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Endocrine Cells of the Colon in Hirschsprung's and Control Children

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Summary. The large intestine resected from 6 Hirschsprung's patients and surgical colonic biopsies from 6 control children were examined with light and electron microscopy. The presence and the relative distribution of various endocrine cell types in both groups of mucosa were determined. In light microscope studies endocrine cell data were expressed as number of cells per unit area of mucosa using a quantitative method after argentaffin and Grimelius's argyrophilic techniques and an immunoperoxidase reaction with glucagon and somatostatin (SRIF) antisera.

The results indicate that endocrine cells are apparently not involved in Hirschsprung's disease, since their number and frequency did not differ significantly between the ganglionic and aganglionic segments of Hirschsprung's patients nor between the latter and control children. Glucagon immunoreactive cells were, on the average, 5–6 times and 7–9 times more numerous that SRIF cells in the rectum and the sigmoid, respectively. Ultrastructurally, five endocrine cell types could be distinguished. The fifth type, probably a transition type, apparently disappears in adults.

Key words: Megacolon – Large intestine – Endocrine cells – Immunohistochemistry – Electron microscopy.

Introduction

Nervous system involvement in Hirschsprung's disease (congenital megacolon) has been investigated since Whitehouse and Kernohan (1948) and Bodian et al. (1949) reported the absence of nerve cell bodies in the myenteric plexus of the distal colon. The disease has been considered as resulting from defective embryogenesis of this plexus. In the framework of this hypothesis there is some disagreement concerning the origin of enteric ganglion cells. A presumptive

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endodermal or mesodermal origin is no longer admitted, but it is generally held that the embryonic neural axis (neural crest and/or neural tube) give rise to the ganglion cells of the intestinal wall. The level of the neural axis from which these ganglion cells are derived, however, is still not clear. Thus, Van Campenhout (1932) suggested that they arose only from the trunk neural crest in chick embryos, while Yntema and Hammond (1954) proposed a vagal neural crest origin. An excellent survey of this complex problem has been published by Andrew (1971), who admits, as do Le Douarin and Teillet (1973), that the parasympathetic enteric ganglion cells arise from two different levels of the embryonic neural axis, corresponding to the vagal and lumbo-sacral parasympathetic centers. Okamoto and Ueda (1967) also studied the problem in human embryos and related it to Hirschsprung's disease. They found that the myenteric plexus is formed from neuroblasts which migrate cranio-caudally from the central nervous system into the alimentary tract. They also considered that Hirschsprung's disease was a developmental anomaly in which neuroblast migration along the gut ceased at various stages before the twelfth week of gestation: the earlier this cessation, the longer would be the resulting aganglionic segment.

In addition to the absence of ganglion cells, Smith (1972) reported that unmyelinated highly cholinergic fiber networks replace the plexus in the contracted segment. Baumgarten et al. (1973) described ultrastructural abnormalities of neuronal profiles, especially synaptic contacts with smooth muscle cells by varicosities. These observations could explained an uncoordinated motor activity. Ehrenpreis and Pernow (1952) demonstrated by extraction techniques that in Hirschsprung's disease substance P exists in smaller concentrations in the aganglionic than in the ganglionic segment. Recently, Pearse and Polak (1975), Nilsson et al. (1975), and Heitz et al. (1976) localized substance P immunohistologically in ganglionic cells, in the nerve fibers of the myenteric plexus and in certain mammalian gut endocrine cells, reported as probably being enterochromaffin cells.

Embryogenesis of gut endocrine cells is also a controversial subject. One of the most recent and widely discussed theories for these endocrine cells belonging to the APUD system of Pearse, proposes a common neural crest origin and their posterior migration into the gut (Pearse, 1969, 1973; Weichert, 1970). Works of Le Douarin and Teillet (1973) and Andrew (1974), however demonstrated that enterochromaffin cells are apparently not derived from the neural crest. In view of these reports and the particular pathogenesis of Hirschsprung's disease proposed by Okamoto and Ueda (1967) it might be asked if endocrine cells are also involved in this disease.

