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The neuroendocrine system of normal human appendix, ileum and colon, and in neurogenic appendicopathy

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Summary. Epithelial endocrine cells containing 5-hydroxytryptamine, substance P, somatostatin, enteroglucagon and vasoactive intestinal polypeptide-immunoreactivity were localized by immunocytochemistry in the mucosa of normal appendices, ileum and proximal colon, and in neurogenic appendicopathy.

In neurogenic appendicopathy a large number of proliferating nerves were visualized independently of neurotransmitters by immunostaining for neuron-specific enolase. A large number of nerve fibers were shown to contain substance P-immunoreactivity and to be of intrinsic origin.

Stromal endocrine cells containing 5-hydroxytryptamine, somatostatin- and possibly substance P-immunoreactivity, were observed in substantial numbers in neurogenic appendicopathy. Substance P may be involved as a neurotransmitter and/or as a paracrine/endocrine peptide in the pathogenesis of spastic contractions and abnormal peristalsis of the appendix, which are characteristic of neurogenic appendicopathy. Stromal endocrine cells may be considered to be the origin of certain carcinoids in the appendix.

Key words: Neuron-specific enolase – Neuropeptides – Immunocytochemistry – Neurogenic appendicopathy – Microcarcinoids

Pierre Masson (1921 and 1932) used the term "appendicite neurogène" for a proliferation of nerve fibers resembling a neuroma in the appendix. He was the first to recognise the close relationship of proliferating nerves and argentaffin cells occurring in the stroma of the mucosa.

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On the basis of the proliferation and swelling of axons in the mucosa observed by electron microscopy by Auböck (1969) and Ratzenhofer et al. (1969), Höfler (1980) proposed the following classification of neurogenic appendicopathy[1] (for review see Höfler et al. 1982):

1) *mucosal neurogenic appendicopathy* is the most frequent variant occurring in approximately 10.4% of all excised appendices, 2) *central or axial neuroma* occurs after obliteration of the top of the appendix and results in a large central nerve bundle, surrounded in typical cases by the muscularis mucosae. In both forms the number of endocrine cells in the mucosal stroma is increased when compared with normal tissue. Most of them display argyrophilia, argentaffinity and a strong formaldehyde-induced-fluorescence 3) in the uncommon *neuromuscular proliferation* there is a striking proliferation of smooth muscle fibers and axons adjacent to the mucosa or muscularis propria layers resulting in neuromuscular tangles. In contrast to other forms of the disease neuromuscular proliferation occurs exclusively following inflammation and there is no endocrine cell proliferation.

The purpose of this study was firstly to demonstrate the extent of the neural network of normal appendix, ileum and proximal colon independently of specific neurotransmitters by immunocytochemical localization of neuron-specific enolase (NSE). This is an isoenzyme of the glycolytic enzyme enolase (E.C.4.2.1.11) specific for neurons and neuroendocrine cells (Schmechel et al. 1978). We also wished, secondly, to visualize peptides and amines in nerves and endocrine cells in the intestine by immunocytochemistry, and thirdly, to compare the extent of the occurrence of peptides possibly relevant to clinical symptoms of neurogenic appendicopathy in neural fibers occurring in the normal tissue and in neurogenic appendicopathy. Finally, we wished to gain insight into the possible route of development of carcinoid tumors of the appendix from stromal endocrine cells.

Material and methods

We investigated 5 appendices with mucosal neurogenic appendicopathy, 6 central neuromas, 6 normal appendices, and ileal mucosa in 5 patients. In addition, 6 appendices and 5 surgical samples of each ileum and proximal colon found to be intact on histology were analyzed. The specimens were fixed in phosphate-buffered or unbuffered liquid formaldehyde (4%). Further samples of 3 appendices with mucosal neurogenic appendicopathy, of 2 central neuromas, of 2 normal appendices and of one appendix with chronic inflammation were quenched in melting isopentane (2-methyl-butane) at $-160°$ C, freeze-dried overnight at $-40°$ C in a thermoelectric freeze-dryer, vapor fixed with paraformaldehyde, p-benzoquinone or diethylpyrocarbonate for 3 h at $+60°$ C and embedded in paraffin. Additional samples of one normal appendix and of one mucosal neurogenic appendicopathy were fixed in freshly prepared paraformaldehyde (4%) in phosphate buffer (0.1 M) postfixed in osmium tetroxide (1%) and embedded in Epon 812. The Epon was removed by immersion of semithin sections (1 μm) into sodium ethoxide (Mayor et al., 1961; Lane and Europa, 1965), the osmium black was bleached with periodic acid (5%) (Baskin et al. 1979). The sections were then immunostained for neuron-specific enolase and substance P.

