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Calretinin Immunohistochemistry versus Acetylcholinesterase Histochemistry in the Evaluation of Suction Rectal Biopsies for Hirschsprung Disease

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ABSTRACT

Diagnosis of Hirschsprung disease (HSCR) relies on histologic and/or histochemical staining of sections from suction rectal biopsies. Acetylcholinesterase histochemistry (AChE) facilitates diagnosis but is not universally employed, in part because it requires special tissue handling. Calretinin immunohistochemistry (IHC) may be a useful alternative, because loss of calretinin immunoreactive nerves reportedly correlates spatially with aganglionosis. We investigated the patterns of calretinin IHC in suction rectal biopsies from HSCR and non-HSCR patients and compared the diagnostic value of calretinin IHC with a widely used rapid AChE method. In suction rectal biopsies that contain ganglion cells, small nerves in the lamina propria, muscularis mucosae, and superficial submucosa contain granular aggregates of calretinin immunoreactivity. Immunolabeling of these nerves is completely absent in the aganglionic biopsies of HSCR patients. Multiple observers independently reviewed calretinin IHC and AChE sections of suction rectal biopsies from 14 HSCR patients and 17 non-HSCR controls. Five observers, blinded to the correct diagnosis, scored each patient's calretinin IHC and AChE slides as HSCR, not HSCR, or equivocal. The frequencies of major and minor discrepant diagnoses were compared. Calretinin IHC yielded no misdiagnoses or major discrepancies between observers. In contrast, 2 misdiagnoses and significantly more interobserver disagreement resulted from the AChE-stained sections. Calretinin IHC appears to be a reasonable, and potentially superior, alternative to AChE as an adjunctive diagnostic method for evaluating suction rectal biopsies for HSCR.

Key words: acetylcholinesterase, aganglionosis, calretinin, Hirschsprung disease, rectal biopsy

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INTRODUCTION

Hirschsprung disease (HSCR) is a congenital malformation characterized by absent submucosal and myenteric ganglion cells in the distal rectum and a variable length of contiguous bowel [1,2]. The disease affects 1 in 5000 individuals [3,4], the majority of whom present during infancy with signs of intestinal obstruction [5]. The severity of intestinal dysmotility varies considerably, and some patients are diagnosed later in life, usually with a chief complaint of chronic constipation [5,6].

Hirschsprung disease is typically diagnosed or excluded by suction rectal biopsy, a procedure that samples a 2- to 4-mm nodule of rectal mucosa and underlying submucosa [7]. Different approaches are used to evaluate suction rectal biopsies (such as histochemical analysis of frozen sections [8]), but the foundation for diagnosis or exclusion of HSCR in most practices is microscopic examination of hematoxylin and eosin (H&E)-stained sections. Positive identification of one or more submucosal nerve cell bodies (ganglion cells) excludes HSCR, whereas absence of ganglion cells in an adequate sample is considered diagnostic, particularly if multiple hypertrophic submucosal nerves (diameter $\geq$40 μ) are present [9]. However, adequate sampling is a serious concern for which limited published standards exist [10].

Several anatomic details complicate H&E-based histopathologic diagnosis of HSCR. First, the terminal rectum, 1 to 2 cm proximal to the dentate line (anorectal junction), is physiologically hypoganglionic in many otherwise normal individuals [11,12]. Histopathologic studies of autopsy samples from patients with no known intestinal dysmotility disorder established that ganglion cells in this region are extremely sparse and often singular (as opposed to grouped in ganglia). To avoid this normally hypoganglionic segment, clinicians are instructed to obtain a biopsy 2 cm proximal to the dentate line. However, the procedure is experience dependent, and it is not uncommon for pathologists to receive biopsies of the anus or anorectal junction. Second,

submucosal ganglia are relatively widely separated from one another, even proximal to the hypoganglionic zone, such that time-consuming preparation and evaluation of numerous H&E sections is required in some cases to identify unequivocal ganglion cells [7,10,13]. Finally, hypertrophic extrinsic nerves, which characterize the terminal rectum in most cases of HSCR, are not present in some patients and therefore are an incompletely sensitive marker of the malformation [9].

A number of ancillary methods have been introduced in efforts to simplify the challenges posed by H&E-based evaluation of suction biopsies. Perhaps the most widely applied ancillary technique is acetylcholinesterase histochemistry (AChE), which exploits the observation that the density and thicknesses of AChE-positive nerve fibers are significantly greater in the aganglionic rectal biopsies of most HSCR patients, particularly in the muscularis mucosae [14]. Since its original description, multiple methods for AChE have been introduced, including a "rapid" technique (rapid AChE) that is used by many laboratories [14]. The abnormal patterns of AChE in HSCR are qualitative and quantitative but not absolute [15–17]. They require experience to recognize and may be influenced by improper tissue handling and staining quality [18]. Acetylcholinesterase histochemistry is generally not used in other clinical contexts and requires a frozen specimen in addition to the paraffin-embedded biopsies for standard H&E evaluation. For these reasons, AChE is not universally available.

Immunohistochemistry is the other major ancillary approach utilized by some laboratories to complement H&E-based histopathology. Many different antigens have been investigated in this context (reviewed by Kapur [19]), most of which are expressed on both extrinsic and intrinsic nerves. The latter may facilitate recognition of ganglion cells but add little to the H&E-based interpretation of an experienced pathologist and do not overcome the sampling issues noted above. Calretinin is a possible exception.

Calretinin, a calcium-binding protein that may function as a calcium sensor/modulator [20], is expressed by a subset of submucosal and myenteric ganglion cells, some of which project nerve terminals into the mucosa [21,22]. Barshack and others [23] examined colonic resections from HSCR patients and reported that calretinin immunoreactivity correlated inversely with aganglionosis. In contrast to portions of the resections that contained ganglion cells, aganglionic segments completely lacked calretinin immunoreactivity in enteric nerves. The authors speculated that calretinin immunohistochemistry (IHC) might be a useful diagnostic adjunct in the evaluation of suction rectal biopsies [23].

