Presence of intramucosal neuroglial cells in normal and aganglionic human colon

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The enteric nervous system (ENS) is composed of neural crest-derived neurons (also known as ganglion cells) the cell bodies of which are located in the submucosal and myenteric plexuses of the intestinal wall. Intramucosal ganglion cells are known to exist but are rare and often considered ectopic. Also derived from the neural crest are enteric glial cells that populate the ganglia and the associated nerves, as well as the lamina propria of the intestinal mucosa. In Hirschsprung disease (HSCR), ganglion cells are absent from the distal gut because of a failure of neural crest-derived progenitor cells to complete their rostrocaudal migration during embryogenesis. The fate of intramucosal glial cells in human HSCR is essentially unknown. We demonstrate a network of intramucosal cells that exhibit dendritic morphology typical of neurons and glial cells. These dendritic cells are present throughout the human gut and express Tuj1, S100, glial fibrillary acidic protein, CD56, synaptophysin, and calretinin, consistent with mixed or overlapping neuroglial differentiation. The cells are present in aganglionic colon from patients with HSCR, but with an altered immunophenotype. Coexpression of Tuj1 and HNK1 in this cell population supports a neural crest origin. These findings extend and challenge the current understanding of ENS microanatomy and suggest the existence of an intramucosal population of neural crest-derived cells, present in HSCR, with overlapping immunophenotype of neurons and glia. Intramucosal neuroglial cells have not been previously recognized, and their presence in HSCR poses new questions about ENS development and the pathobiology of HSCR that merit further investigation.

enteric nervous system; enteric glial cells; Hirschsprung disease

THE ENTERIC NERVOUS SYSTEM (ENS) comprises a complex network of neurons and glial cells that reside in the wall of the gastrointestinal tract. These cells have been shown to arise from progenitors that migrate from the vagal level of the neural crest and populate the entire length of the intestine (10, 13, 26, 33), with a smaller contribution of sacral crest-derived cells to the distal intestine (5). Cell bodies of the ENS neurons, also known as “ganglion cells,” reside principally in two ganglionated plexuses: 1) the myenteric plexus, extending from the esophagus to the anus, and 2) the submucosal plexus, extending from the duodenum to the anus (7). Rare solitary ganglion cells, considered to be “ectopic” by some, may be seen in the lamina propria (3, 27), but the extensive mucosal neuronal networks are believed to derive from the cellular processes or “neurites” of the submucosal or myenteric ganglion cell bodies of the ENS or from the extrinsic plexuses of the autonomic nervous system (3, 7, 8).

Failure of enteric neural crest-derived cells (ENCCs) to migrate and colonize the entire length of the gut results in Hirschsprung disease (HSCR), a congenital condition that is pathologically characterized by variable lengths of aganglionic distal gut, most commonly limited to the rectosigmoid and rarely extending proximally into the small intestine (21, 26). Diagnostic pathology of HSCR has been the subject of some controversy, particularly as it relates to the expression of various histochemical and immunohistochemical markers of various constituents of the ENS (11, 16, 17, 22, 29).

In addition to neurons, the ENS contains a network of glial cells that have been morphologically compared with the astrocytes of the central nervous system and are often described as “irregular, stellate-shaped cells that are associated with neuron cell bodies in enteric ganglia” (12). Largely on the basis of various histochemical and immunohistochemical markers (24). Intramucosal glial cells were largely excluded from original descriptions of glial components of the ENS, but recent studies demonstrate the presence of a distinct network of astrocyte-like mucosal enteroglial cells that likely contribute to epithelial cell differentiation, regulation of barrier function, and mucosal inflammation and healing (12, 31, 32, 34, 37). Enteric glial cells also appear to have a significant role in modulating neural signaling and the remarkable ability to generate functional neurons in culture, either through dedifferentiation into progenitor cells followed by differentiation into neurons or by transdifferentiation into enteric neurons (15, 20).

Despite significant progress in understanding the pathophysiology associated with enteric neurons and enteroglial cells separately, the intersection between these two cell types has remained enigmatic. Sensory and motor activities of the gastrointestinal tract are largely attributed to neuronal function, while the enteroglial cells are thought to play an important role in maintaining epithelial homeostasis, as described above.
Enteroglial cells are phenotypically described as stellate cells expressing specific glial markers such as S100 and GFAP, while neurons are described as dendritic cells with a prominent cell body expressing specific neuronal markers such as neuron-specific class III β-tubulin (Tuj1) and the calcium-binding protein calretinin (33). HSCR is often described as the quintessential disorder of intestinal innervation in which intraganglionic and extraganglionic neural cell bodies are thought to be absent, but the fate of extraganglionic glial cells is largely unknown.