The aim of the present work was to determine if any anomaly of the endocrine cell population of the colon (absence of one or of all specific type(s), or a decrease of their frequency in the different segments) might accompany the interruption of the development of the myenteric plexus in congenital megacolon. Sequential biopsies performed on the resected colons in 6 cases of Hirschsprung's disease were studied with correlated immunohistological and electron microscopic techniques. Similar studies were conducted in parallel on the normal large bowel of children.

Material and Methods

Intestines which were resected because of Hirschsprung's disease (6 children: 2 long form and 4 short form) and surgical biopsies of normal colons as controls (6 children: 4 with urinary bladder anomaly, 1 with an angioma and 1 with high imperforate anus) were studied with histochemistry, immunohistology and electron microscopy. The childrens' ages ranged from one month to three years.

Histological Procedures

Two adjacent specimens which included entire mucosal rings were immediatly removed, at different levels, from both the ganglionic and aganglionic portions of the megacolon. They were streched on cardboard and fixed in Bouin's solution or in 10% formol for 24 h, then dehydrated, embedded in paraffin and cut in 4μ thick serial sections. Masson's argentaffin reaction was performed on formol fixed material; Grimelius's argyrophilic silver technique and immunoperoxidase reactions were performed on Bouin fixed serial sections. The same procedure was carried out on the mucosa of normal colons.

Immunohistology

White female New Zealand rabbits were immunized using glucagon (SIGMA, extracted from a mixture of bovine and porcine pancreases) and synthetic somatostatin or "somatotropin release inhibiting factor" (SRIF) (generously supplied by J. Rivier and R. Guillemin).

To avoid non-specific labeling of polymorphs, macrophages and epithelial cells, all sera were absorbed with bovine albumin (1%) and IgA (1%) prior to the indirect immunoperoxidase reaction. Serial sections were first incubated with the glucagon antiserum (1:10 dilution) or with SRIF antiserum (1:20 dilution), and secondly with peroxidase-labeled sheep antirabbit IgG serum (Institut Pasteur, Paris) diluted to 1:50. Peroxidase activity was revealed with 3-3′-diaminobenzidine tetrahydrochloride (DAB) (Merck, Germany).

Controls utilized included a) incubation with normal rabbit serum as first layer, b) prior absorption of the first layer antisera with an excess of glucagon or of somatostatin (10 μg of synthetic somatostatin and 50 μg of glucagon/ml of diluted antiserum). Controls were negative when sera were inactivated by the corresponding antigen.

Electron Microscopy

Small fresh pieces were taken from the same regions studied with light microscopy (in 4 megacolons and 5 controls) (Table 1) and were immediatly fixed for 2 h in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, post-fixed for 1 h in 1% OsO_4, dehydrated and embedded in Epon 812. Thin sections were observed with a Siemens Elmiskop IA electron microscope after double staining with uranyl acetate and lead citrate.

Table 1. Sites of biopsies for electron microscopy in the congenital megacolon (19 biopsies) and in normal colon (8 biopsies) of children

	Long form disease		Short form disease		Controls
	Patient 1	Patient 2	Patient 3	Patient 4	
Rectum	2	3	2	3	3
Sigmoid	3	2	1		3
Descending colon	2	1			2

Granule Morphometry

For all endocrine cells, the diameters of the largest granule profiles were measured at a magnification of 54,000 × (20–40 granules per cell). Histograms of granule diameter were constructed with a 50 nm frequency.

Quantitative Endocrine Cell Studies

1. Light Microscopy. Only nucleated endocrine cells were taken into consideration. Argentaffin cells, Grimelius argyrophilic cells, somatostatin and glucagon-immunoreactive (GLI) cells, were counted (using oil immersion at a magnification of 1,000 ×) on the entire length of the mucosal ring. Exactly comparable, well oriented regions were examined on all adjacent sections. It was thus possible to establish true ratios between different types of endocrine cells. The length of mucosa in which cells were counted was measured with a micrometer scale (between 1.5 to 6 cm). Cell counts were then refered to a mucosal length of 1 cm, corresponding to a theoretical mucosal surface of 40,000 μ^2 (10,000 $\mu \times 4\ \mu$, the thickness of the section). Average endocrine cell numbers were expressed per unit area thus defined. Data obtained from several mucosal rings belonging to the same region of the colon were averaged and only the final mean cell count for this entire region is given in the results.