In order to investigate the diagnostic utility of calretinin IHC, we conducted a comparative study between calretinin IHC- and rapid AChE-stained sections of suction rectal biopsies from the same cohort of HSCR patients and controls. In addition, we examined calretinin immunoreactivity in a series of "inadequate" biopsies that contained anal mucosa, anorectal mucosa, or insufficient submucosa to exclude HSCR based on examination of H&E sections alone. Our findings suggest that the sensitivity and specificity of calretinin IHC is equivalent, if not superior, to rapid AChE and that calretinin IHC may be informative when inadequate tissue is available to establish an H&E diagnosis.

MATERIALS AND METHODS

Case and Control Selection

Hirschprung disease cases (n = 18) and controls (n = 18; diagnosis of HSCR excluded based on H&E identification of ganglion cells) were identified from Children's Hospital and Regional Medical Center pathology reports of suction rectal biopsies that were evaluated between 2002 and 2007. Review of surgical pathology reports from resections following each biopsy diagnosis of HSCR confirmed all the biopsy diagnoses as correct. For the comparative study of rapid AChE versus calretinin IHC, archival rapid AChE-stained sections (4 per case, plus original HSCR and normal controls) and paraffin blocks were retrieved. Cases and controls were selected in a nonbiased manner. All of the HSCR cases along with the 18 most recent non-HSCR biopsies between December 2002 and December 2007 were retrieved. The AChE patterns were not reviewed prior to inclusion in the study, and no effort was made to select cases with "unusual" or "challenging" AChE results. Paraffin blocks were used to prepare calretinin IHC slides (2 sections per slide). Four HSCR cases and 1 control were excluded because insufficient tissue remained in paraffin to perform calretinin IHC.

To investigate calretinin immunoreactivity in non-HSCR biopsies considered "inadequate" for H&E diagnosis, a computerized search of the pathology records between 2002 and 2008 was conducted to identify suction rectal biopsies reported as "inadequate" because they contained either anal mucosa (squamous or transitional) or insufficient submucosa. Twenty-six cases were identified, 17 of which had sufficient residual paraffin-embedded tissue for calretinin IHC and were parts of multiple biopsy specimens in which other biopsies contained ganglion cells. The original H&E-stained sections from these cases were reviewed, and paraffin blocks corresponding to those cases were used to prepare calretinin IHC slides.

Acetylcholinesterase Histochemistry

Labels on the original rapid AChE slides from each of the HSCR and non-HSCR patients were replaced with unique identifiers. For each patient, the AChE set consisted of 4 sections from the patient's biopsy(ies) on 2 slides, along with the concurrent HSCR and normal controls (histochemically stained in parallel with the patient's sections). Our laboratory uses the rapid AChE protocol of Kobayashi and colleagues [24] with hematoxylin counterstain.

Calretinin Immunohistochemistry

Calretinin immunolocalization was performed using an automated immunostainer (Benchmark XT, Ventana Medical Systems, Tucson, AZ, USA). A rabbit polyclonal antibody (Zymed, Laboratories, San Francisco, CA, USA) was used under the immunostainer's "standard" conditions (60 minutes, cell conditioner 1) at a dilution of 1:100 and an

incubation time of 32 minutes. Slides were counterstained with hematoxylin.

Independent Acetylcholinesterase Histochemistry and Calretinin Immunohistochemistry Slide Review by Multiple Investigators

Five pathologists independently reviewed the rapid AChE and calretinin IHC slides and for each technique scored the case as HSCR, not HSCR, or equivocal. Equivocal diagnoses required a written explanation. Different code numbers were used for the AChE and calretinin IHC sets to prevent biased interpretations influenced by the results of the other method. All 5 observers had between 1 year (observer E) and 15 and 25 years (observers A–D) of experience interpreting rapid AChE stained sections as part of routine handling of suction rectal biopsies at our institution. Only 1 (observer A) of the 5 observers had prior experience interpreting suction biopsies with calretinin IHC. Therefore, in conjunction with the calretinin IHC slide set, observers were provided with a "training module" of 4 HSCR and 4 non-HSCR cases (representative calretinin-immunostained suction biopsies from patients who were not part of the study) along with the correct diagnosis for each training slide.

After all 5 pathologists had completed their independent reviews, cases with interobserver disagreements were reviewed simultaneously by the entire group to clarify reasons for different interpretations. In addition, calretinin-stained sections from 4 more HSCR patients and 12 more non-HSCR patients were acquired after completion of the comparative study to include in a general review of the immunostaining patterns observed in the 2 groups.

The study was conducted in accordance with federal patient privacy regulations and with approval from the center's institutional review board.

RESULTS

Comparative Study of Rapid Acetylcholinesterase Histochemistry and Calretinin Immunohistochemistry for the Diagnosis/Exclusion of Hirschprung Disease in Suction Rectal Biopsies

The interpretations of each pathologist are listed in Table 1, along with correct diagnoses based on analysis of H&E-stained sections. In general, interobserver agreement was good with either technique, in that all observers agreed about the diagnosis for the majority of the cases. Unequivocal diagnoses of either HSCR or not HSCR were made by the majority of observers for every case. A relatively small number of discrepancies resulted, primarily due to an equivocal reading by a single observer of a given case. No one observer appeared more prone to equivocal diagnoses than another, because equivocal interpretations were rendered by every observer for at least 1 example of each staining method. Interobserver agreement about equivocal diagnoses was poor for either method; 1 of 5 observers for 11 cases (9 AChE, 2 calretinin IHC), 2 of 5 observers for 4 cases (2 AChE, 2 calretinin IHC), and 3 of 5 observers for 1 case (AChE).

