Virchows Arch A (1987) 411:369–377

Virchows Archiv A

Increased neuropeptide Y-immunoreactive innervation of aganglionic bowel in Hirschsprung's disease

Y. Hamada [1], A.E. Bishop [1], G. Federici [2], M. Rivosecchi [2], I.C. Talbot [3], and J.M. Polak [1]

[1] Department of Histochemistry, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road London W12 OHS, UK
[2] Department of Surgery, Ospedale Bambino Gesu, Rome, Italy
[3] Department of Pathology, Leicester Royal Infirmary, Leicester, UK

Summary. The pathophysiology of Hirschsprung's disease has not been fully elucidated but is known to have a neurogenic basis. In recent years, new neural proteins and peptides have been discovered and our aim in this study was to use immunocytochemistry to investigate their involvement in the neuronal abnormalities associated with this condition. Large bowel samples from 9 children undergoing surgery for Hirschsprung's disease were compared with those taken from 8 children with other gastrointestinal diseases but no aganglionosis. Immunocytochemistry was carried out using antibodies to a wide range of neuron specific proteins and peptides. Examination of sections immunostained for the general neuronal markers, protein gene product 9.5, neuron specific enolase and neurofilament triplet proteins, allowed rapid identification of aganglionic segments. Nerves containing vasoactive intestinal polypeptide/peptide histidine methionine (VIP/PHM), galanin, substance P, somatostatin, met-enkephalin or calcitonin gene-related peptide (CGRP) showed a marked reduction in all layers of the aganglionic bowel. However, scattered VIP/PHM immunoreactive fibres were also found in the hypertrophied nerve bundles. In contrast with these reduced peptide-containing nerves, fibres displaying NPY immunoreactivity showed a marked increase in all aganglionic segments, particularly in the circular muscle where few are found normally. Our findings shed further light on the neurobiology of aganglionic bowel and suggest that immunostaining of neural proteins and the peptide NPY can aid rapid histopathological diagnosis of congenital aganglionosis.

Key words: Hirschsprung's disease – Neuropeptides – Immunocytochemistry

Offprint requests to: J.M. Polak at the above address

Introduction

Little is known about the pathophysiology of congenital aganglionosis, although it is widely considered to have a neuropathic basis (Whitehouse and Kernohan 1948; Zuelzer and Wilson 1948; Bodian et al. 1949; Webster 1973; Okamoto et al. 1982). The nervous lesions observed in this disease are not confined solely to a lack of intrinsic ganglion cells. Affected bowel has hypertrophied nerve bundles (Whitehouse and Kernohan 1948; Kluck et al. 1984; Vinores and May 1985), increased adrenergic (Bennett et al. 1968) and cholinesterase-positive (Bodian et al. 1951) nerves and a reduction of intrinsic serotoninergic nerves (Rogawski et al. 1978). A large number of different bioactive peptides have been localised to the nervous system of the human gut (Bishop et al. 1982; Ferri et al. 1982, 1984; Keast et al. 1984; Llewellyn-Smith et al. 1984) and some of these peptide-containing nerves have been shown to be abnormal in aganglionic bowel (Ehrenpreis and Pernow 1953; Tafuri et al. 1974; Freund et al. 1979; Dupont et al. 1980; Bishop et al. 1981; Tsuto et al. 1985).

Since the previous studies, additional types of peptide-containing nerves have been discovered, some of which have been shown, by animal experimentation to have at least a partially extrinsic origin (Sundler et al. 1983, 1986; Ferri et al. 1984; Bishop et al. 1984; Clague et al. 1985; Mulderry et al. 1985; Melander et al. 1985; Bishop et al. 1986; Su et al. 1986), and new general neuronal markers, such as protein gene product 9.5 (Thompson et al. 1983; Gulbenkian et al. 1987), have been isolated.

The aim of the present study was to determine whether, in congenital aganglionosis, any immunocytochemically detectable changes occur in nerves containing these neuron specific proteins and pep-

Table 1. Details of antisera used

Antiserum to	Dilution		Concentration of corresponding antigen required for quenching of immunostain [nmol/ml]	Source
	IMF	*PAP*		
CGRP	1:200	1:2000	0.01	HH
Galanin	1:500	1:5000	0.1	HH
Met-enkephalin	1:400	1:4000	0.1	RIA U.K Ltd
Neurofilaments	1:400	1:4000	NK	VL/JT
Neuropeptide Y	1:200	1:2000	0.01	HH
Neuron specific enolase	1:200	1:1000	1.0	PJM
PGP 9.5	1:500	1:2000	NK	RJT
PHM	1:400	1:4000	0.1	HH
S-100	1:800	1:4000	NK	DC
Somatostatin	1:800	1:800	0.1	RIA U.K Ltd
Substance P	1:500	1:5000	0.01	HH
[a] Tyrosine hydroxylase	1:400	1:400	NK	Boehringer
VIP	1:2000	1:10000	0.01	HH

HH = Hammersmith Hospital; IMF = immunofluorescence; PAP = peroxidase antiperoxidase; NK = not known; VL/JT = V. Lee/J Trojanowski; PJM = P.J. Marangos; DC = D. Cocchia

[a] Bovine

tides. In view of the reported increase in aminergic fibres in aganglionic bowel, particular attention was paid to nerves which contain NPY as they have been shown to have a dual origin from local cell bodies and from extrinsic, catecholaminergic sources (Lundberg et al. 1982; Ferri et al. 1984; Ekblad et al. 1984; Furness et al. 1983; Lee et al. 1985a; Sundler et al. 1986).

