

Increased Submucosal Nerve Trunk Caliber in Aganglionosis

A "Positive" and Objective Finding in Suction Biopsies and Segmental Resections in Hirschsprung's Disease

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• **Objective.**—To establish the diagnostic usefulness of submucosal hypertrophic nerve trunk morphology in Hirschsprung's disease as a quantifiable parameter supportive of aganglionosis on hematoxylin-eosin-stained sections.

Design.—We retrospectively evaluated size and density of submucosal nerves on hematoxylin-eosin-stained sections and S100 protein-stained sections of resected segments from 13 patients with Hirschsprung's disease, and in sections of 20 aganglionic and 50 ganglionic rectal suction biopsies.

Setting.—All patients were seen at Childrens Hospital Los Angeles (Calif), a tertiary-care pediatric center; the age of patients at diagnosis or resection ranged between 2 days and 3 years.

A frequent and important role of surgical pathologists is the evaluation of rectal suction biopsies (RSB) and open seromuscular biopsies to determine adequacy of intestinal innervation in children with constipation. As we advance our understanding and classification of Hirschsprung's disease (HD) and other gastrointestinal innervation disorders, a thorough assessment of morphologic features is important to better define the spectrum of HD and other dysganglionoses. The use of adequate RSB, a method whose high degree of accuracy has been proven over the last 30 years, has greatly facilitated the evaluation of children with disorders of gastrointestinal motility.¹⁻⁹ Criteria for evaluation of RSB vary among institutions and even among pathologists, depending on individual preference and experience.¹⁰ The evolving approach to a one-step procedure in some patients with HD by our surgical colleagues, as well as current economic health management issues, make the attainment of a rapid and reliable diagnosis of abnormal innervation highly desirable.^{11,12}

Since the absence of ganglion cells is still the standard for diagnosis of HD, an objective additional criterion apparent in hematoxylin-eosin (H&E)-stained sections

Results.—Aganglionic segments contain many distinct nerve trunks greater than 40 μ m in diameter. Ganglionic segments/biopsies showed no nerve trunk larger than this threshold value ($P \sim .0000$). Nerve trunks of such caliber are rarely encountered in pathologic transition zones and sites of colostomy.

Conclusions.—Submucosal nerve trunks that are 40 μ m or greater in diameter strongly correlate with abnormal innervation/aganglionosis. Use of this objective parameter in evaluating suction biopsies should be helpful in the morphologic diagnosis of Hirschsprung's disease in infancy and early childhood.

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would be of diagnostic utility. The presence of submucosal "hypertrophic" or "prominent" nerve trunks is consistently recognized, as has been shown in recent publications with the use of immunohistochemistry.^{13,14} The present study confirms the diagnostic value of this important criterion on a quantitative basis.

MATERIALS AND METHODS

All cases were retrieved from the archives of the Childrens Hospital Los Angeles (Calif). Thirteen consecutive patients with HD confirmed by pull-through procedure were studied. The patients ranged in age from 2 days to 3 years at the time of suction biopsy, and from 1 week to 3 years at the time of pull-through procedure. The extent of involvement in all patients was limited to the rectosigmoid region and/or colon distal to splenic flexure. Only the most distal portions of the mucosal sleeves and the most proximal ganglionic region, either from the stoma or as a separate specimen identified as "distal end of pull-through," were studied. All tissues were fixed in formalin and embedded in paraffin. To ensure that only nerve structures were measured, deparaffinized 5- μ m sections were stained with S100 protein immunohistochemical stain (Dako Corporation, Carpinteria, Calif), using an automated stainer according to established protocols.

The H&E slides of rectal suction biopsies negative for ganglion cells from 20 consecutive patients (including the 13 with pull-through procedures) and the suction biopsy slides from 50 consecutive patients with ganglion cells identified in the submucosa were reviewed. For morphometric determination, only 1 section was evaluated; for validation of obtained parameters, all available serial sections were examined.