1 The term neurogenic appendicopathy is preferred to "appendicite neurogène" because the latter term implies a primary inflammatory disorder

The following primary antisera were used:

Antibody raised against:	working dilution
neuron-specific enolase, rat (NSE)[2a]	1:4,000
substance P (synthetic)—antibody SP 67[2b]	1:1,000
substance P (synthetic)—antibody $SPRd_2$[2c]	1:1,000
substance P (synthetic)—antibody SPf_2[2c]	1:1,000
somatostatin (synthetic)	1:7,500
gastrin (G-17, synthetic)	1:5,000
cholecystokinin, porcine (CCK-33)[2d]	1:10,000
pancreatic glucagon, procine	1:5,000
intestinal glucagon (enteroglucagon), porcine	1:3,000
13—norleucine—motilin (synthetic)[2e]	1:5,000
5-hydroxytryptamine (synthetic)	1:2,000
neurotensin, bovine (synthetic)	1:40,000
pancreatic polypeptide, bovine (BPP)[2f]	1:80,000
vasoactive intestinal polypeptide (VIP), porcine — antibody B	1:400
VIP, porcine—antibody 77[2b]	1:500
corticotropin, human (ACTH)	1:500
glial fibrillary acidic protein, bovine (GFA)[2g]	1:5,000

2 These antisera were generous gifts of [a] Dr. P.J. Marangos, Bethesda, USA, [b]Prof. A.G.F. Pearse, Dr. J.M. Polak, London, [c]Prof. F. Lembeck, Graz, [d] Dr. W. Schlegel, Münster [e] Dr. P. Mitznegg, Erlangen, [f] Dr. R.E. Chance, Indianapolis, [g] Dr. L.F. Eng, Palo Alto

All reactions were carried out using the unlabeled antibody enzyme method (Sternberger 1979). All reagents were diluted with phosphate buffered saline (0.14 M or 0.5 M; Grube 1980). The histochemical reaction for peroxidase was carried out using a medium containing 3,3′-diaminobenzidine-tetrahydrochloride (0.05% w/v) and hydrogen peroxide (0.01% v/v) in Tris-buffer (0.05 M). Some sections were then postfixed in aqueous osmium tetroxyde (1%).

In additional sections the contrast in the peroxidase reaction was enhanced by adding cobalt chloride (1%) and nickel ammonium sulfate (1%) to the incubation medium (Adams 1981). An other series of liquid formaldehyde-fixed sections was pretreated with protease, type VII (Serva Heidelberg, FRG), 0.06–0.1%, 10 min–2 h at +37° C (Denk et al. 1977; Finley et al. 1981). Sections (5 μm) of freeze-dried formaldehyde vapor-fixed samples of normal ileum and appendix were used for formaldehyde-induced fluorescence (Falck 1962) and photographs. After photography the fluorescence was quenched by sodium borohydride (Corrodi et al. 1964). Subsequently the same sections were immunostained for substance P or neurone-specific enolase and photographed again. The localization and number of cells displaying formaldehyde-induced fluorescence, substance P- or neuron-specific-enolase-reactivity were then compared on the micrographs.