Materials and methods

Specimens of large bowel were obtained fresh at surgery from 9 children with Hirschsprung's disease (ages 1 year 5 months to 4 years 10 months; 4 males, 5 females). Diagnosis was based on the results of radiological examination, rectal manometry and histology. In each case a narrow strip was taken along the length of each resection specimen and cut into 1 cm long pieces. For comparison, macroscopically and histologically normal samples of large bowel were obtained from 8 children (1 year to 6 years; 5 males, 3 females) with no evidence of aganglionosis.

After dissection, the samples were immersed in 0.4% p-benzoquinone (Koch-Light) in 0.01 M phosphate buffered 0.15 M saline (PBS, pH 7.1–7.4) (Bishop et al. 1978) for one hour or in Bouin's solution for four hours. The benzoquinone solution-fixed tissues were thorougly rinsed in PBS containing 15% sucrose and 0.01% sodium azide, cryostat blocks were prepared and 10 μm sections cut at −20° C. Bouin's-fixed material was dehydrated embedded in wax and 5 μm sections were cut. The sections were picked up on glass slides coated with poly-L-lysine (Huang et al. 1983), air-dried for 30 min and stained with haematoxylin and eosin or immunostained using the techniques of indirect immunofluorescence (Coons et al. 1955) for cryostat sections and peroxidase anti-peroxidase (Sternberger 1979) for wax sections. For indirect immunofluorescence, the primary rabbit antisera (Table 1) were applied at appropriate dilutions and left for 16–20 h at 4° C in a moist atmosphere. After three rinses in PBS, the second layer of FITC-conjugated goat anti-rabbit IgG (Miles Laboratories Ltd, Stoke Poges, UK) was applied to the sections at a dilution of 1:200. After one hour's incubation at room temperature, the sections were again washed three times in PBS, mounted in PBS: glycerine (1:9 v/v) and examined under an Olympus Vanox ultraviolet microscope (filter 450–490 nm). Photographs were taken using FP4 black and white film (speed 100 ASA) (Ilford Ltd., UK). For the peroxidase anti-peroxidase technique, the wax sections were immersed in 0.03% (w/v) hydrogen peroxide in PBS for 30 min to remove endogenous peroxidase activity. Possible background staining was also reduced by the application of normal goat serum, diluted 1:30, for 30 min. Primary antisera (Table 1) were applied as for indirect immunofluorescence. The second layer of unconjugated goat anti-rabbit IgG was used at a dilution of 1:200 for 30 min. The final layer of rabbit PAP complex, diluted 1:500 was applied for 30 min at room temperature. Visualisation of the PAP complex was achieved by the diaminobenzidine method (Graham and Karnovsky 1976). When developed, the sections were dehydrated, mounted in DPX and examined under a transmitted light microscope (Reichert-Jung). Photographs were taken using Technical Pan black and white film (speed (150 ASA) (Kodak Ltd. UK).

Results

On the basis of conventional histology and the immunostains for general neuronal markers, three segments could be distinguished in the 9 specimens taken from children with Hirschsprung's disease. In the cryostat sections, an area could be discerned in the proximal portion of each specimen which had a pattern of innervation indistinguishable from that in the controls. Ganglion cells were present in both plexuses and a normal population of peptidergic nerves was seen containing VIP/PHM, NPY (Fig. 1a), CGRP, galanin, substance P, so-