2. Electron Microscopy. All nucleated cells of the different endocrine cell types observed were counted, care being taken not to count the same cell twice. Data are expressed as percentages.

Results

Morphological Data

The morphology of the rectal and colonic mucosa in both the ganglionic and the aganglionic regions in Hirschsprung's disease was no different from that observed in controls: there are many lymphoid follicles; epithelial cells have large and indented nuclei with a prominent nucleolus. No ganglia were seen in the intramural plexus of the aganglionic region in light or in electron microscopy.

In addition to goblet and undifferentiated cells, endocrine cells were seen in normal colons of children as well as in the two regions of megacolon. They were preferentially located in the lower part of the crypt but were sometimes seen in the upper portion of the mucosa. They were characterized ultrastructurally by their basal secretory granules. Prominent desmosomes were present at various points along the contact surfaces and lysosomes surrounding granular structures were occasionally observed. Exocytotic figures of secretory granules were sometimes noted at the basal membrane. The four endocrine cell types previously described for normal adults (Cristina et al., in press) were found in colons of children, i.e. type I (enterochromaffin cell) and types II, III and IV with rounded granules whose diameter frequencies peak for largest granules were 200–300, 150–200 and 300–400 nm, respectively (Fig. 1). Nevertheless, another cell type was observed with a granule diameter distribution peak of 400–500 nm, clearly separated from that of type IV cells (Fig. 2). It was thus called type V. The mean granule diameter ($\pm$SE) was 463.12 ± 3.22 nm for the congenital megacolon and 445.78 ± 5.08 nm for controls (range: 220 to 750 nm). In addition, one cell resembling the F-cell described by Forssmann et al. (1977) was also identified (Fig. 3). It presented slightly granular, almost translucent granules (mean diameter 440 nm).

