B. J. GANNON *ET AL.*: ADRENERGIC INNERVATION OF BOWEL IN HIRSCHSPRUNG'S DISEASE

FIG. 1.—Fluorescent histochemical localization of catecholamines in nerves in the muscle layers of the human colon in Hirschsprung's disease. One hour's incubation with paraformaldehyde at 80° C. A, C, and E are tissue samples removed at operation. B, D, and F are suction biopsy samples. Horizontal bar = 100 μ .

(A) Transverse section of muscularis externa of normal bowel above the aganglionic zone. Circular (c) and longitudinal (l) muscle layers of normal bowel. A few nerve fibre bundles (n) can be seen cut transversely in the circular layer.

(B) Transverse section of mucosa (m) and submucosa (sm) of normal bowel from a control patient showing a non-fluorescent ganglion (g) surrounded by fluorescent terminals, mixed nerve bundles (mnb), and varicose fibres traversing the muscularis mucosae (mm) to the mucosa (m). Note the enterochromaffin cells (ec) in the mucosal epithelium.

(C) Circular and (E) longitudinal muscle of distal aganglionic segment heavily innervated by nerve bundles containing fluorescent varicose fibres (nb).

(D) Transverse section of mucosa from distal aganglionic segment. Large number of nerve bundles (nb) containing fluorescent fibres run in the muscularis mucosae (mm) and some in the submucosa (sm).

(F) Frontal section of muscularis mucosae from distal aganglionic segment. Note the plexus of small to medium nerve bundles (nb) in the plane of the muscularis mucosae varicose fibres (f); and blood vessel (v) and nerve trunk (t) in superficial submucosa.

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FIG. 2.—Regional differences in adrenergic innervation of bowel in one patient (2 years) with Hirschsprung's disease as shown by the fluorescent histochemical method for localization of catecholamines. A, C, D, and F are samples of muscle removed at resection. B, E, and G are suction biopsy samples. Horizontal bar = 100 μ .

(A) Normal bowel above aganglionic zone. Note fluorescent terminals about non-fluorescent ganglion cells (ft) in Auerbach's plexus. There are no adrenergic nerves in circular (c) or longitudinal (l) muscle.

(B) Normal bowel above aganglionic zone. Note fluorescent terminals about non-fluorescent ganglion cells (ft) in Meissner's plexus and the mixed nerve bundles (mnb) connecting them. The muscularis mucosae (mm) is not innervated by adrenergic nerves.

(C) Muscle from proximal aganglionic zone. Note some varicose nerve fibres (f) in longitudinal (l) and circular (c) coats.

(D) Large faintly fluorescent nerve trunks (nt) in Auerbach's plexus replacing ganglion cell groups normally seen.

(E) Transverse section of biopsy specimen from proximal aganglionic zone. Note that there is some innervation of the muscularis mucosae (mm) and the incidence of fluorescent nerve bundles (nb) in the submucosa (sm).

(F) Innervation of circular (c) and longitudinal (l) muscle layers from distal aganglionic region by fluorescent nerve bundles (nb). Note that these are denser than in proximal region. Note fluorescent nerve trunk (nt) in the position normally occupied by Auerbach's plexus.

(G) Oblique transverse section of biopsy specimen from distal aganglionic zone. Note the massive innervation of muscularis mucosae (mm) by fluorescent nerve bundles (nb).

Preliminary Communications

Adrenergic Innervation of Bowel in Hirschsprung's Disease

[WITH SPECIAL PLATE BETWEEN PAGES 330 AND 331]

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Summary: The adrenergic innervation of normal and aganglionic regions of bowel from patients with Hirschsprung's disease was investigated by a fluorescent histochemical technique. In normal bowel the adrenergic nerves end about intramural ganglion cells. In aganglionic bowel the adrenergic nerves form a dense varicose plexus in both muscularis externa and muscularis mucosae. It is suggested that the cause of megacolon in Hirschsprung's disease is due to a lack of nervous pathways controlling the intrinsic reflexes, which is probably congenital in origin.

INTRODUCTION

The involvement of the nervous system in Hirschsprung's disease has been the subject of increasing investigation since the reports of the absence of nerve cell bodies in Auerbach's plexus in the segment of bowel distal to the megacolon.^{1,2} Some authors³ suggested that the bowel constriction in Hirschsprung's disease may be the result of an associated aplasia of the sympathetic nerves which normally inhibit the bowel.