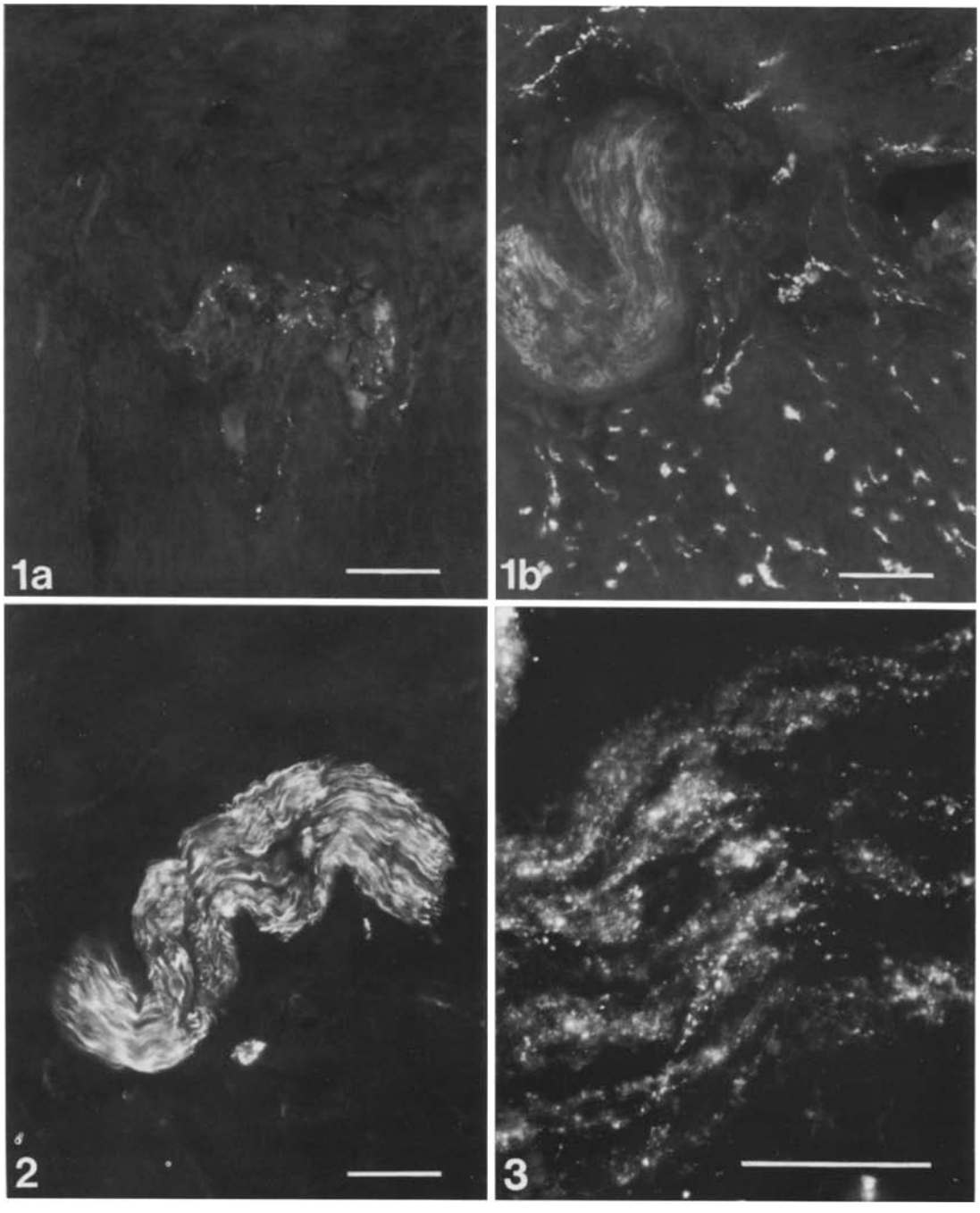

Fig. 1. NPY-immunoreactive fibres. **a** Scattered in the myenteric plexus in normal bowel. **b** Densely immunostained and present in high numbers in a similar region of aganglionic bowel. Indirect immunofluorescence technique on BQS-fixed 10 μm cryostat section. Bar = 70 μm

Fig. 2. An hypertrophied nerve bundle in the region of the myenteric plexus showing strong immunoreactivity for protein gene product 9.5. Processing as for Fig. 1

Fig. 3. VIP-immunoreactive nerves in an hypertrophied nerve bundle like that is seen in Fig. 2. Processing as for Fig. 1

matostatin and met-enkephalin. Several of the ganglion cells in the submucous plexus showed immunoreactivity for VIP/PHM and a few also contained galanin.

Distal to this portion, a hypoganglionic segment, of variable length, could be identified. Apart from the reduced numbers of ganglion cells in both the myenteric and submucous plexuses, this area was characterised by the presence of hypertrophied nerve bundles lying in the serosa and running

