loading of 5-HTP or 5-HT could demonstrate not only serotonergic nerves (5-HT handling nerves), but also non-5-HT-handling nerves (Furness and Costa 1978; Costa et al. 1982) and adrenergic nerves (Snipes et al. 1968; Robinson and Gershon 1971). Griffith and Burnstock (1983) provided direct evidence that serotonergic nerves are present in the ileum and colon of normal human fetuses. The use of highly sensitive immunoenzymatic techniques involving the avidin-biotin interaction led to a direct demonstration of 5-HT-like fibers in the human bowels. The present study confirmed 5-HT-like immunoreactivity in neuropils within the myenteric plexus of human infants, without the application of 5-HTP. However, we found no immunoreactive perikarya in the bowels under our experimental conditions. In aganglionic

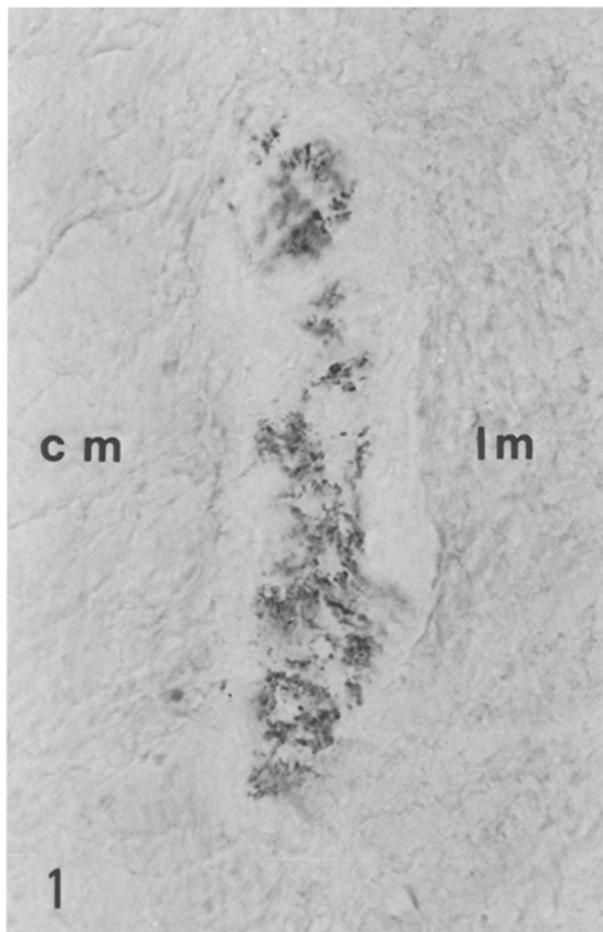


Fig. 1. Neuropils with 5-HT-like immunoreactivity are seen in association with the myenteric plexus of normoganglionic region following treatment with pargyline. $\times 200$

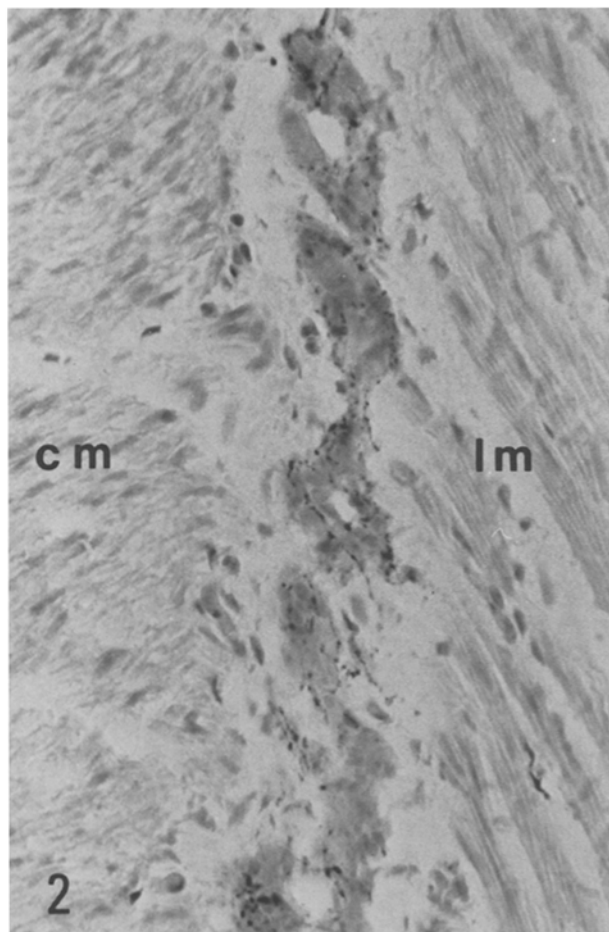


Fig. 2. 5-HT-like immunoreactive fibers form a basket-shaped network around the ganglion cells of the myenteric plexus in normoganglionic region treated with 5-HTP. The section is lightly counterstained with Mayer's hematoxylin. $\times 200$