Equivocal and unequivocally incorrect diagnoses were more frequent with rapid AChE (Table 2). Reasons cited for equivocal diagnoses varied but included variable staining between the 4 AChE-stained sections in a given case, concern about the density (as opposed to the coarseness) of nerve fibers, and focal coarseness of nerve fibers in the muscularis mucosae. Two unequivocally incorrect diagnoses were rendered based on AChE interpretation. In 1 case with ganglion cells in H&E-stained sections (patient #17), AChE slides were interpreted correctly by 3 pathologists, read as unequivocal HSCR and equivocal by the 2 others (Fig. 1A,C,E,G). The AChE findings for a second case (HSCR patient #31) were correctly interpreted by only 1 pathologist; 2 read the same AChE sections as not HSCR, and 2 interpreted them as equivocal (Fig. 1B,D,F,H). Both of the major discrepancies resulted from an intermediate pattern of AChE staining in which the density and thickness of nerve fibers in the muscularis mucosae was in between the positive and negative controls. For both patients, observer A consistently interpreted this pattern as HSCR and other observers interpreted the pattern as either not HSCR or equivocal. No major discrepancies existed for the interpretations of the calretinin IHC from these 2 cases, although 2 observers felt the calretinin immunostaining in the HSCR biopsy of patient #31 was equivocal because large submucosal nerves (Fig. 1H, inset), but not smaller nerves elsewhere in the biopsy (Fig. 1H), contained some immunoreactivity, a pattern that was not represented in the "training module."

Interpretations of calretinin IHC in this series of biopsies were remarkably congruent for the 5 observers with no major discrepancies (unequivocally incorrect diagnoses) and only 6 of 155 (4%) equivocal readings. Reasons cited for equivocal findings were faint immunoreactivity of deep submucosal nerves (despite lack of any other neural staining) in HSCR patients #24 (Fig. 4) and #31 (Fig. 1H, inset), lack of nerve fiber staining (despite strong perikaryal staining of submucosal ganglion cells) in control #16 (Fig. 2C), and limited amount of submucosa (despite complete lack of immunoreactivity) in HSCR patient #25 (not shown).

In addition to the multiobserver blinded comparison discussed above, calretinin IHC sections from 59 additional suction rectal biopsies from 37 patients (4 HSCR, 33 not HSCR) were reviewed. The latter, combined with the comparative study cases, constitute our experience to date with calretinin IHC in this diagnostic context and form the basis for the following observations.

Calretinin Immunoreactivity in Biopsies That Contain Ganglion Cells

Although some neural calretinin immunoreactivity was found in every biopsy that contained ganglion cells, the density and distribution of calretinin immunoreactive fibers varied significantly. Most biopsies demonstrated moderate numbers of intensely immunolabeled nerve fibers in the full thickness of the submucosa, muscularis mucosae, and

Table 1. Comparative analysis of rapid acetylcholinesterase histochemistry (AChE) and calretinin immunohistochemistry (IHC)

Patient	Hematoxylin and eosin diagnosis[a]	Age/gender (male, female)	AChE[b]					Calretinin IHC[c]				
			A	B	C	D	E	A	B	C	D	E
1	Not HSCR	7 months/M	N	N	N	N	N	N	N	N	N	N
2	Not HSCR	3 days/F	N	N	N	N	N	N	N	N	N	N
3	Not HSCR	2 months/F	N	N	N	N	N	N	N	N	N	N
4	Not HSCR	25 days/M	N	N	N	N	N	N	N	N	N	N
5	Not HSCR	3 months/F	N	N	N	N	N	N	N	N	N	N
6	Not HSCR	28 days/M	N	N	N	N	N	N	N	N	N	N
7	Not HSCR	11 days/F	N	N	N	N	N	N	N	N	N	N
8	Not HSCR	8 weeks/F	N	N	N	N	N	N	N	N	N	N
9	Not HSCR	4 weeks/M	N	N	N	N	N	N	N	N	N	N
10	Not HSCR	8 weeks/M	Eq	N	N	N	N	N	N	N	N	N
11	Not HSCR	10 weeks/F	Eq	N	N	N	N	N	N	N	N	N
12	Not HSCR	1 day/M	N	N	N	Eq	N	N	N	N	N	N
13	Not HSCR	12 days/M	N	Eq	N	N	N	N	N	N	N	N
14	Not HSCR	10 days/M	N	N	N	N	Eq	N	N	N	N	N
15	Not HSCR	2 days/M	N	Eq	N	N	N	N	N	N	N	N
16	Not HSCR	4 days/F	N	Eq	N	N	Eq	N	N	N	Eq	Eq
17	Not HSCR	3 days/F	**H**	N	Eq	N	N	N	N	N	N	N
18	HSCR (18 cm)	6 weeks/F	H	H	H	H	H	H	H	H	H	H
19	HSCR (6 cm)	2 days/M	H	H	H	H	H	H	H	H	H	H
20	HSCR (7 cm)	3 days/M	H	H	H	H	H	H	H	H	H	H
21	HSCR (2 cm)	3 years/M	H	H	H	H	H	H	H	H	H	H
22	HSCR (8 cm)	2 days/M	H	H	H	H	H	H	H	H	H	H
23	HSCR (8 cm)	2 days/M	H	H	H	H	H	H	H	H	H	H
24	HSCR (3 cm)	9 days/M	H	H	H	H	H	H	H	Eq	H	H
25	HSCR (7 cm)	2 days/M	H	H	H	H	H	H	H	H	H	H
26	HSCR (20 cm)	4 years/M	H	H	H	H	H	H	Eq	H	H	H
27	HSCR (7 cm)	13 months/M	H	H	H	H	H	H	H	H	H	H
28	HSCR (7 cm)	2 days/M	H	H	H	Eq	H	H	H	H	H	H
29	HSCR (6 cm)	4 days/M	H	H	H	Eq	H	H	H	H	H	H
30	HSCR (15 cm)	8 days/M	Eq	H	Eq	Eq	H	H	H	H	H	H
31	HSCR (4 cm)	3 days/M	H	Eq	Eq	**N**	**N**	Eq	H	Eq	H	H

HSCR indicates Hirschprung disease; N, not HSCR; Eq, equivocal; H, Hirschsprung.

[a]Original diagnosis arrived at by evaluation of hematoxylin and eosin sections. Not HSCR indicates that HSCR was excluded because ganglion cells were present in the biopsy(ies); HSCR indicates that aganglionosis was diagnosed and confirmed later when bowel was resected; lengths in parentheses indicate the length of the aganglionic segment in the resection specimen.