The nerve trunks were quantified by one observer (H.M.). Only clearly defined nerves in the H&E-stained specimens and nerve structures identified with S100 antibody in resection segments

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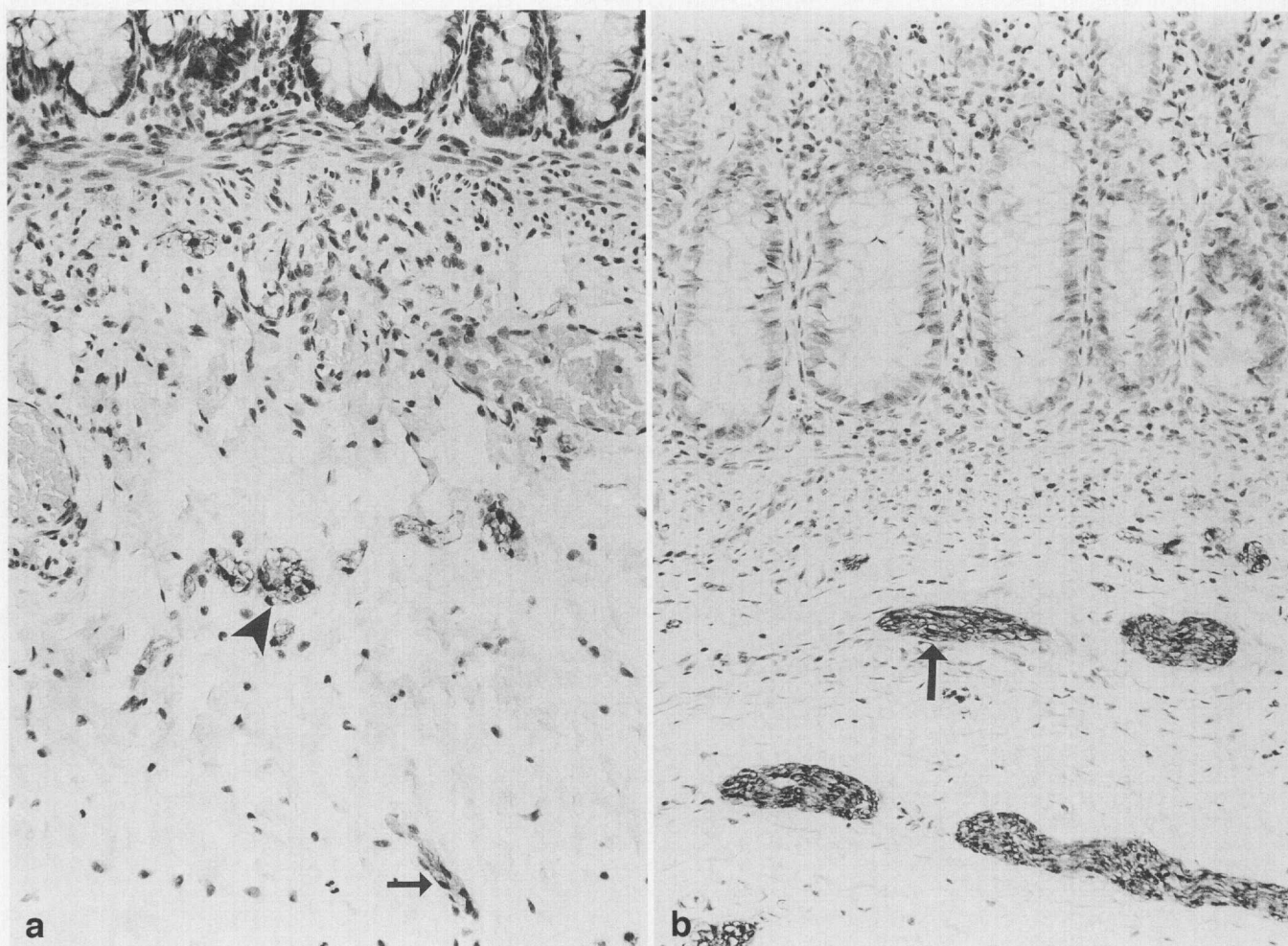


Fig 1.—*a*, Ganglionic submucosa with inconspicuous thin nerve fibers (arrow) and ganglion cell cluster (arrowhead) of the submucosal plexus. *b*, Aganglionic submucosa. Note readily apparent nerve trunks of varying calibers, including many that are 40 μm in diameter (arrow) (S100, original magnification $\times 50$).

were evaluated. Their diameter was measured perpendicular to the direction of the well-oriented, parallel Schwann cells at the widest segment of the nerves. Their density was counted over contiguous 10-mm² segments of colon; a segment of nerve surrounded by connective tissue counted as 1 nerve. Determination of total area of submucosa in an H&E-stained section of RSB specimens and the number of nerves were similarly counted.