Based on a series of observations in normal and aganglionic human intestine, we show a significant overlap between what has been traditionally described as two separate populations of intramucosal neurites and intramucosal enteroglial cells, suggesting that the ENS microanatomy in human intestinal mucosa may differ from previously proposed models. Based on our findings, we propose that the human intestinal mucosa contains a distinct population of interconnecting dendritic cells with overlapping neuroglial differentiation. Moreover, these intramucosal neuroglial cells (INCs) are present in the aganglionic colon, challenging our current understanding of ENS pathophysiology and suggesting that human HSCR is only a partial defect in ENCC migration and differentiation or that the ENS is not uniquely derived from the classic rostrocaudal migration of neural crest-derived cells.

**MATERIALS AND METHODS**

*Patients and specimens.* All studies were conducted under a protocol approved by our Institutional Review Board. A case series of surgical resections in three children with confirmed HSCR, as well as archival frozen sections on diagnostic rectal suction biopsies. All studies were conducted under a protocol approved by our Institutional Review Board. Technetium-99m pertechnetate scintigraphy was performed according to standard clinical protocol (28).

**Immunohistochemistry.** Sections (5 µm) of formalin-fixed, paraffin-embedded tissues were deparaffinized with xylene and hydrated through a graded series of alcohol. After antigen retrieval in 0.1 M sodium citrate (pH 6.0), endogenous peroxidase activity was inhibited with 3% hydrogen peroxide in methanol. The sections were incubated with the primary antibodies and then with a biotinylated secondary antibody and the avidin-biotinylated horseradish peroxidase complex. Staining was developed using diaminobenzidine (DAB; Sigma, St. Louis, MO) or Fast Red Chromogen (Covance, Dedham, MA). Mayer’s hematoxylin (Sigma) was used for nuclear counterstaining.

**Double immunohistochemistry**

<table>
<thead>
<tr>
<th>Antibody (Clone)</th>
<th>Source</th>
<th>Dilution</th>
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</thead>
<tbody>
<tr>
<td>Calretinin (232A)</td>
<td>Cell Marque (Rocklin, CA)</td>
<td>Predilute</td>
</tr>
<tr>
<td>CD56 (MRQ-42)</td>
<td>Cell Marque</td>
<td>Predilute</td>
</tr>
<tr>
<td>Synaptophysin (336A)</td>
<td>Cell Marque</td>
<td>Predilute</td>
</tr>
<tr>
<td>Calretinin (SP65)</td>
<td>Ventana (Tucson, AZ)</td>
<td>Predilute</td>
</tr>
<tr>
<td>CD117 (T595)</td>
<td>Novocastra (Buffalo Grove, IL)</td>
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</tr>
<tr>
<td>CD1a (EP3622)</td>
<td>Ventana</td>
<td>1:100</td>
</tr>
<tr>
<td>CD21 (EP3093)</td>
<td>Ventana</td>
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<tr>
<td>CD31 (JC70)</td>
<td>Ventana</td>
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<tr>
<td>CD34 (QBEnd/10)</td>
<td>Ventana</td>
<td>Predilute</td>
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<tr>
<td>Desmin (DE-R-11)</td>
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<tr>
<td>Langerin (12D6)</td>
<td>Novocastra</td>
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</tr>
<tr>
<td>Neu-N (A60)</td>
<td>Chemicon (Billerica, MA)</td>
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<td>S100 (polycanal)</td>
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<td>Ventana</td>
<td>Predilute</td>
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<td>Chemicon</td>
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<td>Vimentin (V9)</td>
<td>Chemicon</td>
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<td>VIP (polycanal)</td>
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<tr>
<td>GFAP (polycanal)</td>
<td>Dako (Carpinteria, CA)</td>
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<tr>
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<tr>
<td>S100 (polycanal)</td>
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<td>Neuronal class III β-tubulin (Tuj1)</td>
<td>Covance (Dedham, MA)</td>
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<td>Neuronal class III β-tubulin (Tuj1/TU-20)</td>
<td>Novus Biologicals (Littleton, CO)</td>
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GFAP, glial fibrillary acidic protein.