[b,c]Diagnoses from independent "blinded" review of [b]AChE sections and [c]calretinin IHC sections; listed for each of the 5 observers (A–E); major diagnostic discrepancies are bolded and underlined.

lamina propria (Fig. 2A). If the cell bodies of submucosal ganglion cells were present in the section, strong nuclear and cytoplasmic immunoreactivity was usually observed in a subset. However, because ganglion cell bodies are often not present, it is recognition of immunoreactive nerves in the superficial submucosa and muscularis mucosae that is the key to interpretation of calretinin IHC. In most instances, nerves have a beaded or granular pattern of immunoreactivity (Fig. 2B). Under the labeling conditions used in this study, immunoreactivity was usually visible at low magnification (×100) and could not be confused with the absent superficial neural staining of suction biopsies from aganglionic rectum.

Table 2. Frequency of correct, equivocal, and incorrect diagnoses with rapid acetylcholinesterase histochemistry (AChE) versus calretinin immunohistochemistry (IHC)

Method	Interpretations[a]	Correct	Equivocal	Incorrect
AChE	155	136	16[b]	3[c]
Calretinin IHC	155	149	6[d]	0[e]

[a]Refers to total number of individual interpretations rendered by each pathologist (31 cases × 5 pathologists).

Fisher exact test results: $^{(b\text{ vs. }d)}P = 0.04$, $^{(c\text{ vs. }e)}P = 0.25$, $^{[(b+c)\text{ vs. }(d+e)]}P = 0.01$.

In a minority of biopsies (2 of 57, 4%) that contained ganglion cells, calretinin immunoreactivity was observed in only sparse nerve fibers in the superficial submucosa or muscularis mucosae, which might be missed by casual inspection at low magnification. In 1 such case (patient #16), ganglion cell bodies and adjacent nerves were positive but showed weaker staining than all other non-HSCR patients (Fig. 2C). Variability was observed occasionally between

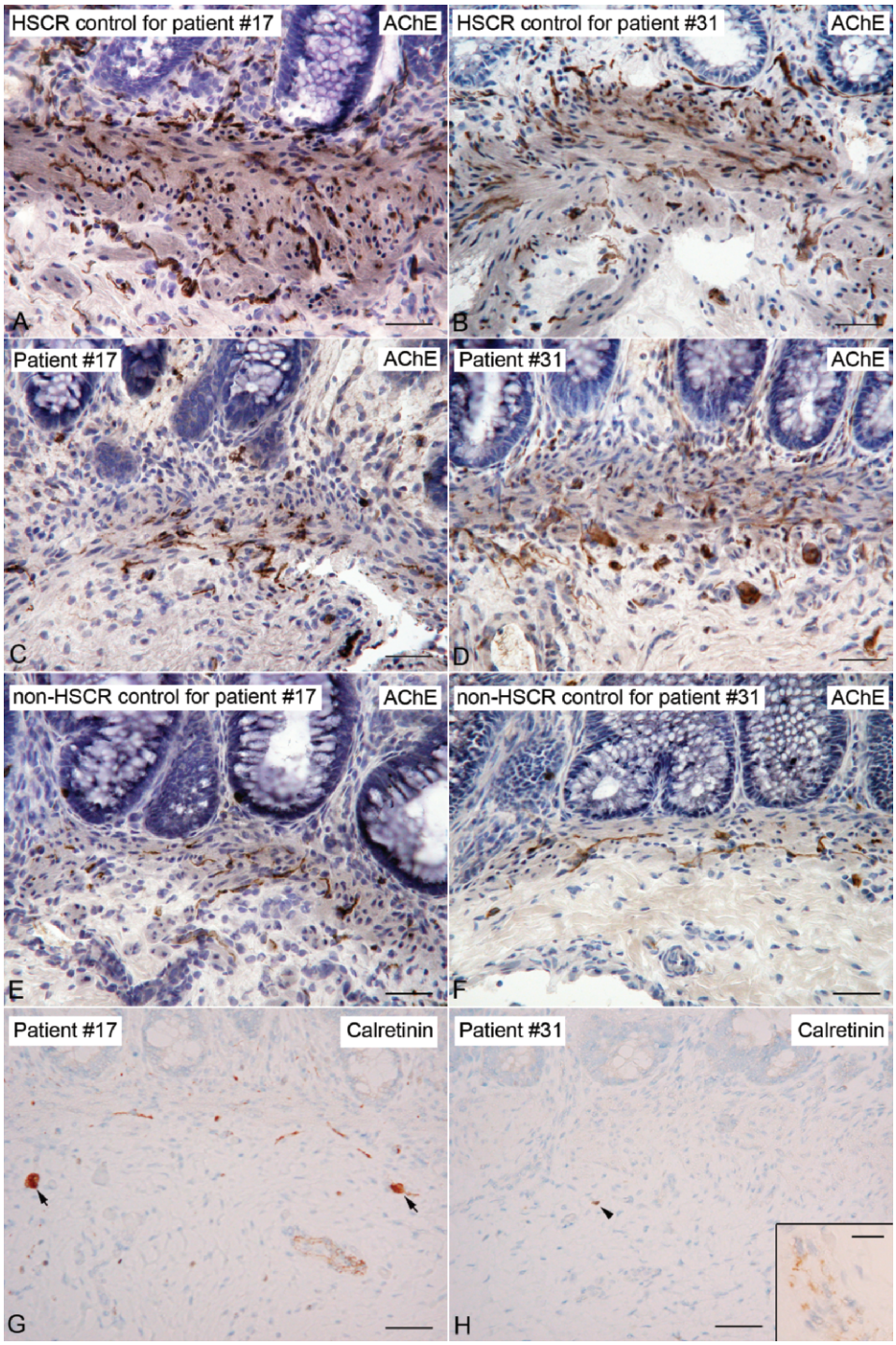

Figure 1. Rapid acetylcholinesterase histochemistry (AChE) (**A–F**) and calretinin immunohistochemistry (**G,H**) from 2 patients with major discordant diagnoses. In the suction rectal biopsies from patient #17, who did not have Hirschprung disease (HSCR) (left column), and HSCR patient #31 (right column), the density and coarseness of AChE nerves (**C,D**) was intermediate between that observed in the HSCR controls (**A,B**) and non-HSCR controls (**E,F**), which were run in parallel. One observer interpreted this intermediate pattern as diagnostic of HSCR, whereas other observers interpreted it as either not HSCR or equivocal (see Table 1). (**G**) Calretinin IHC from the non-HSCR patient showed a normal immunoreactivity of many nerve fibers in the muscularis mucosae and rare ganglion cell bodies (arrows). (**H**) No nerve fiber immunoreactivity in the muscularis mucosae was observed in the biopsy from the HSCR patient, although some background staining was present in rare non-neuronal cells (arrowhead, probable mast cell), and some axonal immunoreactivity was present in large submucosal nerves (inset). Bar = 50 μ; inset bar = 25 μ.