Controls

1) Non-immune rabbit serum, sheep gamma globulin and phosphate buffered saline as first and second layers respectively.
2) Omission of hydrogen peroxide from the incubation medium for peroxidase.
3) Absorption of the antibodies with the apropriate antigen (10 μM) at +4° C for 24 h prior to use; preabsorption of antibodies SP 67, $SPRd_2$ and SPf_2 with the following antigens: Synthetic substance P (Beckman München, FRG; 75 nM, 7,5 μM, 7,5 mM and 75 mM), synthetic eledoisin (Bachem Budendorf, Switzerland; 84 nM, 8,4 μM, 8,4 mM, 84 mM), synthetic physalaemin (Bachem Budendorf, Switzerland; 79 nM, 79 μM, 79 mM), synthetic cyclic somatostatin (Serono Freiburg, FRG; 61 mM) and pancreatic glucagon (Calbiochem San Diego, Ca, USA; 30 mM).

The following tissues were used as controls for the reaction of neuron-specific enolase:

rat spinal cord and cerebellum, human cerebellum and cerebral cortex, ganglioneuromas (4), pheochromocytomas (3), medullary thyroid carcinomas (6), bronchial adenomas (6), intestinal carcinoids (10), pancreatic endocrine tumors (155), melanomas (10), Merkel-cell tumours of the skin (7), astrocytomas and other non-neuroendocrine tumours (40). The characterization of the antineuron-specific enolase serum has been published elsewhere (Schmechel et al. 1978). Rat cerebellum and spinal cord and human cerebral cortex were also used as controls for the reaction of glial fibrillary acidic protein (GFA).

Results

Normal appendix, ileum and colon. Epithelial endocrine cells containing 5-hydroxytryptamine, substance P, enteroglucagon, somatostatin and vasoactive intestinal polypeptide-(VIP)-immunoreactivity were observed in all appendices, ileum and proximal colon. Additionally, in the ileum we found epithelial endocrine cells displaying neurotensin-immunoreactivity. All cells with substance P-immunoreactivity also showed formaldehyde-induced fluorescence (enterochromaffin cells, Fig. 1a and b). This reaction was generally weak for neuron-specific enolase (Fig. 2). In contrast, the great density of the neural network in the wall of appendix, ileum and colon was revealed by the strong reaction for neuron-specific enolase. Many nerve fibers were seen in the basal part of the mucosa and, to a lesser degree, in the submucosa.

A large number of neurons and nerve fibers in the muscle layers, submucosa and mucosa were shown to contain substance P-immunoreactivity. The distribution of substance P-containing nerve fibers and neurons was similar in the normal appendix, ileum and colon. 10–20% of neurons in Auerbach's plexus, and 5–10% of neurons in the plexus submucosus displayed a clear-cut substance P-immunoreactivity using antibodies SP Rd_2 and f_2 (Table 1). Nerve fibers containing substance P were particularly numerous in the vicinity of the pericarya in Auerbach's plexus and in the longitudinal and circular muscle layers, running parallel to the muscle fibers. Voluminous bundles of substance P-reactive nerve fibers were seen interconnecting the groups of neurons of Auerbach's plexus. The innermost layers of the submucosa were rich in substance P-containing nerve fibers, particularly in the areas surrounding lymphatic tissue. Fine substance P-containing nerve fibers surrounded small blood vessels. VIP-immunoreactive nerves could be identified in the mucosa and in the innermost layers of the submucosa. They were often seen in close contact with small blood vessels and with the basement membrane of the epithelium. All other reactions were found to be consistently negative in pericarya of neurons as well as in axons. No staining for GFA could be found.

No stromal endocrine cells could be observed in the normal appendix in this study.

Abnormal appendices. In the *mucosal form of Neurogenic appendicopathy* the immunoreaction for neuron-specific enolase revealed an obviously increased number of nerve fibers in the mucosa, not detectable by current tinctorial stains. The mucosal plexus was markedly enlarged, separating the crypts of the mucosa (Fig. 3). There was also an increase in the number