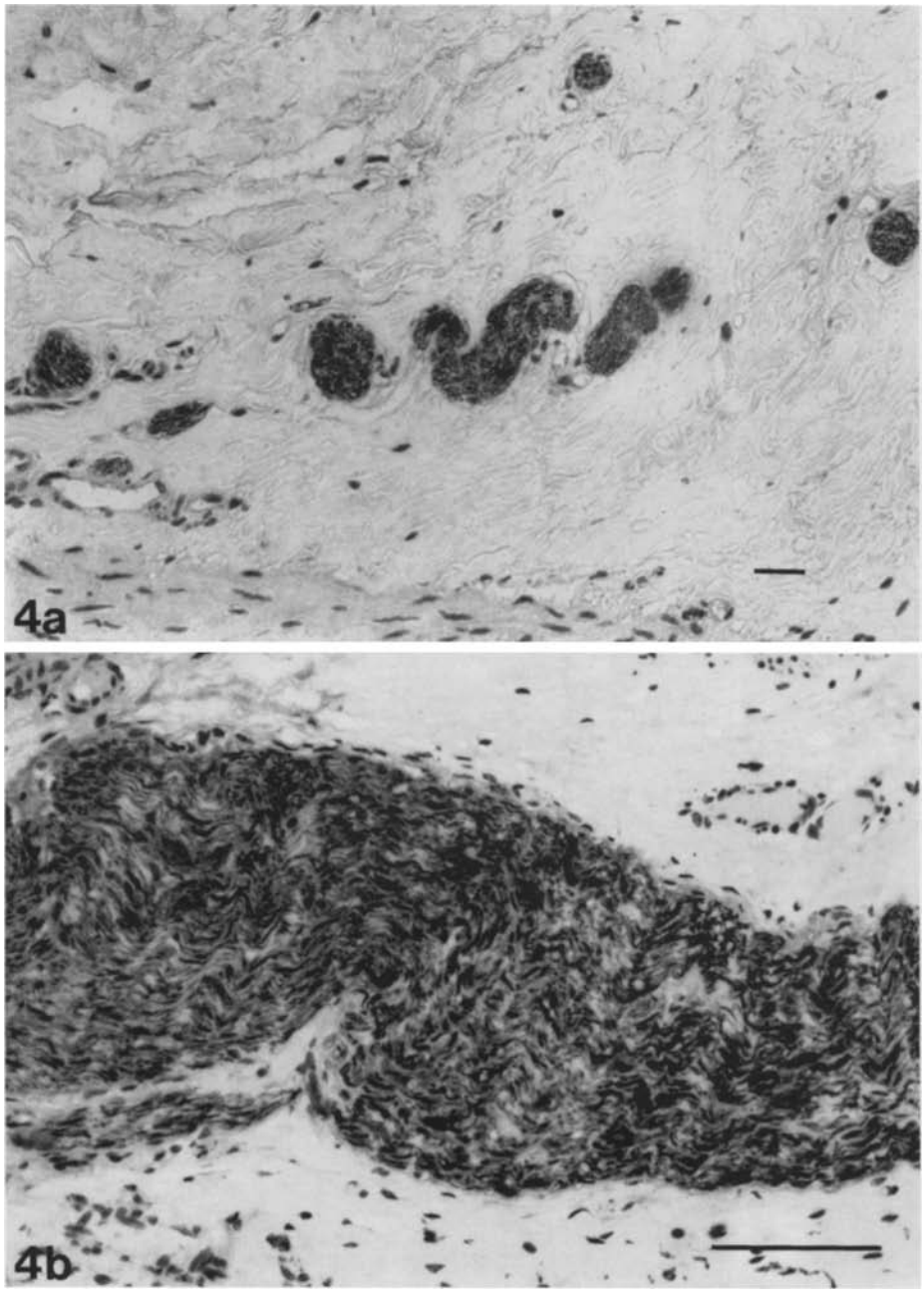

Fig. 4. a PGP 9.5 immunoreactivity clearly visible in hypertrophied nerve bundles of aganglionic submucosa. **b** High-power micrograph of an hypertrophied nerve bundle between the circular and longitudinal muscle coats of aganglionic bowel immunostained for PGP 9.5. PAP technique on Bouin's solution fixed 5 μm wax-embedded sections

through the intermuscular spaces to the submucosa. These bundles showed immunoreactivity for protein gene product 9.5 (Fig. 2), neurofilaments, neuron specific enolase and protein S-100. Only VIP/PHM of all the peptides studied could be visualised in these bundles, in scattered fine fibres (Fig. 3). VIP/PHM-containing nerves also showed a slight reduction in the muscle and mucosa of this segment, unlike the other peptides which showed an almost normal profile of nerves.

The distal portions of the specimens showed aganglionosis, to various extents and increased numbers of hypertrophied nerve bundles which displayed strong immunoreactivity for general neuronal markers and also scattered VIP/PHM nerves similar to those seen in the hypoganglionic segment. Immunoreactivity for general neuronal markers was seen in fibres in all layers of the bowel wall. The differences in the results for these markers between normal and aganglionic bowel

appeared to be confined to absence of ganglion cells and presence of hypertrophied nerve bundles. The pattern of peptidergic innervation in this segment, however, was entirely different from that in the proximal hypoganglionic or normal portions. Nerves containing VIP/PHM, galanin, substance P, somatostatin, met-enkephalin or CGRP all showed a reduction when compared with normal bowel. Subjectively, this reduction appeared to be related to the length of the aganglionic segment, with only few immunoreactive fibres being found in extensively aganglionic bowel. In contrast, a completely opposite pattern of staining was observed for NPY-immunoreactive nerves which showed increased density in all muscle layers, particularly the circular muscle coat (Fig. 1b). Where in normal bowel few immunoreactive fibres can be found lying in the circular muscle, in the aganglionic segments very densely immunostained, intertwined groups of NPY nerves were spread throughout the layer (Fig. 1b). Immunoreactivity for tyrosine hydroxylase was present in nerves of ganglionic and aganglionic segments of bowel, mainly between the circular and longitudinal muscle coats. However, the level of immunostaining was variable both within and between specimens. Two cases showed an increase in immunoreactive fibres in the aganglionic portion.

The information gained from the wax-embedded tissues was limited. The antiserum to PGP 9.5 gave the best results but there was a reduction in the amount of nervous tissue visualised in comparison with the cryostat sections. In particular, few small bundles or individual fibres were seen. In the diseased specimens, the thick, contorted nerve bundles, however, were densely immunostained and clearly visible (Fig. 4) and aganglionic areas could be distinguished easily. Two specimens showed numerous fibres in the muscularis mucosae but few nerves were seen in the mucosa. This apparent reduction in sensitivity of immunostaining in wax-embedded tissues was worse for the other general neuronal markers, neurofilaments, NSE and protein S-100, which were found only weakly in nerve bundles and in scattered nerves or glia. The antisera to peptides and tyrosine hydroxylase gave very weak, inconsistent results which were not comparable to those obtained in the cryostat sections.

Discussion

The aim of our study was to expand our previous work on peptide-containing nerves in Hirschsprung's disease (Bishop et al. 1981) by examining more recently described neuronal proteins and peptides. For optimal preservation of neuronal antigens we have studied cyrostat sections and shown that, as well as VIP/PHM- and substance P-immunoreactive nerves, those containing galanin, somatostatin, met-enkephalin or CGRP are reduced in the aganglionic segment of bowel. In contrast with these, nerves with NPY immunoreactivity were present in much higher numbers in the aganglionic portion than in normal bowel. They were found particularly in the circular muscle where only few fibres can be seen normally. Three general neuronal markers, neuron specific enolase, neurofilaments and protein gene product 9.5, were also immunostained. These allowed rapid differentiation between ganglionic and aganglionic bowel by giving clear demonstration of the lack of ganglion cells and the presence of hypertrophied nerve bundles in the latter. Immunostaining of neural antigens in wax-embedded tissue gave poor results in comparison with those obtained using cryostat sections and fixation in benzoquinone solution. This labile nature of substances in nerves is well known. Of all the antigens immunostained in wax sections, only PGP 9.5 gave satisfactory results, suggesting that antibodies to this protein may be of use in diagnosis of routinely processed tissues. However, as no consistent changes in nerves could be detected in the mucosa of aganglionic bowel, whether wax or cryostat sections, immunostaining of PGP 9.5 is unlikely to be much assistance in the examination of mucosal biopsies.