biopsies from the same patient (Fig. 2D,E). No relationship was resolved between the density of immunoreactive nerve fibers and patient age, rapid AChE staining, proximity of the biopsy to the anorectal junction (see below), or gender, but the number of cases was too small to exclude an imperfect correlation with 1 or more of these factors.

Calretinin Immunoreactivity in Terminal Rectum of Patients without Hirschprung Disease

Submucosal hypoganglionosis is considered a normal variant in the terminal 1 to 2 cm of the rectum. In some instances, 90% or more of sections from a suction biopsy with generous submucosa may not reveal ganglion cells (Table 3), and those ganglion cells that are present are often solitary and challenging to find in H&E-stained sections. For this reason, an effort is usually made to obtain diagnostic biopsies 2 to 3 cm proximal to the anorectal junction, although the actual biopsy location is seldom precise. Calretinin immunostaining potentially could be very useful to exclude HSCR in biopsies of terminal rectum, where ganglion cells are normally sparse. Calretinin IHC was examined in suction biopsies that either were labeled as from the terminal 1 cm of rectum and had

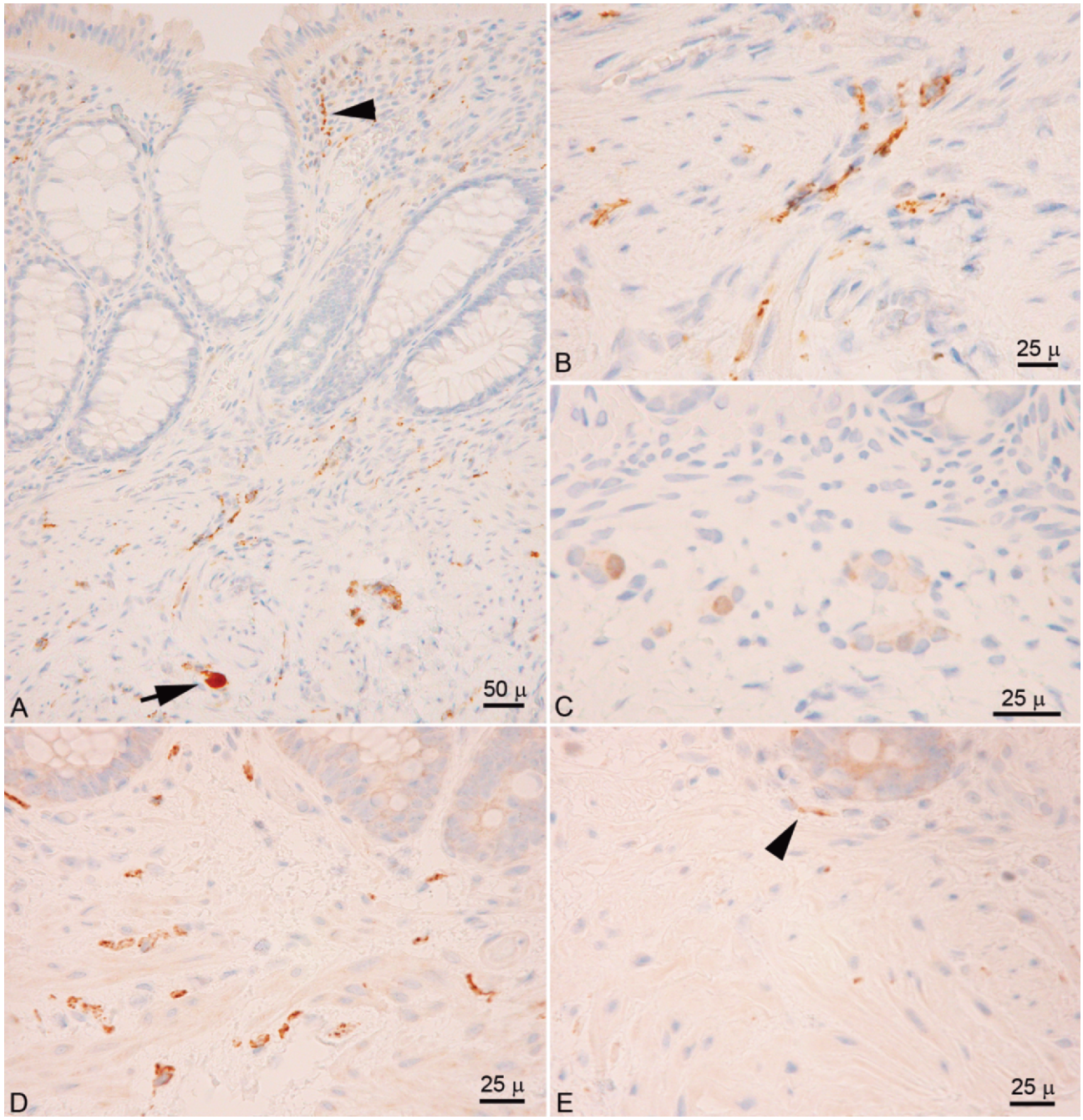

Figure 2. Calretinin immunohistochemistry in biopsies that contain ganglion cells. (**A**) A photomicrograph of the biopsy from patient #4, who did not have Hirschprung disease (HSCR), illustrates the most commonly observed pattern of calretinin immunoreactivity. Dense granular immunolabeling is present in many nerves of the muscularis mucosae and superficial submucosa, as well as less numerous fibers in the lamina propria (arrowhead) and ganglion cell bodies (arrow). (**B**) The granular appearance of the immunoreactive nerves is more apparent in this higher magnification image of the muscularis mucosae. (C) In rare non-HSCR patients (patient #16), few immunoreactive nerves were present in the muscularis mucosae, but ganglion cells and adjacent nerves in the submucosa were immunoreactive. Two observers interpreted this pattern as equivocal (see Table 1). (**D,E**) Separate biopsies from the same patient occasionally showed very different densities of calretinin immunoreactive nerves in the muscularis mucosae. This set demonstrates the common pattern of numerous calretinin-positive nerves in a biopsy taken 1 cm from the anorectal junction (**D**) and only rare fibers (arrowhead) in a biopsy taken 2 cm from the anorectal junction (**E**). A color version of this figure is available online.