segments, pretreatment with 5-HTP revealed additional 5-HT-positive nerve fibers in the circular and longitudinal muscle layers as well as in the hypertrophic nerve bundles. It is noteworthy that the muscular 5-HT-like fibers were found in the aganglionic segments, but not in the normoganglionic ones. Histochemical studies have shown that the myenteric serotonergic neurons of normoganglionic segments are small and may send processes to other elements within the ganglia (Dreyfus et al. 1977; Gershon et al. 1977). Therefore, these neurons have been referred to as the intrinsic elements in the myenteric plexus. An autoradiographic study showed that myenteric 5-HT-containing neurons are absent in the aganglionic segments in Hirschsprung's disease (Rogawski et al. 1978). This finding was supported by our present experiment. In addition, the muscular 5-HT-positive nerve fibers were observed in the diseased bowel following treatment with 5-HTP, as described

above. In contrast to the myenteric neuropils, the muscular 5-HT-like nerve fibers attained a great length and sometimes emitted branches or collaterals. We are not aware of other reports demonstrating this type of serotonin-positive fibers in the aganglionic segments. Griffith and Burnstock (1983) reported that in the human fetal bowel, nerve fibers with 5-HT-like immunoreactivity are present in the deep muscular plexus of the circular muscle, whereas the fibers are generally not found within the longitudinal muscle layer, and further, they showed that the plexus is made up of fine, single, unbranching, varicose fibers. It is also of interest to note that both the circular and longitudinal muscle layers of the aganglionic segment contained long, branching, varicose, 5-HT-positive fibers.

Richardson (1975) stated that only extrinsic autonomic nerve fibers are distributed in a chaotic fashion throughout the smooth muscle layers of aganglionic

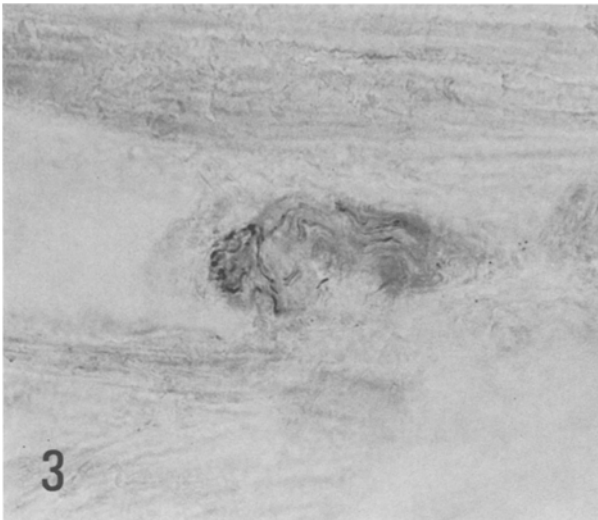


Fig. 3. 5-HT-like immunoreactive fibers are evident in the hypertrophic nerve bundle located in the longitudinal muscle layer of the aganglionic segment treated with 5-HTP. $\times 200$

Fig. 4. 5-HT-like immunoreactive fibers within the longitudinal muscle layer of the aganglionic region treated with 5-HTP. They are varicose and occasionally emit collaterals (*arrow*). $\times 200$

segments, while the intrinsic elements are absent (see Rogawski et al. 1978). Some enteric 5-HT nerves are considered to be of extrinsic origin (Griffith and Burnstock 1983). It seems likely that extrinsic 5-HT fibers are distributed throughout the muscle layers, in a manner similar to both the sympathetic and parasympathetic fibers in aganglionic segments in case of Hirschsprung's disease. There is the possibility that the appearance of immunoreactive fibers could have resulted from entry of 5-HTP into axons other than serotonergic elements. If such is the case, 5-HT-positive fibers should have been present in the corresponding portions of the normoganglionic segments; however, 5-HT-fibers were not observed in the muscle layers of the normoganglionic segments. Although no definite statement can be made as to the origin of the muscular 5-HT fibers, they may be a candidate for extrinsic fiber. This view is supported by the finding that 5-HT-like fibers are present in hypertrophic nerve bundles considered to be of extrinsic origin.

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