Measurements and counts were performed using ocular reticles and were calibrated with an objective micrometer (Olympus, Tokyo, Japan) using an Olympus BH-2 microscope with S-Plan objectives was wide-field 10 $\times$ ocular eyepieces. Nerve trunks were tabulated by their caliber into groups of fibers equal to or larger than 10, 20, 40, and 60 μm .

The mean, median, and standard deviation (SD) were calculated for each fiber size group in both specimen types. A one-sample *t* test was performed to determine whether the mean number of nerve trunks greater than 40 μm was statistically different from zero for both the biopsy and resection specimens.

RESULTS

The segments of colon showed striking differences in the submucosal plexus in ganglionic versus aganglionic regions in every patient (Fig 1). Most nerve trunks in ganglionic areas appeared inconspicuous and simple. The vast majority has a diameter between 10 and 20 μm . The largest single nerve trunk measured 32 μm . The density of these nerve trunks ranged from 23 to 34 nerves/10 mm²,

with an average of 27.5 nerves/10 mm² (median 26, SD 3.7). No nerve trunk greater than 40 μm in diameter was identified in the ganglionic segments of colon. The nerves in the aganglionic segments were more numerous, in part owing to their marked tortuosity, and their caliber varied greatly, ranging from 10 to 100 μm (Fig 2). There was increased density in the aganglionic segments, ranging from 23 to 183 nerves/10 mm²; the average density was 83.5 nerves/10 mm² (SD 38.1) (Fig 3). The mean number of nerve trunks greater than 40 μm was 20.6, the median 19, the SD 10.4, and standard error of the mean (SEM) 2.9 nerves/10 mm². In transition (aganglionic-ganglionic) zones, the nerves were less numerous but similarly enlarged and associated with sparse density of ganglion cells.

The ganglionic RSB specimens showed findings similar to those of the resected segments. No nerve trunk larger than 40 μm could be found in the multiple serial sections examined (Fig 4). All aganglionic suction biopsies showed increased numbers of nerves with striking nerve trunk hypertrophy, and 90% contained nerve trunks greater than 40 μm in diameter. The mean number of nerve trunks larger than 40 μm was 3.1 (SD 2.6, SEM 0.6). Presence of nerve trunks of this size in the submucosa of rectosigmoid