In normal bowel the majority of adrenergic fibres in the gut wall have been shown with the fluorescent histochemical technique for the localization of monoamines⁴ to form terminal networks about the nerve cell bodies in the enteric plexuses, both in man⁵⁻⁸ and in other mammals.⁹⁻¹¹ As enteric ganglion cells are absent in the spastic segment of Hirschsprung's disease, an investigation of the pattern of adrenergic innervation in this tissue has been undertaken with the fluorescence method. Reports by other investigators using this technique have given evidence for an abnormal adrenergic innervation of the muscularis externa⁸ as well as lack of terminal networks in the region of the plexuses.^{5,6,8}

The present work, while confirming these observations, presents evidence of an associated pathological innervation of the muscularis mucosae and submucous plexus which has not been reported previously. In addition, regional differences in the aberrant innervation of the aganglionic segment are reported. These observations provide the basis of an additional non-traumatic diagnostic procedure which may be useful in young children.¹³ The pattern of the innervation of the aganglionic segment is discussed with reference to the normal innervation of the mammalian bowel.

MATERIALS AND METHODS

As a screening procedure for Hirschsprung's disease rectal suction biopsy specimens obtained by using a technique recently described¹⁴ were taken at distances of 1-6 cm. (usually 4 cm.) above the anorectal line from 29 young patients suffering from constipation. Hirschsprung's disease was diagnosed by histological¹⁵⁻¹⁸ and histochemical¹³ criteria in nine patients and ruled out in the other 20. In all, 53 specimens, each comprising one to three biopsies, were examined.

In those cases of Hirschsprung's disease with colostomies established suction biopsy specimens were taken from normal bowel above and from aganglionic bowel a short distance below the colostomy. Full-thickness biopsy specimens were taken from bowel removed at Duhamel resection and at colostomy closure.

To prevent diffusion of catecholamines from their cellular sites all samples were rapidly frozen by immersion in liquid propane (cooled with liquid nitrogen) and the water was sublimed by freeze-drying for 8 to 30 hours at -35°C . and a vacuum of 10^{-7} torr. After returning the tissues to room temperature the specimens were incubated for one hour in formaldehyde vapour at 80°C . (at a relative humidity of 70%¹⁵) to produce the highly fluorescent fluorophore characteristic of catecholamines condensed with formaldehyde.⁴ The tissue was embedded in 63°C . m.p. paraffin wax in vacuo and sectioned at 8-10 μ , the sections being mounted on a hot paraffin oil film on a glass slide. The sections were observed with a Leitz Ortholux fluorescent microscope equipped with a heated stage to prevent wax crystal formation. Dark-field illumination was from an Osram HBO 200 high-pressure mercury lamp with a 410-mm. excitation filter and a 530-mm. barrier filter. Selected areas were photographed with a Leitz Orthomat microscope camera on Kodak Tri-X film.

In studies of the adrenergic innervation of tissues the presence of highly fluorescent beaded or varicose nerves is interpreted as evidence of a functional innervation—for example, in the heart, vas deferens, and iris. The varicosities of adrenergic nerves contain high levels of noradrenaline and are thought to be the points of transmitter release for nervous transmission. Preterminal nerves do not have this varicose appearance.

RESULTS

MUSCULARIS EXTERNA AND MYENTERIC PLEXUS

Ganglionic Bowel.—In samples of muscle taken proximal to the aganglionic segment there were almost no fluorescent fibres seen in the circular and longitudinal muscle coats (Special Plate, Figs. 1A and 2A). Groups of large numbers of non-fluorescent ganglion cells in the myenteric plexus showed pericellular networks of fluorescent nerve terminals (Fig. 2A). No adrenergic nerve cell bodies were seen in the myenteric plexus.

Aganglionic Segment.—There were no fluorescent varicose pericellular terminals in the region of Auerbach's plexus as seen in normal tissue. Histological analysis of the same or alternate sections confirmed the absence of nerve cell bodies. Large nerve trunks, some of which contained faintly fluorescent, non-varicose, preterminal nerve fibres, were seen in the site normally occupied by the myenteric ganglia (Special Plate, Fig. 2D—compare with normal plexus, Fig. 2A). While in the most proximal region of the aganglionic segment the innervation of the muscle layers was not noticeably different from controls, in the distal aganglionic region both longitudinal and circular layers of the muscularis externa contained considerable numbers of fluorescent nerve fibre bundles, which were not seen in normal tissue (compare Figs. 1C and 1E with normal muscle Fig. 1A). Varicose fibres could be seen in the smaller nerve bundles.