**Immunofluorescence**

**Flow cytometry**

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**RESULTS**

Normal colon mucosa contains inconspicuous submucosal nerve fibers that are visualized by hematoxylin-eosin staining of formalin-fixed, paraffin-embedded tissue sections (Fig. 1a, arrowhead). Occasional ganglion cells, with their characteristic abundant cytoplasm and round, eccentric nuclei, can also be seen (Fig. 1a, arrow and inset). Immunohistochemical staining for the calcium-binding protein calretinin reveals delicate dendritic cellular processes in the lamina propria (Fig. 1b). Interestingly, these calretinin-positive cellular structures, which are commonly referred to as neurites (cellular processes), emanating from the submucosa (4, 11, 17), appear to contain cell nuclei within the lamina propria (Fig. 1b, arrows and inset). Histochemical staining for ACE is generally negative in normal colon mucosa (Fig. 1c), although delicate neurites may occasionally be seen.

Aganglionic colon from a patient with HSCR shows the absence of ganglion cells on multiple serial sections (not shown), as well as the characteristic neural hypertrophy (Fig. 1d, arrow). In contrast to the ganglionic normal colon, calretinin-immunoreactive cellular processes appear to be absent from the mucosa of aganglionic colon (Fig. 1e), while ACE-positive dendritic cellular processes are now prominent (Fig. 1f). Interestingly, as noted with the calretinin-immunoreactive cellular processes in the normal colon, the ACE-positive fibers in aganglionic colon appear to be associated with cell nuclei located within the lamina propria (Fig. 1f, arrows and inset). As such, immunoreactivity and enzyme histochemical characteristics of the intramucosal ENS are not entirely explained by extrinsic neurites and appear to be associated with a cellular network, the cell bodies of which are located within the lamina propria.

We performed additional immunohistochemical staining to characterize the immunophenotype of dendritic cells within the lamina propria. The calretinin-positive processes and cell bodies shown in Fig. 1b are again readily apparent in normal colon mucosa (Fig. 2a), where a direct cell-cell connection can also be seen between two neighboring cells, the nuclei of which are marked by arrows (Fig. 2a, arrows). Cells with similar morphology are immunoreactive for the neural cell adhesion molecule, also known as CD56 (Fig. 2b), the neuronal synaptic vesicle glycoprotein synaptophysin (Fig. 2c), and the enteric glial cell marker S100 (Fig. 2d). Occasional cells also react with the neuropeptide VIP (Fig. 2e). A similar expression pattern was also seen with intermediate filament vimentin (not shown), although vimentin expression was widely distributed in various cell types and was not specific to the dendritic cells. No immunoreactivity in INCs was observed with multiple additional immunohistochemical markers that have been associated with a dendritic cell morphology, including CD21, CD31, CD34, CD57, CD117, CD1a, langerin, D240, tryptase, Neu-N1, and desmin. GFAP was degraded or lost in formalin-fixed, paraffin-embedded sections but was detectable on frozen sections (see below). Calretinin-positive dendritic cells in the mucosa are also present in the appendix (Fig. 2f), small intestine (Fig. 2g), and stomach (Fig. 2h), and CD56 stains cells with similar morphology at these locations (not shown).

To determine whether the cells seen in Fig. 2, a–d, represent a single or an overlapping population, double-immunohistochemical staining was performed using calretinin-CD56 (Fig. 3a) and calretinin-synaptophysin (Fig. 3b). Overlapping coexpression of markers is observed within the same cells, suggesting that a single cell population within the lamina propria coexpresses calretinin, CD56, and synaptophysin. Rare intra-
mucosal ganglion cells, which are morphologically distinct and separate from the intramucosal dendritic cells being studied, also coexpress calretinin and synaptophysin (Fig. 3b, inset).

As shown in Fig. 1e, aganglionic colon mucosa from a patient with HSCR shows no calretinin expression. This is repeated in another patient with HSCR (Fig. 4a) and is consistent with our clinical experience and the literature (4, 11, 17, 19). However, expression of other neural markers, including CD56 (Fig. 4b) and synaptophysin (Fig. 4c), remains intact in HSCR. In conjunction with the calretinin-CD56 and calretinin-synaptophysin coexpression results shown in Fig. 3, these findings suggest that the absence of calretinin staining in HSCR may not be due to the absence of neurites derived from calretinin-positive submucosal ganglion cells, as often suggested (4, 17). In contrast, these results suggest that calretinin expression may be downregulated in intramucosal dendritic cells in the context of HSCR, resulting in loss of detectable calretinin by immunohistochemistry, with preservation of CD56 and synaptophysin.