ganglion cells in less than 10% of H&E-stained sections (5 biopsies) or were obtained from an unspecified distance from the anorectal junction but contained anal (squamous; 5 biopsies) or anorectal (mixed of squamous and columnar or transitional epithelium; 4 biopsies) mucosa. Sections stained with H&E from the latter did not contain ganglion cell bodies (Table 3). Although neural calretinin immunoreactivity was absent in the stroma underlying squamous

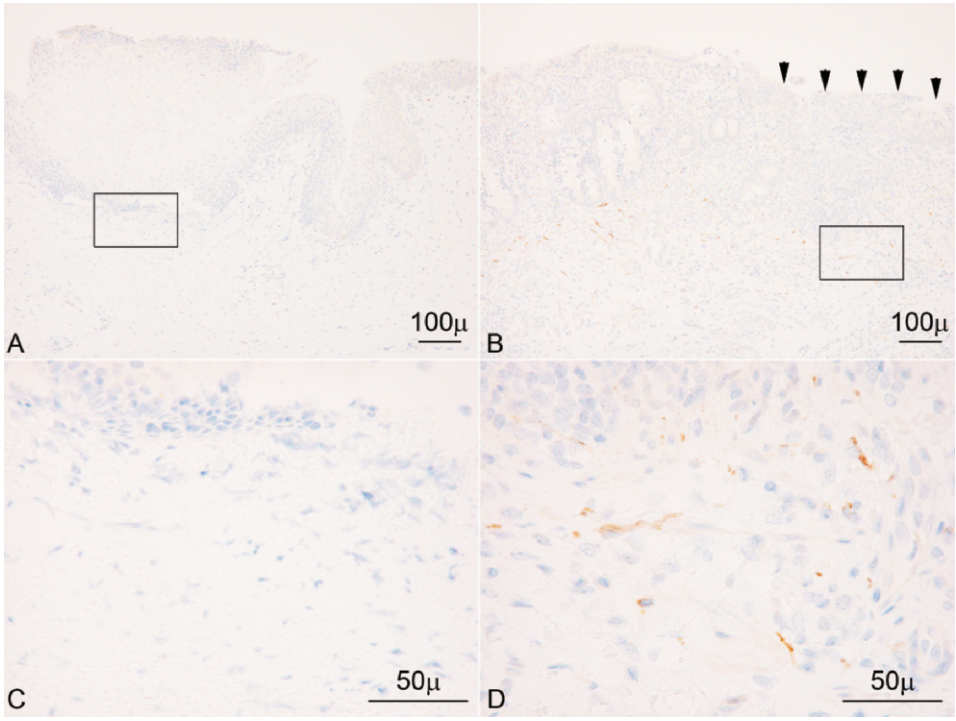

Figure 3. No calretinin-positive nerves are evident in suction biopsies of anus (**A,C**), but many calretinin-immunoreactive nerves are present in a biopsy of anorectal mucosa (**B,D**). The latter is covered partially by crypts lined by colonic epithelium and partially by transitional epithelium (arrowheads). Both biopsies are from patients with ganglion cells in other biopsies that contained rectal mucosa. The boxed areas in **A** and **B** correspond with the higher magnification images shown in **C** and **D**, respectively. A color version of this figure is available online.

mucosa (Fig. 3A), a normal pattern of rectal mucosal staining was observed deep to anorectal mucosa (Fig. 3B), consistent with the fact that rare ganglion cells were present in other biopsies from these patients. Even in the context of physiologically hypoganglionic distal rectum, calretinin immunoreactivity predicts the existence of submucosal ganglion cells.

Calretinin Immunoreactivity in Aganglionic Biopsies from Hirschprung Disease Patients

Absolutely no neural calretinin immunoreactivity was observed in the muscularis mucosae of aganglionic biopsies from HSCR patients. However, large nerves in the submucosa may contain immunoreactive axons (Fig. 4A), as in rare examples that were interpreted as equivocal by 1 or 2 observers in the multiobserver comparison study (discussed above). Immunostaining in large submucosal nerves in HSCR consists of punctate evenly spaced axons, identical to calretinin immunoreactivity in normal serosal nerves (Fig. 4B) and some nerves in the normal deep submucosa (Fig. 4C) but distinctly different from the confluent dark granular immunostaining of small-caliber nerves in the normal superficial submucosa (Fig. 2). Weak nonspecific cytoplasmic or nuclear immunoreactivity was present in sparse ovoid cells (probably mast cells) in the submucosa of all biopsies independent of ganglion cells (Figs. 1H, 4A). Although this immunoreactivity could potentially have caused confusion for the uninitiated, it was faint and homogeneous and easily distinguished from the dark, granular immunoreactivity of slender nerve processes that intensely label in biopsies that contain ganglion cells. A summary of the calretinin immunoreactivity observed in biopsies from non-HSCR and HSCR patients is provided in Table 4.