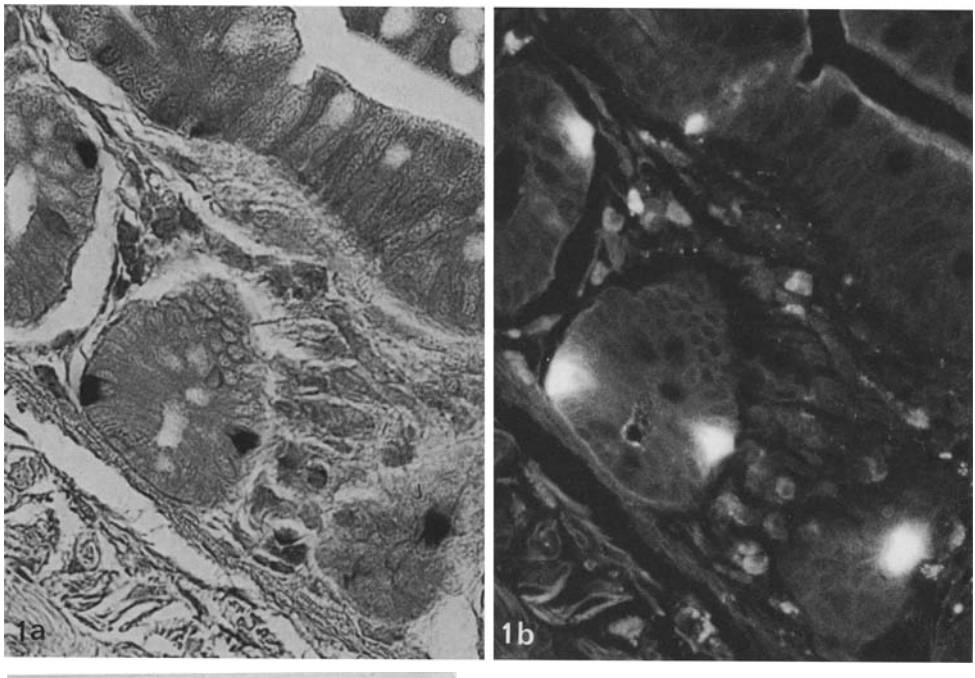


Fig. 1. a Mucosa of normal ileum with endocrine cells displaying substance P-immunoreactivity. Anti-substance P-serum 67, diluted 1:1,000. (×312). **b** Same section as **a**. Formaldehyde-induced fluorescence (×212)

Fig. 2. Normal appendix: endocrine epithelial cell containing neuron-specific enolase. Differential interference contrast optics (×788)

Table 1. Distribution pattern of substance P-containing neurons

	Appendix, ileum, colon	Neurogenic appendicopathy
Neurons		
Auerbach's plexus	+ (+)	+ (+)
Meissner's plexus	+	+
Nerve fibers		
Muscle longitudinal layer	+	+ +
Periganglionic layer	+ + +	+ + +
Circular layer	+ (+)	+ +
Submucosa		
Outer layers	+	+
Inner layers	+	+ + +
Mucosa	+	+ + +

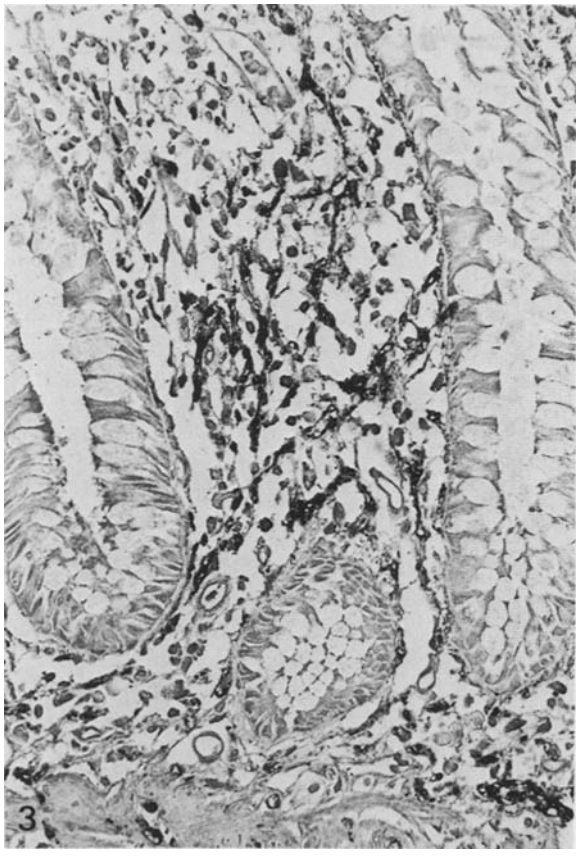


Fig. 3. Mucosal neurogenic appendicopathy: mucosal proliferation of substance P-immunoreactive nerves. Semithin section. Incubation with antibody SP Rd_2. (×300)