INNERVATION OF SUBMUCOSA AND MUCOSA

Normal Bowel.—Samples taken from above the aganglionic segment and suction biopsy specimens taken from control

patients showed small groups of non-fluorescent ganglion cells in the superficial submucosa surrounded by pericellular networks of fluorescent nerve fibres (Figs. 1B and 2B). These groups were connected by mixed nerve bundles containing both fluorescent varicose and non-fluorescent nerve fibres. Similar mixed nerve bundles, but occasionally larger ones, were seen deeper in the submucosa. Blood vessels in the submucosa exhibited a fluorescent perivascular adventitial plexus typical of normal tissue. Non-specific fluorescence of connective tissue was not a problem in the material from young individuals, unlike the situation in adults, where it often obscures visualization of structures exhibiting specific fluorescence.⁷ Moderate non-specific fluorescence, however, was observed in submucosal connective tissue from a 12-year-old patient, and some in samples from a 5-year-old patient. Some fluorescent varicose nerve fibres were seen in the muscularis mucosae of normal bowel, but these were usually associated with small blood vessels and oriented at right angles to the plane of the muscularis mucosae, apparently in transit to the mucosa (Fig. 1B).

Aganglionic Segment.—The absence of basket-like pericellular networks around non-fluorescent ganglion cells of Meissner's plexus was apparent. The small mixed nerve bundles seen in control tissue were absent but great numbers of highly fluorescent nerve bundles of varying size containing a higher proportion of fluorescent fibres were seen (compare Fig. 1D with control field Fig. 1B, and Fig. 2E with normal Fig. 2B). The varicose nature of many of the fibres was discernible only in the smallest of these nerve bundles (see Fig. 1F), probably because of the large number of fibres packed closely together. Some larger nerve trunks, seen deeper in the submucosa, contained large numbers of fluorescent fibres, some of which appeared to be varicose. These nerve bundles were hypertrophied as compared with those seen in normal tissue. The perivascular plexus about submucosal vessels also appeared to be hypertrophied when compared with that about vessels of normal bowel. Fluorescent nerve fibre bundles were seen throughout the muscularis mucosae which were not seen in normal tissue (compare Fig. 1D with normal tissue Fig. 1B). In the distal region of the aganglionic segment a massive plexus of small fluorescent nerve bundles was present in the plane of the muscularis mucosae (Fig. 1F). In the smaller nerve bundles varicose fibres could be distinguished. In summary, in the distal aganglionic region all muscle layers were heavily and abnormally innervated by fluorescent nerve fibres.

MUSCLE LAYERS

In the muscularis externa and muscularis mucosae of normal or ganglionic bowel above the aganglionic segment very few fibres were seen in the muscle tissues, as stated above (Figs. 2A and 2B).

In the proximal region of the aganglionic segment no fluorescent terminals were seen around ganglion cells, and a few nerve fibre bundles were seen in the circular, longitudinal, and mucosal muscle coats (Figs 2C and 2E). In the distal region of the aganglionic segment from the same patient a much greater number of fluorescent nerve bundles were seen in all three muscle coats (Figs. 2F and 2G). Thus there was a proximodistal (oral-aboral) gradient in the density of the aberrant adrenergic innervation of all muscular layers.

DISCUSSION

The absence of ganglion cells in the myenteric plexus of the aganglionic segment in Hirschsprung's disease is accompanied by aganglionosis of the submucous plexus.^{2 16-18} Evidence has been presented that in normal development the submucous

plexus arises by neuroblast migration from the myenteric plexus.^{19 20}

Thus agenesis of myenteric ganglia will cause absence of submucous ganglia, though absence of submucous ganglia may not necessarily indicate absence of myenteric ganglia. In cases exhibiting the clinical symptoms of Hirschsprung's disease, however, aganglionosis of the submucous plexus has been used to diagnose the disease.^{2 18}

In the present study it was found that the lack of fluorescent terminals about ganglion cells of the myenteric plexus in the aganglionic segment of Hirschsprung's disease^{5 6 8 12} is accompanied by a similar deficiency in the submucous plexus. Furthermore, the massive proliferation of adrenergic nerves into the muscularis externa of the aganglionic segment shown in the present work and by others⁸ is accompanied by a similar pathological innervation of the muscularis mucosae, not previously shown. Observation of this aberrant innervation of the muscularis mucosae in rectal section biopsy samples has been used as a non-traumatic corollary diagnostic method for the disease.¹³

The cause of the massive proliferation of sympathetic adrenergic nerves into the musculature of the aganglionic segment is as yet unknown. In normal development the adrenergic nerve fibre grows into synaptic contact with the intramural nerve cell body and then growth of the nerve fibre is arrested. In aganglionic bowel, however, where the ganglion cells are not present, the adrenergic nerve fibre continues to grow and invades the muscle tissue. These fibres, entering with the blood supply along the colon, grow distally towards the anal region. Thus in sections of aganglionic tissue a greater number of fibres is seen in the more distal samples of the segment. The intramural ganglion cells may release a locally effective antitrophic substance, inhibiting continued growth of nerve fibres which come into contact with them. Such a contact inhibition of cell growth has been reported in tissue culture of hamster fibroblast cells,²¹ where a macromolecular factor was implicated.²² The pathological adrenergic innervation of the muscle is reminiscent of the massive proliferation of sympathetic innervation seen in adrenergically innervated tissues of mice injected postnatally with sympathetic nerve growth factor.²³ Furthermore, an anti-nerve-growth factor has been prepared,²⁴ which when injected into newborn mice prevents normal development of the sympathetic nervous system.