Interpretation of the neuronal abnormalities in congenital aganglionosis is difficult largely because only incomplete information is available on the developmental pattern of the human enteric nervous system and the sources of the various types of nerve supplying the gut. Thus, it is only possible to speculate on the significance of the present findings. The observed reduction in the frequency of nerves containing VIP/PHM, substance P, galanin, somatostatin or met-enkephalin in the aganglionic segment suggests that these nerves have mainly intrinsic origins in the human bowel and their loss is a direct result of the lack of intrinsic cell bodies. This conclusion is supported both by the observation that the degree of fibre loss often appeared to be in proportion to the length of the aganglionic segment and by results of experiments made in other mammals which show that most of these nerves are enteric in origin (Schultzberg et al. 1980; Jessen et al. 1980; Malmfors et al. 1981). It was interesting to note, however, that VIP/PHM-immunoreactive fibres were seen in hypertrophied nerve bundles, some of which lay in the serosa

and were, presumably, of extrinsic origin. Although VIP is known to occur in enteric ganglion cells in human gut, in cat and rat at least it has been found in pathways linking the pelvic viscera with not only the autonomic but also the sensory centres of the sacral spinal cord (Basbaum et al. 1983; Kawantini et al. 1983; Jansco et al. 1981; Gibson et al. 1986). Thus, an extrinsic origin for some VIP nerves cannot be ruled out.

CGRP-immunoreactive nerves were also reduced in aganglionic segments. This peptide has been localised previously to autonomic, motor and sensory nerves (Rodrigo et al. 1985; Lee et al. 1985b; Wiesenfeld-Hallin et al. 1984; Gibbins et al. 1985) and has been shown in the large bowel of experimental animals to have dual intrinsic and extrinsic (sensory) origins (Clague et al. 1985; Sternini et al. 1986; Su et al. 1986). The reduction of CGRP-immunoreactive fibres suggests an intrinsic origin but no immunoreactive cell bodies could be found in normal, ganglionic bowel. However, the lack of CGRP-immunoreactive ganglion cells in the control tissues does not necessarily mean that nerves containing this peptide do not have an intrinsic origin in human bowel. It is possible that only a minor proportion of intramural neurons produce CGRP and the content of peptide within each soma is too low to be detected by immunocytochemistry.

In complete contrast to the other types of peptide-immunoreactive nerves, those containing NPY showed increased density in aganglionic bowel, particularly in the circular muscle layer where few fibres are normally found. Most NPY-immunoreactive fibres in the human gut are thought to arise from enteric ganglion cells but a minor proportion are suggested to have an extrinsic, adrenergic origin and these latter innervate mainly the vasculature and myenteric plexus (Ferri et al. 1984; Ekblad et al. 1984; Furness et al. 1983; Lee et al. 1985a; Sundler et al. 1986). The change in NPY nerves may, thus, reflect the reported hyperplasia of aminergic fibres in Hirschsprung's disease shown by an induced fluorescence technique (Bennett et al. 1968). In the present study, we have tried to reproduce these results by visualising aminergic nerves using antibodies to tyrosine hydroxylase, the enzyme which converts tyrosine to 3,4-DOPA. It is possible, however, that the variable results obtained may be due to these antibodies not cross-reacting fully with the human enzyme.

The mechanism behind the apparent hyperplasia of nerves reported in Hirschsprung's disease in not clear but some interpretations can be offered. 1. Is this phenomenon analogous to "amputation" neuroma? The increased numbers of cholinesterase-positive and possibly aminergic fibres and the presence of hypertrophied nerve bundles have been postulated to be the result of incoming extrinsic nerves "searching" for intramural ganglion cells with which to synapse and their appearance has invited comparison with amputation neuromas (Okamoto et al. 1967; Okamoto et al. 1982). 2. Is the putative trophic factor, released from "target" tissue to modulate neuronal maturation (for review see Prestige 1974; Hendry et al. 1981), produced by muscle instead of ganglion cells in aganglionic bowel? For the extrinsic NPY/aminergic fibres which normally synapse with myenteric ganglion cells (Sundler et al. 1986), there are no target tissues and, thus, there should be no trophic influences in the aganglionic segment. It has been shown, however, that catecholaminergic nerves which synapse with myenteric ganglia under normal conditions can instead supply the muscle if no ganglion cells are present (Burnstock et al. 1971). Thus, the muscle may be able to provide the stimulus for the incoming aminergic fibres. 3. Is the phenotypic expression of neuronal antigens abnormal in aganglionic bowel? There are numerous examples of experimental models where a selective reduction in one particular type of nerve causes a reciprocal rise in another (Kessler et al. 1983; Schon et al. 1985; Cole et al. 1983; Zhang et al. 1984; Terenghi et al. 1986). Also, transient expression of different phenotypic features has been observed frequently and is clearly under control of the local environment (Le Douarin and Teillet 1974; Le Douarin 1981; Smith et al. 1977; Rothman et al. 1978; Bjorklund et al. 1985; Patterson 1978; Garcia-Arraias et al. 1986; Coulombe and Bronner-Fraser 1986). The characteristics displayed by the different nerve types in aganglionic bowel may be the result of interruption of differentiation, leaving the neurons incapable of fulfilling their normal functional role. Neuronal immaturity in terms of incomplete phenotypic differentiation may be a contributory factor precipitating the intestinal dysfunction.