DISCUSSION

The results of this study suggest that calretinin IHC may be a useful ancillary technique in the pathological evaluation of suction rectal biopsies for HSCR. In our comparative study, unequivocal diagnosis or exclusion of HSCR by calretinin IHC alone was more accurate than rapid AChE alone, with no major errors, fewer equivocal readings, and good interobserver agreement. These results are particularly impressive because 4 of the 5 observers had no prior experience with calretinin IHC of suction rectal biopsies, apart from the 4-case training set and individual positive and negative controls provided with the study set. Another potential advantage of calretinin IHC is its application to sections from the same paraffin-embedded tissue used for conventional H&E-stained sections, which, unlike rapid AChE, does not require an additional frozen biopsy specimen. In this way, H&E and calretinin IHC results can be directly correlated, and the adequacy of a paraffin block for calretinin IHC can be assessed prior to sectioning. Because IHC is widely available, introduction of calretinin staining will not impose a significant technical burden for pathology laboratories, in contrast to AChE histochemistry, which is used only for HSCR diagnosis.

In our comparative study, reliance on rapid AChE alone resulted in a single false-positive diagnosis by 1 reviewer and another case with false-negative diagnoses by 2 reviewers. Others have evaluated the sensitivity and specificity of AChE in their own laboratories [16,25–29]. In general, false-positive findings suggestive of HSCR are extremely rare; most studies report 100% specificity. However, published rates of false-negative diagnoses range from 0% [29] to 40% [16]. False-negative interpretations appear to be more common in very young HSCR patients [25–27]. Some false-negative diagnoses have been attributed to ultrashort-segment disease and biopsies proximal to

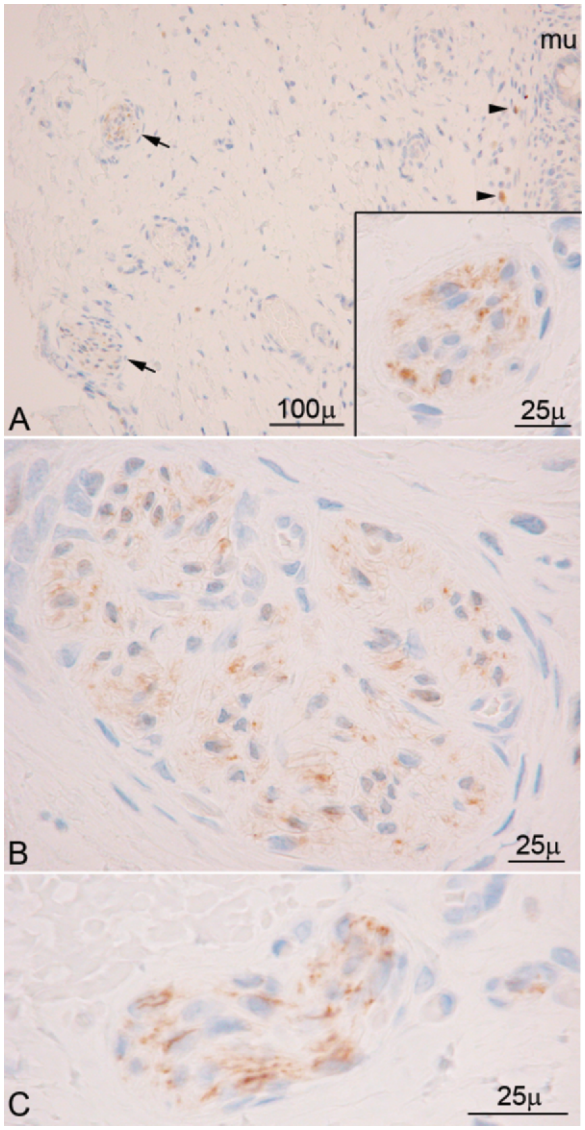

Figure 4. In suction biopsies from aganglionic rectum (patient #24), immunoreactivity is often present in large nerves in the submucosa (arrows). At higher magnification (inset), the punctate immunoreactivity in these nerves is less intense and more aggregated than superficial submucosal or mucosal nerve fibers that stain in specimens without Hirschprung disease (HSCR) (see Fig. 2) and resembles the immunoreactivity of serosal nerves (**B**) and occasional large-caliber, deep submucosal nerves (**C**) in non-HSCR colon. Arrowheads in **A** demonstrate background immunoreactivity of presumptive mast cells. Abbreviation: mu indicates mucosa. A color version of this figure is available online.

the aganglionic zone [30]. Our laboratory, like many others, routinely utilizes a rapid modification of the original AChE method. The observers in this study had 80 years of combined experience with the use of AChE staining, and 4 of the 5 observers have used the rapid AChE modification routinely for more than 10 years. Although alternative AChE staining methods or other observers might yield a different outcome, the discrepant rapid AChE diagnoses in this unbiased study reflect inherent difficulties associated with the approach as it is employed by pediatric pathologists with considerable experience. It seems likely that equivocal or erroneous AChE interpretations are even more likely in laboratories that utilize AChE less often.

Although the results of calretinin IHC are promising, it is premature to rely on calretinin IHC alone for the diagnosis/exclusion of HSCR. Several limitations to the current study need to be acknowledged. The series of HSCR and non-HSCR biopsies used in this study are relatively small, such that neither the sensitivity nor specificity of calretinin IHC or rapid AChE can be estimated precisely. Even a low rate of diagnostic inaccuracy (such as 1%) could lead to an unacceptable incidence of incorrect diagnoses with serious potential consequences. Therefore, we believe that calretinin IHC, like AChE, should be used as an ancillary method in addition to conventional H&E evaluation. As our experience with calretinin grows and empiric data accumulates, we expect that confidence in calretinin IHC will increase and we can safely reduce the number of H&E-stained sections that need to be prepared and reviewed.

Each of the aganglionic suction rectal biopsies from the 22 (14 study set, 4 training set, and 4 additional) HSCR cases showed no calretinin immunoreactive nerve fibers in the muscularis mucosae or mucosa. This aberrant pattern of calretinin IHC was predicted by Barack and others based on their evaluation of HSCR resection specimens, in which the distribution of calretinin-positive fibers correlated spatially with the proximal-to-distal distribution of ganglion cells. Appreciation that *superficial* immunoreactive nerves are *completely* absent in aganglionic biopsies from HSCR patients is important, because even sparse immunoreactive nerves in suction biopsies correlated with the presence of ganglion cells. However, it is equally important to recognize that immunoreactivity exists in axons in large submucosal nerves of HSCR, a pattern identical to serosal nerves in normal rectum. This pattern of axonal staining in hypertrophic nerves differs from the diffuse granular staining of normal superficial submucosal nerves, which is lost in HSCR.