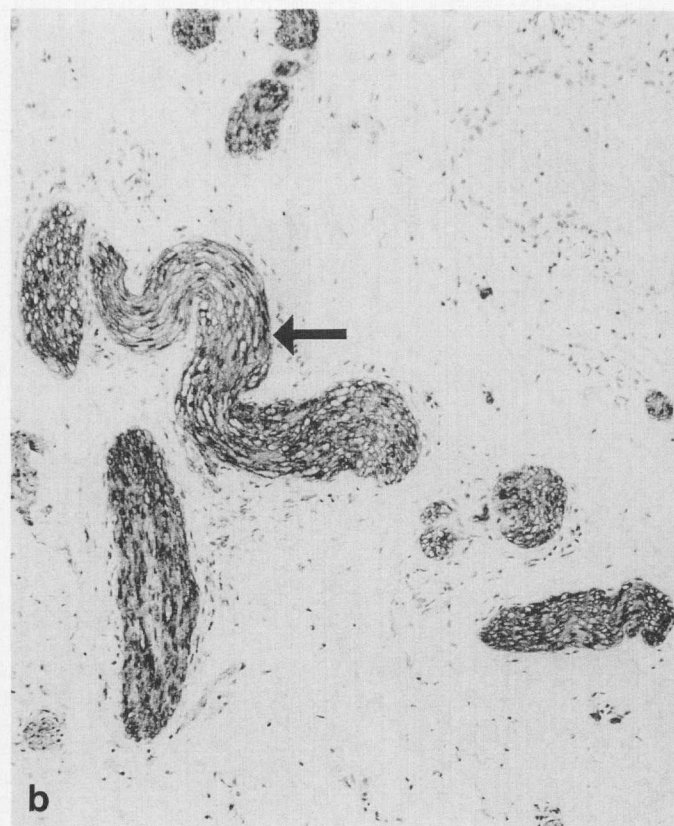
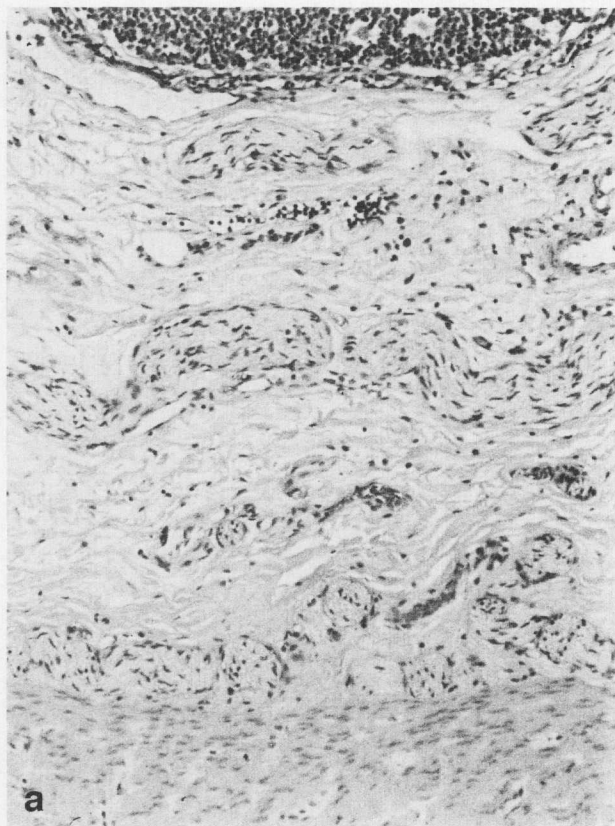


Fig 2.—a, Hypertrophic submucosal nerve trunks are readily apparent and quantifiable on hematoxylin-eosin-stained sections. Tortuous course of nerve cut at different planes accounts in part for apparent increased density (original magnification $\times 25$). b, Spectrum of hypertrophic nerves in deep submucosa; arrow points to nerve segment that is $80\ \mu\text{m}$ in diameter. The direction of the arrow represents the plane of measurement for all nerves evaluated (S100, original magnification $\times 25$).

colonic sections appears to be appreciably supportive of the diagnosis of HD.

The means of nerve trunk numbers of aganglionic intestine showing nerve trunks greater than $40\ \mu\text{m}$ are significantly different from zero ($P \sim .0000$) for both biopsy and resection specimen observations.

COMMENT

Correctly assessing the presence or absence of submucosal ganglion cells is consistently reliable if the surgical pathologist is aware of structures resembling ganglion cells and other pitfalls of RSB interpretation. The number of paraffin section levels of RSB for a "thorough" evaluation can be up to several hundred,^{8,9} making determinations time-consuming. The search for a positive parameter has led to the use of enzyme histochemistry for acetylcholinesterase as evidence of abnormal innervation and to the consideration of various immunohistochemical markers to highlight otherwise "inconspicuous" ganglion cells. The acetylcholinesterase stain warrants a separate frozen tissue mucosal specimen, and the procedure is technically difficult, requiring experience in its performance. Moreover, the interpretation of acetylcholinesterase requires experience and awareness of the variable patterns and their pitfalls. For those institutions that routinely utilize enzyme histochemistry for acetylcholinesterase, it is the most valuable ancillary procedure in the evaluation of HD.^{15,16} While the use of immunohistochemistry for the study of the enteric nervous system is invaluable, its use in RSB is generally impractical. S100 protein antibody was