Our results support the existence of a population of intramucosal cells that exhibits morphological (i.e., cell body with long interconnecting projections) and immunophenotypic (i.e., expression of CD56, synaptophysin, and calretinin) features that are classically associated with ENS neurons. Given the striking morphological similarity of this cell population to intramucosal enteroglial cells described by others (31, 32, 36), as well as the S100 expression pattern shown in Fig. 2d, we hypothesize that these observations point to an intramucosal dendritic cell population with mixed neuroglial differentiation. The neuroglial phenotype of this intramucosal population of dendritic cells is supported by coexpression of the classic enteric neuronal marker Tuj1 and the enteric glial markers S100 (Fig. 5, a–d) and GFAP (Fig. 5, e–h) within the same cell population, as shown by immunofluorescent staining. These images are not intended to show or suggest subcellular molecular colocalization of Tuj1 and S100 but, rather, their coexpression in the same cell, as highlighted in the merged immunofluorescence panels. Finally, we provide supportive evidence for a neural crest origin of the INCs by double-immunofluorescent staining using Tuj1 and the neural crest cell marker HNK1 (CD57), as shown in Fig. 5i (arrows). This observation is not unexpected, given that cellular elements of the ENS are known to be of neural crest origin.

Double-immunofluorescent staining was also performed on colon mucosa from patients with HSCR. The proximal, ganglionicated colon shows Tuj1 and S100 coexpression within the same dendritic cells in the lamina propria (Fig. 6, a–c), as seen in normal colon from individuals without HSCR (Fig. 5). Interestingly, and consistent with data presented in Fig. 4, we find a similar population of cells in the mucosa from the

Fig. 1. Characteristic morphology of normal (a–c) and aganglionic (d–f) human colon. a: Routine hematoxylin-eosin staining of normal colon mucosa after formalin fixation and paraffin embedding shows inconspicuous submucosal nerves (arrowhead) and occasional ganglion cells (arrow, expanded in inset). b: By immunohistochemistry of formalin-fixed, paraffin-embedded tissue, delicate calretinin-positive dendritic cellular processes are seen in the lamina propria, with cell nuclei present in the calretinin-positive regions (arrows, inset). c: By enzymatic histochemistry on a corresponding frozen section of fresh (unfixed) tissue, acetylcholinesterase (ACE)-positive neurites are not seen in the normal colon mucosa. d: Aganglionic colon shows marked neural hypertrophy (arrow) and no submucosal ganglion cells. e and f: calretinin-immunoreactive cellular processes are absent in the aganglionic colon from a patient with Hirschsprung disease (HSCR), while prominent ACE-positive dendritic processes are seen in the lamina propria, some associated with cell nuclei (f, arrows, inset). Morphological features of formalin-fixed, paraffin-embedded tissue (a, b, d, and e) differ slightly, as expected, from frozen sections of fresh tissue (c and f). Scale bar = 100 μm (a and d) and 25 μm (b, c, e, and f).
aganglionic region (Fig. 6, d–f). Notably, however, S100 and, possibly, Tuj1 expression is reduced, which mimics the reduced or absent expression of calretinin in HSCR and provides further evidence in support of an altered expression phenotype in mucosal neuroglial cells in this disease.

Based on the above findings, we hypothesized that at least one population of cells in the colon exhibits dendritic morphology and expresses markers classically associated with ENS neuronal elements (calretinin, CD56, synaptophysin, and, possibly, ACE). Furthermore, intramucosal cells of a similar and overlapping phenotype appear to express markers that are classically associated with ENS glial elements (S100 and GFAP). The body (nucleus) of this cell is morphologically distinct from the body of the ENS ganglion cell, which may be identified on rare occasions in the lamina propria of the normal colon. We recognize that light-microscopic resolution is not sufficient to exclude the possibility that multiple cell types of similar morphology, but different immunophenotype, are inextricably present in the lamina propria, although we consider this scenario unlikely.
To quantify these intramucosal dendritic cells, formalin-fixed, paraffin-embedded sections of normal colon mucosa from 10 patients were immunostained for calretinin and subjected to quantitative image analysis and morphometry. A total of 2.79 mm² of the colonic lamina propria was analyzed for expression of calretinin-positive cells. The analyzed area per patient was 0.279 ± 0.087 (SD) mm². The total number of intramucosal dendritic cell bodies encountered in the analysis area was 268, resulting in an average of 102 ± 45 cell bodies/mm² of lamina propria in the 10 patients analyzed. The average DAB-positive area per patient, representing the area of calretinin-positive cells in the lamina propria, was 0.017 ±