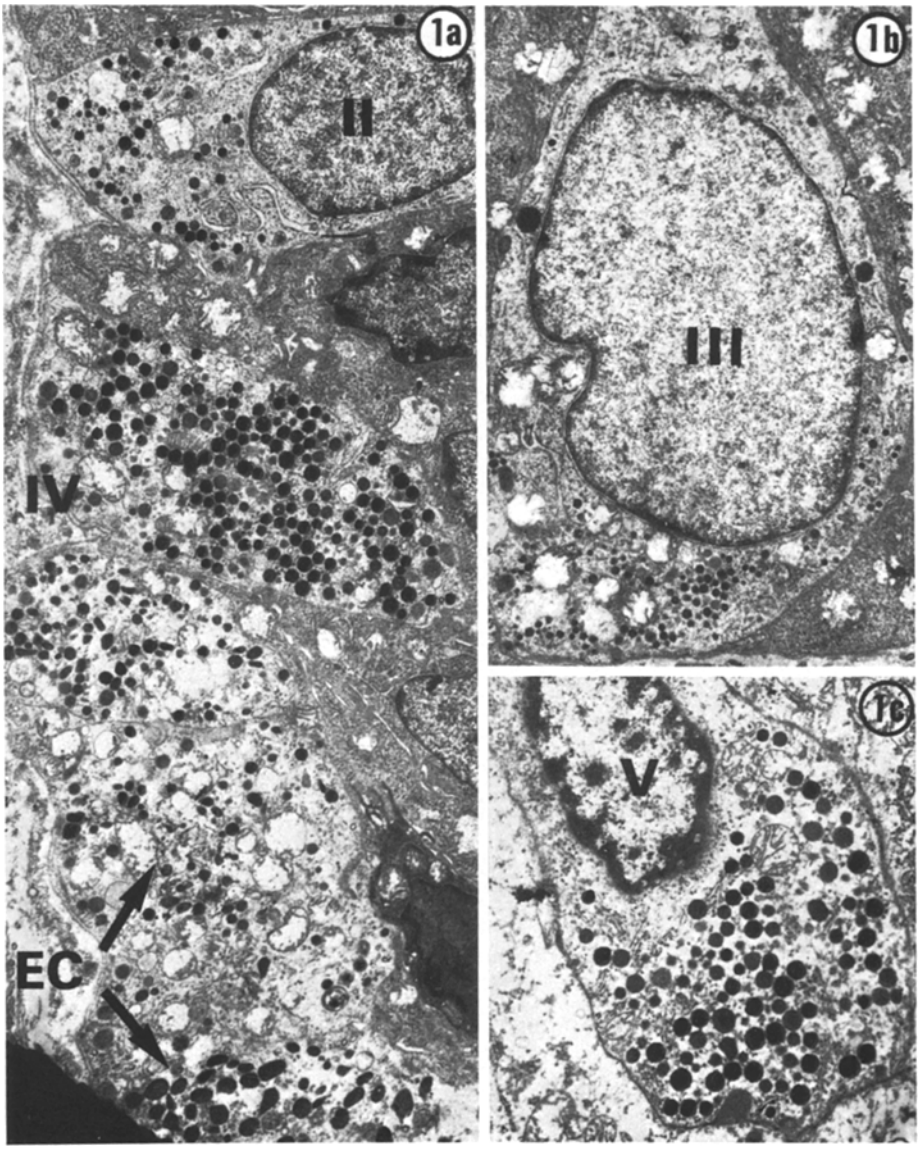

Fig. 1a–c. Different endocrine cell types observed in children's colons. **a** Hirschsprung's disease; rectal mucosa: two ends of EC cells showing granules of different size, one type II cell and one extremity of type IV cell were seen side by side. ×5400. **b** Hirschsprung's disease; sigmoid mucosa: one type III cell. ×5400. **c** Normal children; rectal mucosa: one type V cell (mean diameter 436.12 ± 3.22 nm, range: 220 to 750 nm) (glutaraldehyde-OsO_4 fixation). It should be noted that surgery results in reduced blood flow and so mitochrondria were often altered in spite of immediate fixation, without any change of the ultrastructural morphology of granules

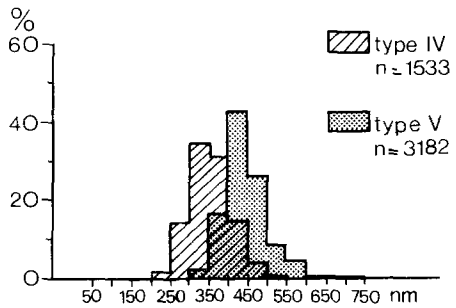

Fig. 2. Histogram with a frequency per class of 50 nm, showing the granule size distribution of type IV (peak of frequency 300–400 nm) and type V cells (peak of frequency 400–500 nm). n = the number of granules measured