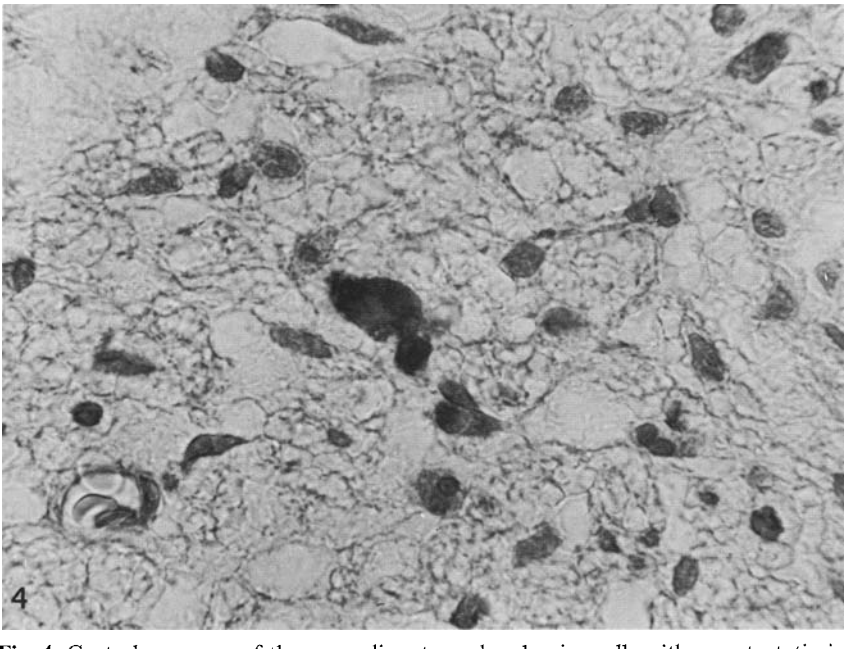


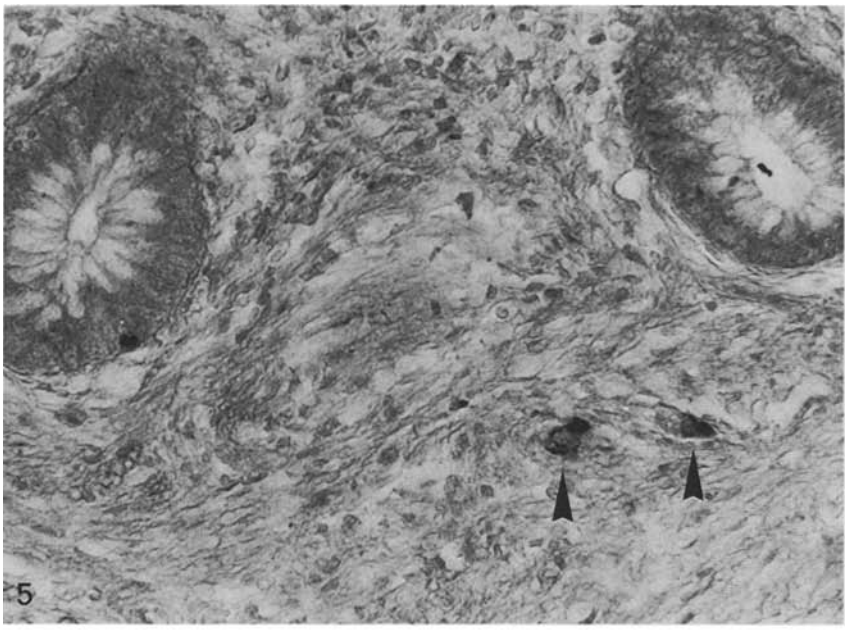
Fig. 4. Central neuroma of the appendix: stromal endocrine cells with somatostatin-immunoreaction. Counterstain with nuclear fast red (× 520)