The proliferation of the sympathetic nerves in Hirschsprung's disease seems to be accompanied by a comparable overgrowth of the parasympathetic nervous system. The cholinesterase levels of samples from aganglionic segment are higher than in normal bowel.³ At the ultrastructural level there is a much higher incidence of cholinesterase-staining nerve fibres in small bundles in the muscularis mucosae of aganglionic than of normal bowel, and cholinesterase-staining ganglion cells seen in normal tissue are, of course, absent in aganglionic tissue samples (J. Houchin, personal communication).

The explanation of the causation of the megacolon of Hirschsprung's disease in terms of denervation hypersensitivity⁵ (Cannon's law) is obviously unsatisfactory in view of these and other findings of innervation of the spastic segment,⁸ since the aganglionic segment in Hirschsprung's disease, at least towards the rectum, is much more heavily innervated than normal muscle, though these nerves are not those which innervate the muscle of normal bowel.

Fluorescent histochemistry of gut tissues has shown adrenergic nerve terminals around non-fluorescent ganglion cells in the bowels of mammals.^{5-13 25-27} With one exception all workers agree that adrenergic nerve cell bodies are not present in the enteric plexuses of mammals (including man). In one report which does claim adrenergic nerve cells in Auerbach's plexus of man²⁸ the micrographs suggest diffusion from fluorescent pericellular terminals into the perikaryon of non-adrenergic neurons during the histochemical procedure.

What then are the nervous elements missing in Hirschsprung's disease? The ganglion cells normally present in the enteric plexuses have been generally thought to be postganglionic parasympathetic neurons, innervated by cholinergic preganglionic parasympathetic fibres. Studies of cholinesterase-staining properties of enteric neurones in the cat,²⁹ however, have shown heavily stained, lightly stained, and almost unstained neurones in the ratio of about 1:1:9, and cytological studies indicate at least two and possibly three different morphological types.³⁰⁻³¹ Physiological and pharmacological studies in this laboratory³²⁻³³ and elsewhere³⁴⁻³⁶ have indicated inhibitory neurones of a non-adrenergic type in the gut of mammals and man.

Bowel from which the extrinsic nerves had been severed several days previously continued to exhibit a peristaltic reflex when stroked with moist cotton-wool.³⁷⁻³⁸ Thus it seems that all the nerve cell bodies for this reflex are present in the enteric plexuses. In Hirschsprung's disease there seems to be a disturbance of sensory as well as motor innervation, since children with the disease often do not have normal sensation [of the bowel], and even if the segment is resected they may never have the normal desire to defecate.³⁹ Thus it seems that in Hirschsprung's disease there are several abnormalities of the enteric innervation, which include:

(1) Absence of intramural postganglionic cholinergic parasympathetic excitatory nerve cell bodies.

(2) Absence of intramural postganglionic non-adrenergic inhibitory nerve cell bodies. These are innervated by vagal preganglionic fibres in the stomach.⁴⁰⁻⁴² Work in this laboratory, however, indicates that non-adrenergic neurones in the colon form part of a completely intrinsic reflex.⁴³

(3) Absence of intramural sensory neurone cell bodies.

(4) Pathological proliferation of postganglionic sympathetic adrenergic and parasympathetic preganglionic cholinergic nerve fibres into the muscle layers, both of which normally end on intramural ganglion cells.

It is suggested that the cause of megacolon in classic Hirschsprung's disease—that is, complete aganglionosis of a section of bowel—is due not to denervation hypersensitivity of the aganglionic zone (Cannon's law),⁴⁴ as suggested by some workers,⁶ but to a lack of nervous pathways controlling intrinsic reflexes. The condition apparently arises in the foetus from lack of normal migration of neuroblasts into the bowel in a particular region. In a more mature individual it is manifested by an absence of at least three types of neurones (interneurones may also be lacking), which in normal tissue would have differentiated from these neuroblasts. All these nerve components are required for the normal co-ordinated reflex contraction and relaxation of the bowel wall which results in peristaltic propulsion of lumen contents. The aberrant adrenergic and cholinergic innervation of the aganglionic segment is pathological and of a secondary nature.

Other disorders of bowel content propulsion, including idiopathic megacolon and pseudo-Hirschsprung's disease, may be explicable in terms of absence or hypertrophy of one or more of the different enteric neurone types involved in the peristaltic reflex. It is hoped that histochemical techniques for distinguishing between these neurones at the light or electron-microscope level will be developed.

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