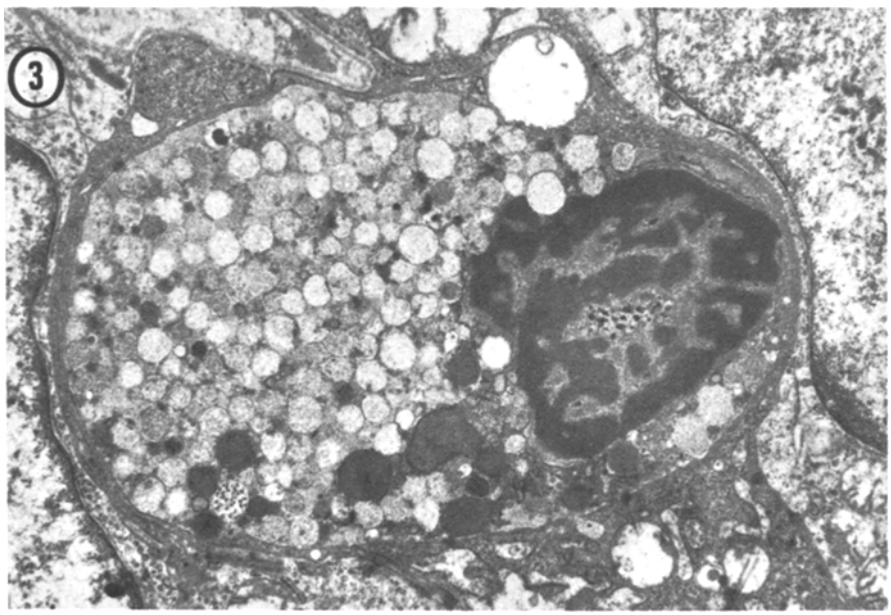

Fig. 3. Hirschsprung's case; descending colon. F cell showing translucent, slightly granular and densely packed secretory granules (glutaraldehyde-OsO_4 fixation) ×9500

In two subjects (one normal and one megacolon) there were some cells at the base of the crypts with poorly developed organelles. Some of them showed several small, undefinable granules resembling the "progranules" or immature granules found in the Golgi apparatus of endocrine cells.

Quantitative Data (Tables 2–3, Figs. 4–5)

The numbers of nucleated argentaffin, Grimelius argyrophilic, GLI- and SRIF-immunoreactive cells per unit area are given in Table 2. There was no significant difference in these numbers between normal gut and resected gut for Hirschsprung's disease, although a decrease in GLI- and argyrophilic cells was observed in the sigmoid of controls compared with the same region of the