Fig. 5. Intramucosal dendritic cells coexpress neural and glial markers. a: Double immunofluorescence performed with a neural marker (Tuj1, green) and an enteric glial marker (S100, red) on frozen sections of normal colon mucosa shows cellular coexpression. Nuclear stain (4',6-diamidino-2-phenylindole dihydrochloride, blue) depicts the crypt and lamina propria anatomy. b–d: High-magnification image of a double-labeled cell. e–h: Immunofluorescence with Tuj1 and glial fibrillary acidic protein (GFAP), also on frozen sections of normal colon mucosa, shows cellular coexpression (e), highlighted in the high-magnification images (f–h). i: Staining with Tuj1 (red) and a neural crest cell marker [human natural killer-1 (HNK1), green] supports coexpression in the same lamina propria cells (arrows), providing evidence in favor of a neural crest origin. Scale bar = 40 μm (a), 20 μm (b and c), 15 μm (d and f–h), 50 μm (e), and 30 μm (i).
Additional information about these two distributions is presented as box-and-whisker plots in Fig. 7, d and e, respectively. As stated previously, we cannot entirely exclude the possibility that S100-positive or GFAP-positive glial elements and Tuj1-positive neural elements are inextricably associated with each other in two distinct cell populations at a submicron resolution below that of confocal fluorescence microscopy, but we propose that this scenario is less likely than mixed neuroglial differentiation in the same cellular network. This conclusion is further supported by flow cytometry on cells isolated from fresh human colon after removal of the muscularis propria to confirm the presence of a dual-staining population of individual cells. With this approach, we find a small, but reproducible, population of Tuj1-positive/S100-positive cells that comprise ≈0.4% of the total number of cells obtained from colon mucosa and submucosa (Fig. 7, f and g). These flow cytometry data are consistent (i.e., same order of magnitude) with morphometric data that showed an average of 102 cell bodies/mm² of the colonic lamina propria (Fig. 7).

Finally, we rely on observations in surgical pathology of the human intestinal tract to demonstrate conditions in which there appears to be an intramucosal proliferation with biphenotypic neuroglial differentiation, consistent with origin from an INC type. Figure 8a shows a rectosigmoid polypectomy specimen from a routine screening colonoscopy for colorectal cancer in an otherwise healthy adult in which an intramucosal neuroma was identified. The spindle cells in this benign neoplasm are limited to the colon mucosa (Fig. 8a) and appear to coexpress calretinin (Fig. 8b) and S100 (Fig. 8c), supporting the existence of an intramucosal neuroglial phenotype. A similar spindle cell proliferation with coexpression of S100 and calretinin is shown within the lamina propria in a case of acute appendicitis with “fibrous obliteration of the lumen” in an appendectomy from a child with abdominal pain (Fig. 8d). The luminal obliteration was due to the presence of a cellular proliferation with coexpression of neural (calretinin) and glial (S100) markers, favoring an origin from a single precursor cell with neuroglial features. While we cannot completely exclude the simultaneous proliferation of two separate cell types in each of these cases, that would be a less likely scenario.

**DISCUSSION**

Based on morphological observations and immunophenotypic characteristics of normal human intestine, we propose the presence of an intramucosal network of interconnected dendritic cells with overlapping neuronal and glial differentiation. We designate this cellular network as INCs, which are distinct in morphology and in number from the occasional intramucosal ganglion cells, which are well characterized (3, 27). While the phenotype of INCs has overlapping features with those previously described as intramucosal enteroglia (12, 31, 36), recognition of INCs as a distinct biphenotypic element of the ENS leads to a more complete understanding of its anatomy and pathobiology. Previous work has drawn a sharp boundary...
between enteric neurons and enteric glia, largely on the basis of historical association of specific cell types with one or two protein markers. For instance, although various S100 protein subtypes are known to be expressed in melanocytes, adipocytes, Schwann cells, chondrocytes, Langerhans cells, and others, S100 is considered to represent a specific marker of enteric glia (12, 31, 33, 35) without definitive proof of its specificity in this regard. On the basis of morphology and immunophenotype, the proposed INC network likely overlaps wholly or in part with what has been considered previously to represent a purely glial network of intramucosal cells.