of nerve fibers in the innermost layers of the submucosa and, particularly, in the muscularis mucosae (Table 1). The majority of the proliferated axons visualized by the immunoreaction for neuron-specific enolase contained substance P-immunoreactivity, particularly those in the innermost mucosal and submucosal layers (Fig. 3).

The distribution pattern of SP-reactive nerve fibers and neurons in neurogenic appendicopathy was found to be essentially normal in the muscle layers. In contrast, a large increase in the number of nerve fibers was observed in the inner layers of the submucosa and the mucosa. At the top of the mucosal folds and of the villi the nerve fibers became larger and the immunocytochemical reaction was finely granular. This is probably due to the swelling of axons as observed on electron microscopy (Auböck 1969). Nerve containing VIP-immunoreactivity were also numerous but much less common than substance P-reactive nerve fibers.

In *central neuroma*, a close contact of proliferating nerves with Meissner's plexus was obvious. In *neuromuscular proliferation* of the submucosa the relationship between nerves and muscle fibers could not be analyzed using tinctorial stains. Immunocytochemical visualization of neuron-specific enolase revealed a predominantly peripheral localization of nerve fibers in the neuromuscular tangles. The majority of the axons showed substance P-immunoreactivity. An immunoreaction for GFA was never observed.

The distribution and number of *epithelial endocrine cells* in the mucosal form of neurogenic appendicopathy and in central neuroma were found



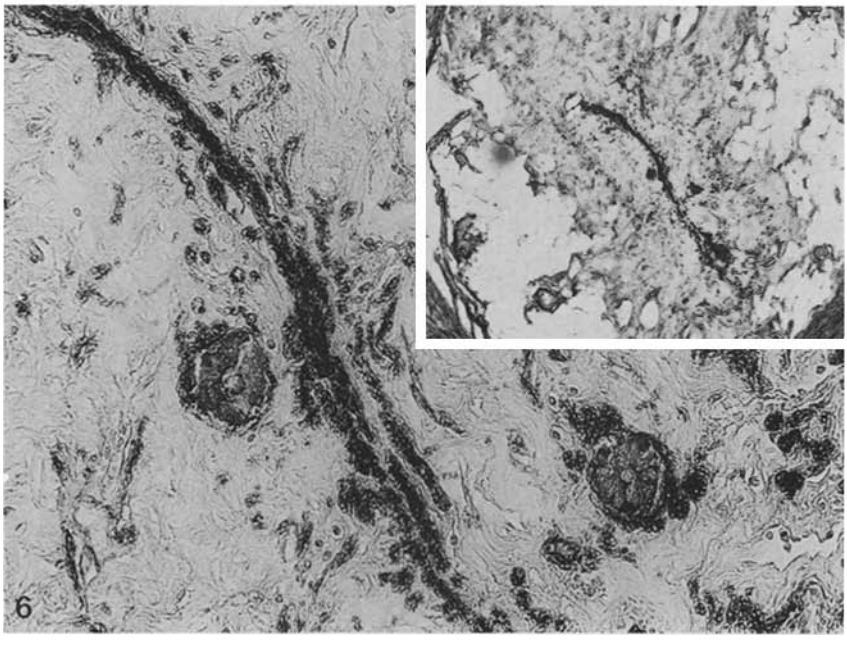


Fig. 5. Mucosal neurogenic appendicopathy. Stromal endocrine cells with 5-hydroxytryptamine-immunoreactivity (*arrows*), epithelial enterochromaffin cell with 5-hydroxytryptamine-immunoreactivity (*left*) (× 312)