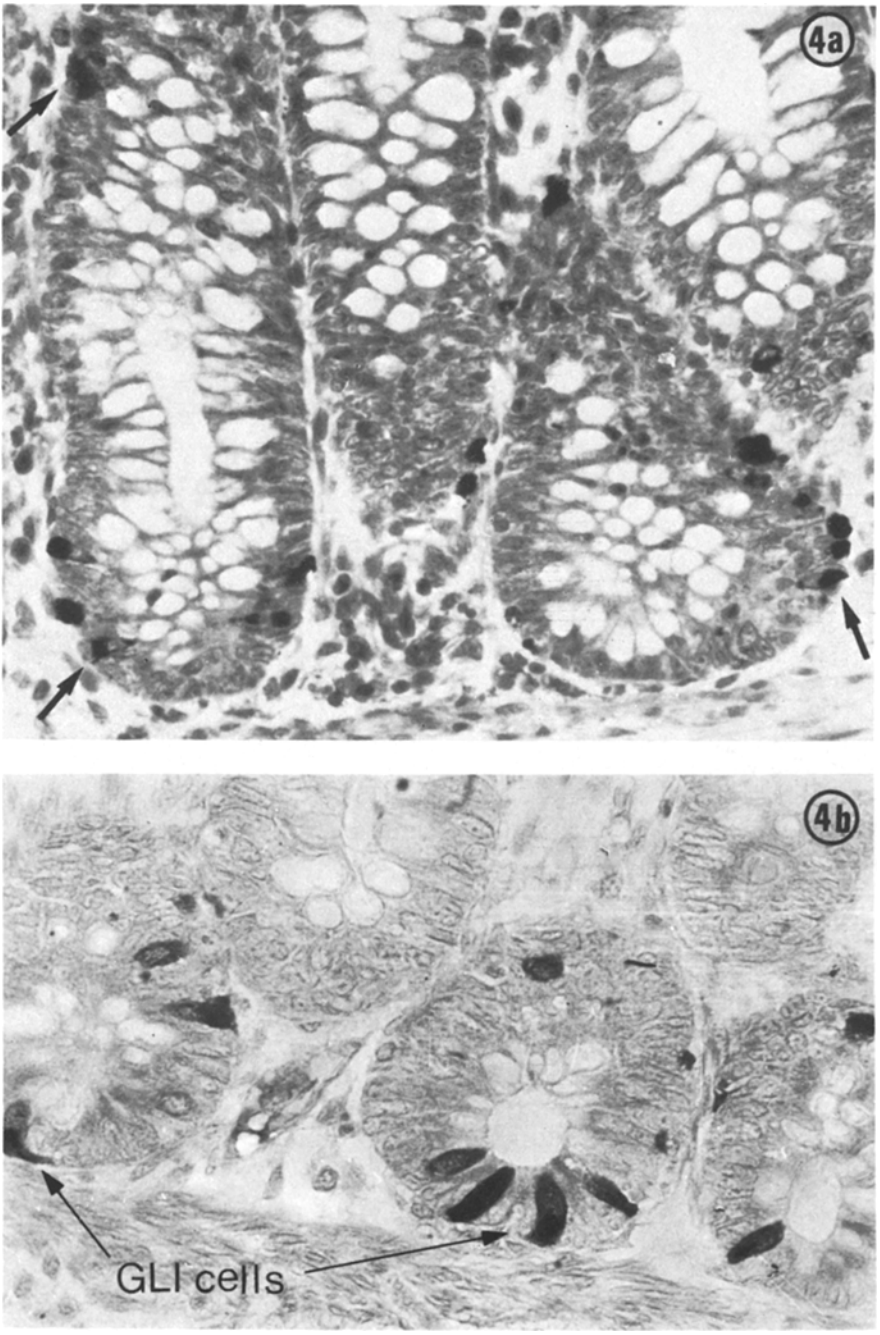

Fig. 4a and b. Colonic mucosa of children (Bouin fixation). **a** Hirschsprung rectal mucosa: argyrophilic cells with Grimelius technique. ×420. **b** Sigmoid mucosa in normal children: immunoperoxidase staining with antiglucagon serum showing several positive cells at the base of the crypts. ×420. Prior absorption of the antiserum with albumin bovine and IgA