The acknowledgment of a novel neuroglial cell type explains several puzzling observations in the literature. Morphologically, the intramucosal glial network that has been described by S100 staining (32, 35) has many similarities to the intramucosal neural network demonstrated by protein gene product 9.5 (PGP9.5) or Tuj1 immunoreactivity (1, 2, 23). Although there is unquestionably a network of axonal processes emanating from the cell bodies of ganglion cells in the wall of the gut and from the extrinsic ganglia, the likelihood that these axons and the enteric glia have a nearly identical dendritic morphology is small. Physiologically, enteric glia are given virtually every neuronal attribute, including neuromodulation, cell-cell synaptic communication, selective response to neurotransmitters, and others (12, 35). As such, intramucosal glia appear to function largely as primary signaling elements, rather than “supporting” cells. Finally, enteric glial cells can generate functional enteric neurons in vitro (15, 20), and stem cells derived from the human intestinal mucosa appear to be bipotent, generating PGP9.5-positive neurons and GFAP-positive/S100-positive glial cells in culture (23). Despite the shared neural crest origin of enteric neurons and glia, there has not previously been a formal attempt to investigate the coexpression or the relationship of various “glial-specific” and “neuron-specific” markers in the human intestinal mucosa. INCs thus require recognition as a novel cell type in the human intestine. Interestingly, we noted that there appears to be cytoplasmic continuity between neighboring INCs, as seen in Figs. 1b, 2a, 3a, and 5a. This raises the intriguing possibility that these cells may form a syncytial network, which would allow synchronized electrical and chemical activity. Functional and physio-

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**Fig. 7.** Quantitative analysis and flow cytometry of intramucosal dendritic cells. *a:* Immunostained sections were scanned, and well-oriented regions where crypts reached the muscularis mucosae were selected for analysis. In each region, 5 separate foci were manually selected by following the epithelial basement membrane (magenta lines within rectangular boxes). *b* and *c:* Each masked focus was subjected to a manual cell count (*b*, blue arrows) and automated measurement of brown diaminobenzidine (DAB) pigment (*c*, orange mask over brown pigment). *d* and *e:* Box-and-whisker plots were generated for the number of cell bodies per unit area (*d*) and for DAB-positive fractional area (*e*) using data from 10 patients, including 5 analysis foci in 2 separate regions per patient. *f:* Representative scatter plot of intestinal cells incubated with anti-rabbit Alexa Fluor 488-conjugated and anti-mouse Alexa Fluor 647-conjugated secondary antibodies (1:500 dilution) without primary antibodies. *g:* Representative scatter plot of cells incubated with anti-Tuj1 and anti-S100, each at 1:100 dilution, and visualized with the same secondary antibodies (1:500 dilution). Axes represent fluorescence intensity for Tuj1 (y-axis) and S100 (x-axis) on a logarithmic scale. Percentage of cells that costain for Tuj1 and S100 (0.02% in the negative control (f) and 0.40% in the presence of primary antibodies (g)) confirms coexpression in a distinct population of cells. Data are representative of triplicate samples.
logical studies, however, are needed to test this hypothesis, to precisely define the physiological role and ontogeny of INCs, and to determine the role, if any, of potential syncytial synchronization in the mucosal ENS.

We find INCs in aganglionic segments of intestine in patients with HSCR, albeit with an altered expression pattern. This finding sheds light on seemingly contradictory observations in surgical pathology of HSCR, where numerous markers, including ACE, PGP9.5, and calretinin, have been proposed as aids to the diagnosis of HSCR in mucosal biopsies, but none has gained universal acceptance because of false-positive and false-negative observations in the subsequent clinical validation. Whereas the field has focused on the presence or absence of specific cellular elements, largely on the basis of the observation of a complete absence of ganglion cells in HSCR, we suggest that all such markers focus on the population of INCs, which we have shown can be present in HSCR with an altered expression profile. The use of a nonquantitative method, such as enzyme histochemistry and immunohistochemistry in the background of an altered expression profile, is sufficient cause for false-positive and false-negative surgical pathology results (29).