In short, Hirschsprung's disease can no longer be viewed as a simple denervation effect. As our knowledge of enteric nerve development increases, further insights into the pathogenesis of the disease will be gained. The findings reported here invite speculation as to the possible consequences of specific forms of enteric neuronal damage. Our observation of increased NPY innervation in aganglionic bowel fits well with previous reports of aminergic nerve hyperplasia but neither observation can

be explained fully at present. The use of antisera to the general neuronal markers neurofilament triplet proteins, neuron specific enolase and, in particular, PGP 9.5 allows rapid differentiation between ganglionic and aganglionic bowel.

Acknowledgements. The authors thank Dr. V. Lee, Dr. J. Trojanowski, Dr. P.J. Marangos, Dr. D. Cocchia, Dr. J. Thibault and Dr. R.J. Thompson for providing antisera. Peptide antisera were raised in collaboration with Professor S.R. Bloom. We are also grateful to Miss S. Lock and Miss A. Williams for secretarial assistance.

References

Basbaum AI, Glazer EJ (1983) Immunoreactive vasoactive intestinal polypeptide is concentrated in the sacral spinal cord: a possible marker for pelvic visceral afferent fibres. Somatosens Res 1:69–82

Bennett A, Garrett JR, Howard ER (1968) Adrenergic myenteric nerves in Hirschsprung's disease. BMJ i:487–489

Bishop AE, Polak JM, Bloom SR, Pearse AGE (1978) A new universal technique for the immunocytochemical localisation of peptidergic innervation. J Endocrinol 77:25–26P

Bishop AE, Polak JM, Lake BD, Bryant MG, Bloom SR (1981) Abnormalities of the colonic regulatory peptides in Hirschsprung's disease. Histopathology 5:679–688

Bishop AE, Ferri GL, Probert L, Bloom SR, Polak JM (1982) Peptidergic nerves. In: Polak JM, Bloom SR, Wright NA (eds) Structure of the Gut. Basic Science in Gastroenterology. Daly M.J. Glaxo Group Research Ltd., England, pp 221–237

Bishop AE, Polak JM, Yiangou Y, Christofides ND, Bloom SR (1984) The distribution of PHI and VIP in porcine gut and their colocalisation to a proportion of intrinsic ganglion cells. Peptides 5:255–259

Bishop AE, Polak JM, Bauer FE, Christofides ND, Carlei F, Bloom SR (1986) Occurrence and distribution of a newly discovered peptide, galanin, in the mammalian enteric nervous system. Gut 27:849–857

Bjorklund H, Hokfelt T, Goldstein M, Terenius L, Olson L (1985) Appearance of the noradrenergic markers tyrosine hydroxylase and neuropeptide Y in cholinergic nerves of iris following sympathectomy. J Neurosci 5:1633–1640

Bodian M, Stephens FD, Ward BLK (1949) Hirschsprung's disease and idiopathic megacolon. Lancet i:6–11

Bodian M, Carter CO, Ward BCK (1951) Hirschsprung's disease. Lancet i:302–309

Burnstock G, Gannon BJ, Malmfors T, Rogers DG (1971) Changes in the physiology and fine structure of the taenia of the guinea pig caecum following transplantation into the anterior eye chamber. J Physiol 219:139–154

Clague JR, Sternini C, Brecha NC (1985) Localisation of calcitonin gene-related peptide-like immunoreactivity in neurons of the rat gastrointestinal tract. Neurosci Lett 56:63–68

Cole DF, Bloom SR, Burnstock G, Butler JM, McGregor GP, Saffrey MJ, Unger WG, Zhang SQ (1983) Increase in SP-like immunoreactivity in nerve fibres of rabbit iris and ciliary body one to four months following sympathetic denervation. Exp Eye Res 37:191–197

Coons AH, Leduc EH, Connolly J (1955) Studies of antibody production. J Exp Med 102:49–60

Coulombe JN, Bronner-Fraser M (1986) Cholinergic neurons acquire adrenergic neurotransmitters when transplanted into an embryo. Nature 324:569–571

Dupont C, Navarro J, Chenut B, Rosselin G (1980) Modifications of VIP intestinal content associated with abnormal nervous myenteric plexus: A biologic feature of chronic intestinal obstruction. J Paediatrics 96:1047–1039

Ehrenpreis T, Pernow B (1953) On the occurrence of substance P in the recto-sigmoid in Hirschsprung's disease. Acta Physiol Scand 27:380–388

Ekblad E, Wahlestedt C, Ekelund M, Hakanson R, Sundler F (1984) Neuropeptide Y in the gut and pancreas. Distribution and possible vasomotor function. Front Horm Res 12:85–90

Ferri GL, Botti PL, Vezzadini P, Biliotti G, Bloom SR, Polak JM (1982) Peptide-containing innervation of the human intestinal mucosa. An immunocytochemical study on whole-mount preparations. Histochem 84:777–786

Ferri GL, Ali-Rachedi A, Tatemoto K, Bloom SR, Polak JM (1984) Immunocytochemical localisation of neuropeptide Y-like immunoreactivity in extrinsic noradrenergic and intrinsic gut neurons. Front Horm Res 12:81–84

Freund HR, Humphrey CS, Fischer JE (1979) Reduced tissue content of vasoactive intestinal polypeptide in aganglionic colon of Hirschsprung's disease. Gastroenterology 76:1135

Furness JB, Costa M, Emson PC, Hakanson R, Maghimzadeh E, Sundler F, Taylor IL, Chance RE (1983) Distribution, pathways and reactions to drug treatment of nerves with neuropeptide Y- and pancreatic polypeptide-like immunoreactivity in the guinea pig digestive tract. Cell Tiss Res 234:71–92