Fig. 6. Central neuroma of the appendix with micro carcinoids. Immunoreaction for neuron – specific enolase (× 150). *Inset:* low power micrograph of central neuroma. Immuno staining for neuron-specific enolase (× 27)

to be essentially normal. *Endocrine cells* containing somatostatin- or 5-hydroxytryptamine-immunoreactivity (Figs. 4, 5) in the stroma of the mucosa (i.e. distant from the epithelium) were observed in both forms of neurogenic appendicopathy. The rare somatostatin cells were sometimes large and bizarre-shaped while 5-hydroxytryptamine cells were rather small. The identity of the latter with stromal cells yielding formaldehyde-induced fluorescence and a weak reaction for neuron-specific enolase was demonstrated. The content of stromal endocrine cells of substance P-immunoreactivity could not be shown consistently because of the variability of reactions obtained using various antisera. In addition no immunoreaction could be obtained in stromal endocrine cells with any of the other antisera used in this study.

In two appendices with mucosal neurogenic appendicopathy and in two appendices containing a central neuroma, *multicentric microcarcinoids* were found. They consisted of small solid cell nests, sometimes containing glandular structures. All of them were seen to be in close contact with proliferated nerve fibers. Carcinoid cells revealed neuron-specific enolase-immunoreactivity (Fig. 6) and sometimes 5-hydroxytryptamine-immunoreactivity, but no reaction with other antisera used.

Control reactions. The immunoreaction for substance P in endocrine cells using serum SP 67 was not quenched after absorption of the diluted antibody with substance P, glucagon, somatostatin, eledoisin and physalaemin. On the other hand, using antisera SP Rd_2 and SP f_2, the reaction was negative after preabsorption with 75 nM to 75 mM substance P and 84 mM eledoisin. Absorption of substance P antibodies with all other antigens prior to incubation did not prevent immunostaining for substance P. The immunoreaction of endocrine cells obtained with anti-VIP-serum was quenched after preabsorption with 30 mM glucagon. All other immunoreactions were negative after preabsorption of the antibody with the appropriate antigen.

Immunostaining for neuron-specific enolase was shown to occur exclusively in neuroendocrine cells and tumors. GFA could invariably be visualized in astrocytes of the human and rat central nervous systems.

Technical aspects. The contrast of the product of the peroxidase reaction was greatly enhanced by using 0.5 M phosphate buffer and, particularly, by using heavy metals instead of osmium tetroxide. This was especially useful for the visualization of nerve fibers in semithin sections. In general freeze-dryed and formaldehyde-vapor fixed sections yielded the best results of the immunoreactions. This procedure proved to be the only suitable pretreatment for the immunoreaction of motilin, neurotensin and VIP. In contrast, substance P immunoreactive nerve fibers were best visualized on tissue fixed in unbuffered liquid formaldehyde. The exposure of the sections to pronase was found to be useful only for the immunoreaction of somatostatin and to be deleterious for the neuron-specific enolase immunoreaction.

Discussion

Using immunostaining for neuron-specific enolase it is possible to visualize specifically and independently of neurotransmitters, the dense network of neuron pericarya and nerve fibers in the intestinal wall. Our results demonstrate the intrinsic origin of many of the intestinal substance P-containing axons in man, as approximately 20% of neurons of Auerbach's plexus and approx. 5% of neurons of the submucosal plexus showed substance P-immunoreactivity. The ratio found in the guinea-pig was to be 11,3% and 3,6% respectively (Costa et al. 1980). It may therefore be assumed that substance P is a widely distributed modulator of activity in the human gastrointestinal tract. These findings parallel those obtained from the intestine of the rat and the guinea-pig (Franco et al. 1979; Schultzberg et al. 1980; Jessen et al. 1980a and b). The physiological role of substance P in the gastrointestinal tract is complex: it may act as a hormone; it may be a neurotransmitter of excitatory interneurons affecting peristalsis (Holzer and Lembeck 1979; Morita et al. 1980); and it may be involved in presynaptic regulation of the release of norepinephrine and acetylcholine (Jessen et al. 1980b).