Based on the expression pattern of INCs, we propose that the abnormal mucosal neurites often referred to in the context of HSCR likely include the dendritic processes of INCs and are not entirely made of abnormal extensions of extrinsic nerve fibers, as commonly suggested (11, 16, 17, 22, 28, 29). The presence of intramucosal nuclei within hypertrophic ACE-positive fibers in HSCR and calretinin-positive cells in normal intestine, in conjunction with the expression of calretinin in submucosal nerves trunks but not in the neurites derived from these trunks (17), favors a contribution from INCs, rather than extrinsically derived neurites, in many of these scenarios. We cannot, however, exclude the possibility of multiple or mixed populations of INCs and extrinsic neurites in our study.

Regardless of the above-stated arguments for an overlapping neuronal and glial phenotype, our data demonstrate a network of mucosal cells that express glial markers in the setting of human aganglionic colon. In a study of glial GFAP expression in the human intestine, Kawana et al. (18) do not address or describe GFAP-positive cells in the mucosa of normal subjects or those with HSCR. Metzger et al. (23) found a rich network of Tuj1-positive cells, but only rare GFAP-positive cells, in the lamina propria of the human intestine. Interestingly, Kawana et al. describe a network of S100-positive dendritic processes in the lamina propria of normal and aganglionic colon, but they attribute this staining entirely to extrinsic nerve fibers, rather than intrinsic mucosal cells. As discussed below, the presence of intramucosal cells with glial or neuroglial differentiation in the setting of HSCR challenges our current understanding of the pathobiology of this disease in humans.
The current understanding of HSCR is based on two fundamental concepts: 1) all intrinsic enteric neurons and glia are derived from the neural crest; and 2) HSCR is caused by a defect in the rostrocaudal migration, survival, or differentiation of these neural crest-derived cells. The identification of INCs in the setting of aganglomosis leads to new hypotheses. 1) INCs may not reach the distal bowel via the rostrocaudal wave of crest cell migration, which appears to be the path taken by ganglion cells. This would be consistent with the observation that glial cells can enter the gut along extrinsic nerve fibers in aganglionic mice (30) or that ENCCs can take alternative pathways to the distal gut (25). 2) Perhaps HSCR represents a selective defect affecting only ganglion cells, rather than all intrinsic cells of the ENS. Further studies on the embryological origins, anatomic location, and density of INCs in HSCR are needed to clarify these important questions.

Our study has several limitations, largely due to the retrospective nature of the data and the constraints posed by the use of human tissue. We primarily relied on specimens from patients with HSCR in a 2-yr period when we were able to control tissue processing without interrupting clinical care. As such, we had little control over clinical variables, such as patient demographics, clinical presentation, surgical approach, or quantity or quality of tissue available for study. We managed to use archival tissue, but the quantity of material and the annotations describing the archived tissue were not sufficient to contribute to these studies. Despite these limitations, we believe that the morphological findings described here are not patient-specific. Our study focuses on fundamental anatomic observations, rather than variations thereof. It is likely that there are variations in density, distribution, or phenotypic expression of INCs based on age, anatomic location, or disease state, but the existence of INCs in the human intestine is certain. Our observations need to be reconciled with the wealth of data available in various animal models of ENS development and disease.

In summary, we propose the recognition of INCs as a novel cell type in the human ENS. Such recognition sheds light on the human HSCR data that would otherwise be incompletely explained by two mutually exclusive populations of intramucosal glia and extramucosal neurons. It also suggests the possibility of an anatomic link between the intestinal epithelium and the nervous system, which until now have been considered to be only anatomic observations, rather than variations thereof. It is likely that there are variations in density, distribution, or phenotypic expression of INCs based on age, anatomic location, or disease state, but the existence of INCs in the human intestine is certain. Our observations need to be reconciled with the wealth of data available in various animal models of ENS development and disease.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

K.B. and A.M.G. are responsible for conception and design of the research; K.B., A.R.T., N.N., D.N., S.A.M., A.A., and J.B.-G. performed the experiments; K.B., A.A., J.B.-G., and A.M.G. analyzed the data; K.B., A.A., J.B.-G., and A.M.G. interpreted the results of the experiments; K.B., N.N., A.A., and A.M.G. prepared the figures; K.B. and A.M.G. drafted the manuscript; K.B. and A.M.G. edited and revised the manuscript; A.M.G. approved the final version of the manuscript.

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