Garcia-Arraris JE, Fauquet M, Chanconie M, Smith J (1986) Coexpression of somatostatin-like immunoreactivity and catecholaminergic properties in neural crest derivatives. Comodulation of peptidergic and adrenergic differentiation in cultured neural crest. Devel Biol 114:247–257

Gibbins IL, Furness JB, Costa M, MacIntyre I, Hillyard CJ, Girgis S (1985) Co-localisation of calcitonin gene-related peptide-like immunoreactivity with substance P in cutaneous, vascular and visceral sensory neurons of guinea pigs. Neurosci Lett 57:125–130

Gibson SJ, Polak JM, Anand P, Blank MA, Yiangou Y, Su HC, Terenghi G, Katagiri T, Morrison JFB, Lumb BM, Inyama C, Bloom SR (1986) A VIP/PHI-containing pathway liked urinary bladder and sacral spinal cord. Peptides 7:205–219

Graham RC, Karnovsky MJ (1976) The early stages of absorption of injected horse-radish peroxidase in the proximal tubules of mouse kidney. J Histochem Cytochem 14:291–302

Gulbenkian S, Wharton J, Polak JM (1987) The visualisation of cardiovascular innervation in the guinea pig using an antiserum of protein gene product 9.5 (PGP 9.5). J Autonom Nerv Syst 18:235–237, 1977

Hendry IA, Hill CE, Bonyhady RE (1981) Interactions between developing autonomic neurones and their target tissues. In: Development of the autonomic nervous system. Ciba Foundation Symposium 83. Pitman Medical: Bath;194–206

Huang WM, Gibson SJ, Facer P, Gu J, Polak JM (1983) Improved section adhesion for immunocytochemistry using high molecular weight polymers of L-lysine as a slide coating. Histochemistry 77:275–279

Jansco G, Hokfelt T, Lundberg JM, Kiraly E, Halasz N, Nilsson G, Terenius L, Rehfeld J, Steinbusch H, Verhofstad A, Elde R, Said S, Brown M (1981) Immunohistochemical studies on the effect of capsaicin on spinal and medullary peptide and monoamine neurons using antisera to substance P, gastrin/CCK, somatostatin, VIP, enkephalin, neurotensin and 5-hydroxytryptamine. J Neurocytol 10:963–980

Jessen KR, Saffrey MJ, Van Noorden S, Bloom SR, Polak JM, Burnstock G (1980) Immunohistochemical studies of

the enteric nervous system in tissue culture and *in situ:* Localisation of vasoactive intestinal polypeptide (VIP)-, substance P- and enkephalin-immunoreactive nerves in the guinea pig gut. Neuroscience 5:1717–1735

Kawatini M, Lowe IP, Nadelhaft I, Morgan C, de Groat WC (1983) Vasoactive intestinal polypeptide in visceral afferent pathways to the sacral spinal cord of the cat. Neurosci Lett 42:311–316

Keast JR, Furness JB, Costa M (1984) Somatostatin in human enteric nerves: Its distribution and characterisation. Cell Tiss Res 237:299–308

Kessler JA, Bell WO, Black IB (1983) Interactions between the sympathetic and sensory innervation of the iris. J Neurosci 3:1301–1307

Kluck P, Van Muijen GNP, Van der Kamp WM, Tibboel D, Van Hoorn WA, Warnaar SO, Molenaar JC (1984) Hirschsprung's disease studied with monoclonal antineurofilament antibodies on tissue sections. Lancet i:652–653

Le Douarin NM (1981) The ontogeny of the neural crest in avian embryo chimeras. Nature (Lond) 236:663–669

Le Douarin NM, Teillet MA (1984) Experimental analysis of the emigration and differentiation of neuroblasts of the autonomic nervous system and of neurorectodermal mesenchymal derivatives, using a biological cell marking technique. Dev Biol 41:162–184

Lee Y, Shiosaka S, Emson PC, Powell KF, Smith AD, Tohyama M (1985a) Neuropeptide Y-like immunoreactive structures in the rat stomach with special reference to the noradrenaline neuron system. Gastroenterology 89:118–126

Lee Y, Takami Y, Girgis S, Hillyard CJ, MacIntyre I, Emson PC, Tohyama M (1985b) Distribution of calcitonin gene-related peptide in the rat peripheral nervous system with reference to its co-existence with substance P. Neuroscience 15:1227–1237

Llewellyn-Smith IJ, Furness JB, Murphy R, O'Brien PE, Costa M (1984) Substance P-containing nerves in human small intestine – distribution, ultrastructure and characterization of the immunoreactive peptide. Gastroenterology 86: 421–435

Lundberg JM, Terenius L, Hökfelt T, Martling CR, Tatemoto K, Mutt V, Polak JM, Bloom SR, Goldstein M (1982) Neuropeptide Y (NPY)-like immunoreactivity in peripheral noradrenergic neurons and the effects of NPY on sympathetic function. Acta Physiol Scand 116:477–480

Malmfors G, Leander S, Brodin E, Hakanson R, Holmin T, Sundler F (1981) Peptidergic neurons are intrinsic to the gut wall. An experimental study in the pig. Cell Tiss Res 214:225–238

Melander T, Hökfelt T, Rokaeus A, Fahrenkrug J, Tatemoto K, Mutt V (1985) Distribution of galanin-like immunoreactivity in the gastrointestinal tract of several mammalian species. Cell Tiss Res 239:253–270

Mulderry PK, Ghatei MA, Bishop AE, Allen YS, Polak JM, Bloom SR (1985) Distribution and chromatographic characterisation of CGRP-like immunoreactivity in the brain and gut of the rat. Regul Pept 12:133–143