used primarily in this project to expedite and validate nerve caliber measurement; it is not necessary for routine use, since nerves are readily apparent on H&E-stained specimens. The question of whether a cell represents a ganglion cell requires confirmation or exclusion by using neuronal versus endothelial, histiocytic, and myogenic markers on a specific or on contiguous levels of the suction biopsy to ensure that the cell or cells in question are addressed. In our opinion, if a cell does not exhibit conclusive ganglion cell features by H&E, it should be best interpreted as one of the alternatives. Furthermore, if only 1 ganglion cell is noted in an otherwise adequate specimen, additional specimens should be requested before rendering an interpretation of "normal innervation," particularly when the cell coexists with abnormal-appearing nerve trunks.

The frequent presence of hypertrophic $40\text{-}\mu\text{m}$ nerve trunks in aganglionic segments of rectosigmoid and descending colon makes this parameter a strong indicator that the lack of ganglion cells is not due to sampling or lack of visualization, but that a real abnormality of innervation is represented in the suction biopsy specimen.

Nerves narrower than $10\ \mu\text{m}$ are difficult to identify and evaluate by H&E, those greater than $20\ \mu\text{m}$ are readily discerned, and nerves that reach the $40\text{-}\mu\text{m}$ diameter threshold comprise the majority of those that we refer to as prominent or hypertrophic (Fig 2, a). Nerves of this size can be found in aganglionic segments as seen in this study, in transition aganglionic-ganglionic zones in HD, at

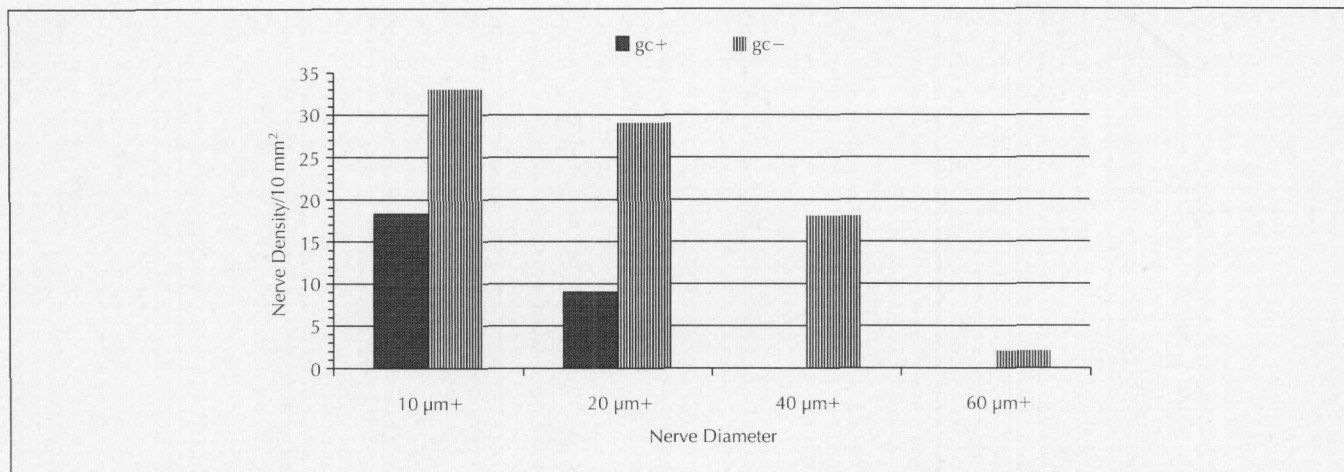


Fig 3.—Tabulation of submucosal nerves in colonic segments demonstrating marked differences in density and spectrum of nerve trunk diameters between ganglionic and aganglionic regions. No nerve trunk greater than 40 µm was identified. gc+ indicates ganglionic; gc-, aganglionic.