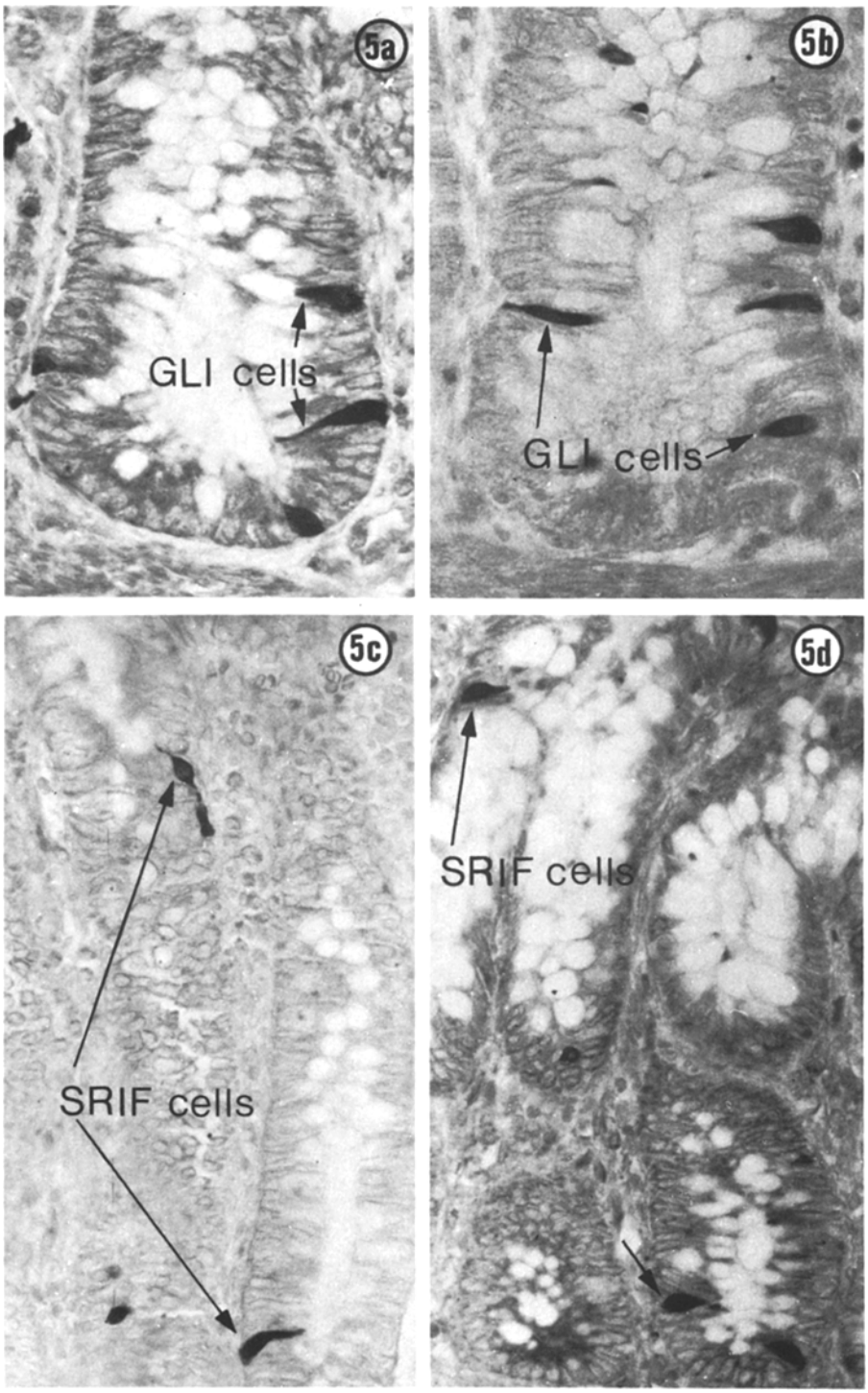

Fig. 5a–d. Colonic mucosa of children (Bouin fixation). Comparison of GLI and SRIF cells in Hirschsprung disease (H.D.) and normal children. GLI cells were seen with the same frequency in the lower part of the crypt in rectal mucosa in H.D. **(a)** and in normal children **(b).** Note the thin process of GLI cells reaching the glandular lumen. ×430. SRIF cells were seen at the base of the crypt as well as at the top of sigmoid mucosa in H.D. **(c)** and normal children **(d).** ×305. Prior absorption of the antisera with albumin bovine and IgA

Table 2. Mean number of nucleated argentaffin, argyrophilic, SRIF and glucagon immunoreactive cells per unit mucosal area (40,000 μ^2) in congenital megacolon and the colons of control children (± 1 SEM)

	Grimelius positive cells	Argentaffin cells	GLI cells	SRIF cells	SRIF/GLI
Rectum					
Hirschsprung (6)	386 ± 50	186 ± 26	218 ± 26	41 ± 8	5.70 ± 0.89
Control child[a] (1)	360	179	193	39	4.95
Hirschsprung +control children[b] (8)	365 ± 40	171 ± 23	208 ± 20	47 ± 9	$5,11 \pm 0.82$
Sigmoid					
Hirschsprung (5)	362 ± 38	188 ± 34	195 ± 42	28 ± 7	8.73 ± 2.87
Control child[a] (1)	340	191	185	32	5.78
Hirschsprung +control children[b] (8)	300 ± 47	158 ± 29	169 ± 30	27 ± 5	7.18 ± 1.84
Descending colon					
Distal Hirschsprung (2)	366 ± 12	123 ± 18	91 ± 33	17 ± 7	5.53 ± 0.53
Proximal control child (1)	93	55	41	16	3.12

Number of patients studied is shown in parentheses

[a] Control child with high imperforate anus (entire intestinal mucosal ring)

[b] Hirschsprung's patients+control children (entire intestinal mucosal ring+small surgical biopsies). Colonic endocrine cell data were not significantly different between these two groups and they were averaged

Table 3. Comparative distribution in Hirschsprung's disease and in normal colons of children. Percentages of different endocrine cell types according to the region of the colon[a]