Okamoto E, Ueda T (1967) Embryogenesis of intramural ganglia of the gut and its relation to Hirschsprung's disease. J Paediat Surg 2:437–443

Okamoto E, Satani M, Kuwata K (1982) Histologic and embryologic studies on the innervation of the pelvic viscera in patients with Hirschsprung's disease. Surg Gynae Obs 155:823–828

Patterson PH (1978) Environmental determination of autonomic neurotransmitter functions. Am Rev Neurosci 1:1–17

Prestige MC (1974) Axon and cell numbers in the developing nervous system. Br Med Bull 30:107–111

Rodrigo J, Polak JM, Fernandez L, Ghatei MA, Mulderry PK, Bloom SR (1985) Calcitonin gene-related peptide-immunoreactive sensory and motor nerves of the rat, cat and monkey oesophagus. Gastroenterology 88:444–451

Rogawski MA, Goodrich JT, Gershon MD, Touloukian RJ (1978) Hirschsprung's disease: absence of serotonergic neurons in the aganglionic colon. J Paediatr Surg 13:608–615

Rothman TP, Gershon MD, Holtzer H (1978) The relationship of cell division to the acquisition of adrenergic characteristics by developing sympathetic ganglion cell precursor. Dev Biol 65:322–341

Schon F, Ghatei M, Allen JM, Mulderry PK, Kelly JS, Bloom SR (1985) The effect of sympathectomy on calcitonin gene-related peptide levels in the rat trigeminovascular system. Brain Res 348:197–200

Schultzberg M, Hökfelt T, Nilsson G, Terenius L, Rehfeld JF, Brown M, Elde R, Goldstein M, Said SI (1980) Distribution of peptide- and catecholamine-containing neurons in the gastrointestinal tract of rat and guinea pig. Neuroscience 5:687–744

Smith J, Le Douarin NM, Fauquet M, Ziller C (1981) The development of autonomic neurons from the neural crest: *in vivo* and *in vitro* studies. In: Garrod DR, Feldman JD (eds) Development in the Nervous System. Cambridge University Press, Cambridge, pp 129–145

Sternberger LA (1979) The unlabelled antibody peroxidase anti-peroxidase (PAP) method. In: Sternberger LA (ed) Immunocytochemistry. John Wiley, New York, pp 104–169

Sternini C, Anderson K, Brecha N (1986) Colocalisation of calcitonin gene-related peptide immunoreactivity with substance P in the enteric nervous system of the guinea pig. Gastroenterology 90:1648

Su HC, Bishop AE, Power RF, Hamada Y, Polak JM (1987) Dual intrinsic and extrinsic origins of CGRP- and NPY-immunoreactive nerves of gut and pancreas: Extrinsic CGRP-immunoreactive sensory neurons project mainly from dorsal root ganglia at T_5-S_1 and NPY-immunoreactive neurons from inferior mesenteric and coeliac ganglia. J Neurosci (in press)

Sundler F, Maghimzadeh E, Hakanson R, Ekelund M, Emson P (1983) Nerve fibres in the gut and pancreas of the rat displaying neuropeptide Y immunoreactivity. Cell Tiss Res 230:487–493

Sundler F, Hakanson R, Ekblad E, Uddman R, Wahlestedt C (1986) Neuropeptide Y in the peripheral adrenergic and enteric nervous systems. Int Rev Cytol 102:243–269

Tafuri WL, Maria TA, Pittella JE, Bogliolo L (1974) An electron microscopic study of the Auerbach's plexus and determination of substance P of the colon in Hirschsprung's disease. Virch Arch (Pathol Anat Physiol) 362:41–50

Terenghi G, Zhang SQ, Unger WG, Polak JM (1986) Morphological changes of sensory CGRP-immunoreactive and sympathetic nerves in peripheral tissues following chronic denervation. Histochemistry 86:89–95

Thompson RJ, Doran JF, Jackson P, Dhillon AP, Rode J (1983) PGP 9.5 – a new marker for vertebrate nervous and neuroendocrine cells. Brain Res 278:224–228

Tsuto T, Okamura H, Fukui K, Obata-Tsuto HL, Terubayashi H, Yanagihara J, Iwai N, Majima S, Yanaihara N, Ibata Y (1985) Immunohistochemical investigations of gut hormones in the colon of patients with Hirschsprung's disease. J Paediatr Surg 20:366–270

Vinores SA, May E (1985) Neuron-specific enolase as an immunohistochemical tool for the diagnosis of Hirschsprung's disease. Am J Surg Pathol 9:281–285

Webster W (1973) Embryogenesis of the enteric ganglia in nor-

mal mice and in mice that develop congenital aganglionic megacolon. J Embryol Exp Morphol 30:573–585
Whitehouse FR, Kernohan JW (1948) Myenteric plexus in congenital megacolon. Archiv Int Med 82:75–111
Wiesenfeld-Hallin T, Hökfelt T, Lundberg JM, Forssmann WG, Reinche M, Tschopp P, Fisher J (1984) Immunoreactive calcitonin gene-related peptide and substance P coexist in sensory neurons to the spinal cord and interact in spinal behavioral responses of the rat. Neurosci Lett 52:199–204
Zhang SQ, Terenghi G, Unger WG, Ermis KW, Polak JM (1984) Changes in substance P- and neuropeptide Y-immunoreactive fibres in rat and guinea pig irides following unilateral sympathectomy. Exp Eye Res 39:365–372
Zuelzer WW, Wilson JL (1948) Functional intestinal obstruction on congenital neurogenic basis in infancy. Am J Dis Child 75:40–45

Accepted April 24, 1987