This study confirms the presence of cells with enteroglucagon-immunoreactivity (Helmstaedter 1979) and of enterochromaffin cells in the epithelium of the mucosa of the appendix, proximal colon and ileum. In addition we found substance P-immunoreactivity in enterochromaffin cells as previously described in enterochromaffin cells of the human duodenal mucosa (Heitz et al. 1976), and somatostatin in distinct endocrine cells. On the other hand we have also found cross-reactions of anti-VIP-sera with substances present in endocrine cells (see Larsson et al. 1979; Dimaline et al. 1980). As described above there was a difference between the reactions for substance P in endocrine cells using various antisera and we were not able to visualize somatostatin immunoreaction in nerves. It is therefore possible that the molecular forms of peptides in endocrine cells are different from those present in nerves (Chayvialle et al. 1980).

We were unable to detect "enteric glial cells" reactive for glial fibrillary acid protein in the human intestinal tract. Therefore, unlike the gastrointestinal system of the rat (Jessen and Mirsky 1980) there are apparantly no glial cells present in the human intestine. By contrast immunoreactivity of protein S-100 could be demonstrated in Schwann cells adjacent to intestinal nerve fibers (Ferry et al. 1982).

In *neurogenic appendicopathy* (mucosal form and central neuroma) the number of nerve fibers and, particularly, of subsubstance P-containing and, to a lesser extent, of VIP-containing mucosal and submucosal nerve fibers is greatly increased. A comparison of the extent and distribution of neuron-specific enolase and substance P-reactive nerve fibers on serial section revealed that substance P is present in at least 50% of all proliferated fibers. The cause of the proliferation is not known, but the localization of the proliferation is not known, but the localization of the proliferation at the very end of axons is striking. In 1951 Feyrter proposed that an impaired

communication between the "adeno- and neurointestine" could be the cause of neurogenic appendicopathy. In 1958 he suggested a neurovascular crisis to be the basic pathophysiologic mechanism leading to symptoms such as irregular peristalsis and spastic contractions of the intestine and hyperaemia of the serosal blood vessels of the appendix. In the light of present knowledge, increased production and secretion of the excitatory neurotransmitter substance P could be responsible for spastic contractions of the intestinal muscles and of abnormal peristalsis in neurogenic appendicopathy (Yau 1978; Leander et al. 1981), while secretion of VIP could cause local hyperaemia.

For unknown reasons the number of endocrine cells in the stroma of the mucosa is obviously greatly increased in neurogenic appendicopathy since it is extremely difficult to detect them in the normal appendix (Auböck and Ratzenhofer 1982; Höfler et al. 1982). Because of the presence of 5-hydroxytryptamine, somatostatin and, possibly, substance P-immunoreactivity, a significant contribution of these cells to the symptoms of neurogenic appendicopathy is conceivable. The invariably close contact of stromal endocrine cells with nerve fibers is of interest (for details see Höfler et al. 1982), since both structures contain identical or at least similar neurotransmitters. The question arises, whether the hyperplasia of stromal endocrine cells in neurogenic appendicopathy is the cause or an effect of an impairment of the local neuroendocrine regulatory mechanisms. It is possible that stromal somatostatin cells are proliferating in response to the increase in substance P-ergic neurons since somatostatin is considered an inhibitory neuromodulator substance. At present we are unable to define the pathophysiologic mechanisms leading to the symtpoms of neurogenic appendicopathy. This will be possible when significant advances are made in the knowledge of the physiology of the enteric neuroendocrine system.

The presence of stromal endocrine cells in neurogenic appendicopathy is perhaps relevant to the histogenesis of tumors since they are considered to be the cells of origin of carcinoid tumors of the appendix (Auböck et al. 1980). Single cells, small cell clusters and small carcinoid tumors can sometimes be observed in the same area. These cells or cell groups always occur in close contact with the dense network of nerve fibers. Confirming these findings obtained by other workers (Rode et al. 1982) we suggest at least some appendix carcinoids – and perhaps other gastrointesinal carcinoids – originate in multiple foci of small endocrine cell groups localized in the mucosal stroma distant from the epithelium.

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