Hirschprung disease is a genetically and phenotypically heterogeneous disorder. One of our patients, an infant with congenital central hypoventilation syndrome (patient #18), carried a pathogenetic trinucleotide expansion in *PHOX2B*. Mutational studies were not conducted on any other patients in this series, all of whom had transitional zones distal to the splenic flexure. It seems likely that calretinin immunoreactive nerves are not present in suction rectal biopsies from the aganglionic bowel of patients with HSCR, independent of genotype or the length of aganglionic gut. However, the possibility that extrinsic innervation of some forms of HSCR may contain calretinin-positive nerves has not been excluded.

A context in which AChE may be particularly important is the diagnosis of ultrashort-segment HSCR (US-HSCR). This type of HSCR is a controversial subject, in part because variable diagnostic criteria have been proposed [31–33]. Conceptually, US-HSCR is functionally significant aganglionosis limited to the distal 2 cm of the rectum, although some authors extend this to 4 cm [31]. Diagnosis of US-

Table 3. Ganglion cell counts, mucosal properties, and calretinin immunoreactivity of biopsies from the distal 1 cm of rectum in patients proven not to have Hirschprung disease

Biopsy	Age/gender (male, female)	Location[a]	Ganglion cell counts/no. of sections examined	Mucosa	Calretinin staining
A	10 days/M	1 cm	9/192 (4.7%)	Rectal	Abundant nerves; no cell bodies
B	6 weeks/M	1 cm	5/26 (8.1%)	Rectal	Abundant nerves; rare cell bodies
C	13 months/F	1 cm	0/76[b] (0%)	Rectal[c]	Abundant nerves; no cell bodies
D	3 days/F	1 cm	6/124 (4.8%)	Rectal	Abundant nerves; no cell bodies
E	9 weeks/M	1 cm	1/207 (0.5%)	Rectal	Abundant nerves; no cell bodies
F	5 years/M	2 cm	0/202[b] (0%)	Rectal ("inadequate submucosa")[d]	Abundant nerves; no cell bodies
G	5 years/M	ns	0/47[b] (0%)	Rectal ("inadequate submucosa")	Abundant nerves; no cell bodies
H	13 months/F	2 cm	0/45[b] (0%)	Rectal ("inadequate submucosa")[c]	Sparse nerves; no cell bodies
I	5 years/M	1 cm	0/78[b] (0%)	Anorectal[d]	Abundant nerves; no cell bodies
J	10 months/M	ns	0/167[b] (0%)	Anorectal	Sparse nerves; no cell bodies
K	6 months/F	ns	0/80[b] (0%)	Anorectal	Sparse nerves; no cell bodies
L	3 years/M	ns	0/161[b] (0%)	Anorectal	Sparse nerves; no cell bodies
M	15 months/M	2 cm	0/18[b] (0%)	Anal	Negative
N	5 years/M	1 cm	0/78[b] (0%)	Anal[d]	Negative
O	15 years/F	ns	0/30[b] (0%)	Anal	Negative
P	11 days/F	1 cm	0/95[b] (0%)	Anal	Negative
Q	3 weeks/M	1 cm	0/71[b] (0%)	Anal	Negative

[a]Distance from anorectal junction as denoted by the clinician who obtained the biopsy; ns indicates not specified.
[b]Another rectal biopsy obtained from the same patient contained rectal mucosa and ganglion cells.
[c,d]Different biopsies from the same patient.

HSCR based on suction biopsies alone may be impossible because autopsy series have shown this part of the gut to be hypoganglionic normally. In the setting of a patient with clinical signs of HSCR and ganglion cells in suction biopsies more than 2 cm proximal to the anorectal junction, absence of ganglion cells with an HSCR-like AChE pattern in suction biopsies from the distal 2 cm of rectum has been used to diagnose US-HSCR [32]. Although calretinin-immunoreactive fibers are present in the distal-most rectal submucosa, it is unclear whether loss of calretinin IHC in the terminal rectum also correlates with US-HSCR. The shortest aganglionic segment in our series was 2 cm (patient #21), and calretinin immunoreactivity was absent in suction biopsies from the aganglionic bowel.

A potential benefit of calretinin IHC relates to investigation of biopsies that are deemed "inadequate" because they contain anorectal mucosa or insufficient submucosa. A small number of biopsies of this type were included in our study because additional biopsies from the same patient contained ganglion cells. The fact that normal calretinin immunoreactivity was present in the otherwise "inadequate" biopsies suggests that the immunohistochemical marker may be used to salvage a pathological diagnosis when only anorectal mucosa or limited submucosa is sampled, thereby avoiding another procedure. Given the small number of cases in our study, we regard the latter conclusion as preliminary with the need for confirmation in larger series. However, anal biopsies, which are lined entirely by transitional or squamous epithelium, normally lack both ganglion cells and any calretinin immunoreactive nerves and therefore are inadequate for the diagnostic evaluation of HSCR.

Among the non-HSCR group, the density of calretinin-immunoreactive nerves varied considerably. The significance of this variability is unclear, but we observed no apparent correlation with age or gender of the patient or the biopsy location. Because the control population in our series was exclusively patients with signs of intestinal dysmotility, it is possible that differences in calretinin IHC may be functionally significant. However, considerable additional research, including long-term clinical follow up and/or physiological studies, will be required to address this hypothesis.

Table 4. Calretinin immunoreactivity in suction rectal biopsies from non-Hirschprung (HSCR) disease versus HSCR disease patients

Location	Non-HSCR	HSCR
Small nerves (<15 μ): lamina propria	Usually present	Always absent
Muscularis mucosae and superficial submucosa	Always present; usually abundant, rarely sparse	Always absent
Ganglion cell bodies	Often present	Always absent
Large submucosal nerves (>20 μ)	Fine/punctate axonal staining	Fine/punctate axonal staining

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