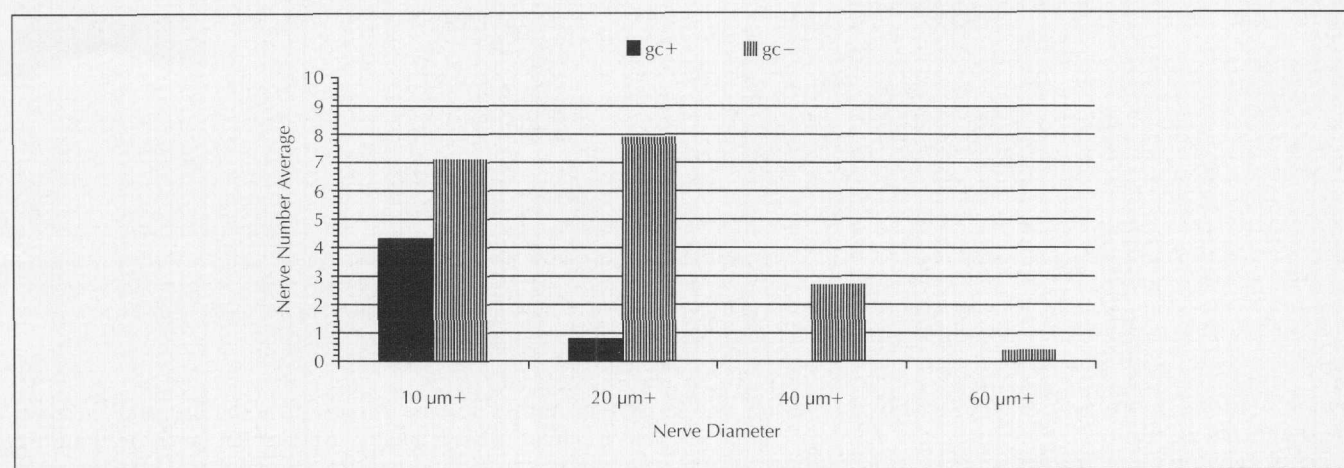


Fig 4.—Tabulation of submucosal nerves in rectal suction biopsy specimens (20 aganglionic and 50 ganglionic) showing similar fiber size distribution as seen in colonic segments. Note disparity in number of nerves greater than 20 µm in diameter. Graph also depicts that rectal suction biopsy material is truly representative of the alterations seen in HD. gc+ indicates ganglionic; gc-, aganglionic.

sites of enterostomy, or at other reparative sites, such as postoperative biopsies in patients with imperforate anus. Reports discussing the pathophysiology of innervation abnormalities in HD¹⁷⁻²¹ show that hypertrophic nerves in aganglionic segments are cholinergic nonmyelinated fibers of extrinsic origin that end blindly in the intestine, as has been demonstrated in intestinal whole-mount studies and indirectly by documenting reduction in synaptic vesicle protein in HD.¹⁷⁻²⁰ The colon distal to the splenic flexure is innervated by the inferior mesenteric and sacral nerves. Various studies have shown that the tortuous and hypertrophic nerves in aganglionic segments show increased acetylcholinesterase activity and absence of adrenergic markers. The concept that these hypertrophic nerves represent unregressed pathways for neuroblast migration during fetal development may be valid.²¹ Recent advances in our understanding of the intestinal microenvironment facilitating colonization by ganglion cell precursors²² theoretically suggest that these nerves remain under the influence of local factors. Evidence implicating nerve growth factor has been reported recently.²³

In evaluating RSB, it becomes apparent that nerve

trunks 40 µm or greater in diameter are highly predictive of aganglionosis and are an integral part of the structural abnormalities of HD involving the descending colon and rectosigmoid. In our material, they were present in 90% (n = 18) of aganglionic rectal suction biopsies and in 100% of resected HD segments distal to the splenic flexure. Although variable, nerve trunks in total colonic aganglionosis are sparse and may be inconspicuous in the rectosigmoid, making 40-µm nerves less useful (M. Reyes-Múgica, MD, oral communication, March 1996). However, we have seen examples of total colonic aganglionosis with sparse but distinct hypertrophic nerve fibers in rectal submucosa biopsy material.

These data strongly advocate use of this parameter as an important ancillary feature for the diagnosis of HD. Additional detailed morphologic studies will be of value in refining diagnostic criteria for HD and other intestinal innervation disorders.

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