	Total cell number	Type I EC	Type II	Type III	Type IV	Type V
Rectum						
Hirschsprung	377	51.7%	4.2%	0.8%	20.7%	14.6%
Control children	61	47.5%	3.3%	0	29.5%	18%
Control adults[b]	179	42%	31.3%	3.3%	14%	0
Sigmoid						
Hirschsprung	135	49.6%	5.2%	3.7%	19.2%	11.8%
Control children	76	47.4%	11.8%	0	21%	7.9%
Control adults[b]	135	53.3%	24.4%	1.5%	9.6%	0
Descending colon						
Hirschsprung	66	68.2%	3%	1.5%	16.6%	6%
Control children	16	68.7%	6.2%	0	25%	0
Control adults[b]	68	78.2%	13.2%	0	3.5%	0

[a] There were no significant differences in Hirschsprung colon between ganglionic and aganglionic regions; data were averaged per anatomic region

[b] Previous data: Cristina et al., Gastroenterology in press

Percentages of non-classified cells were not included in this table

megacolon. Two other facts are evident: first, in children, there were noticeable numbers of GLI- and SRIF-containing cells in the distal large bowel; secondly, the number of argyrophilic cells roughly corresponded to the number of argentaffin cells + the number of GLI-cells. It appears that GLI-cells were on the average 5–6 times more numerous than SRIF-cells in the rectum and 7–9 times more numerous in the sigmoid.

At the electron microscope level, 731 nucleated cells were examined: 578 in congenital megacolon (117, 135, 157 and 169 cells in each of the 4 cases) and 153 cells in the controls. Cells which could not be classified with certainty represent 7.2% of the endocrine cell population of which 80% belonged to the types described in normal adult colon.

Table 3 shows the percentages of each endocrine cell type in the rectum, sigmoid and descending colon for control and Hirschsprung's cases, and compares these data with those previously observed in the normal human adult colon (Cristina et al., in press). It should be noted that the percentages of each type of endocrine cells was not significantly different between the two categories of children. Three major differences exist between children and normal adults: the complete disappearance of type V in the adult, a higher frequency of type II in the adult than in children, and a relative higher and more constant proportion of type IV cells up to the descending colon in children.

Discussion

Our study has established with certainty the presence of endocrine cells along the entire distal (ganglionic and aganglionic) colonic mucosa in Hirschsprung's disease. Other new data are also presented: not only are several endocrine cell types present in the colon of Hirschsprung's disease, but their relative frequencies are similar to controls, whatever the intestinal segment. The comparison of ultrastructural data concerning the colonic endocrine cells of adults and children shows that the latter possess an additional cell type, as well as a different distribution pattern for two other endocrine cell types, i.e. II and IV.

As mentioned above, neither the number of endocrine cells per unit area in light microscopy nor their percentages in electron microscopy were significantly different between Hirschsprung cases and controls (Tables 2 and 3), although the age dispersion of the two groups was different (1 month to 3 years and 6 to 10 months respectively). A slight decrease in the numbers of GLI and of Grimelius argyrophilic cells per unit area was noted only in the smallest control biopsies. In one control case, the child with imperforate anus, the entire mucosal ring was examined, as in Hirschsprung patients and the data were quite comparable.

Our light microscopic immunohistochemical data indicate that Grimelius argyrophilic cells seemed consistently to include argentaffin and GLI cells, confirming that GLI cells in the human intestine react strongly to the Grimelius technique (Grimelius et al., 1976). Knudsen et al. (1975) found an average of 3.3 GLI cells (range 1–5) per crypt in the human colon, mostly in the median and deepest portions. In our material they were often observed at the upper

portion of the mucosa. Besides, our data do not agree with those of Capella et al. (1976) who found no D (SRIF) cells in the human colon and rectum. Another attempt to quantitate GLI and SRIF cells in the human intestine (Pearse et al., 1977) did not state subject ages or the colonic segments studied: these authors found 24.74 GLI, and 0.5 SRIF cells per mm^2 which give a GLI/SRIF cell ratio of about 50. To our knowledge, no data concerning endocrine cells in the distal colon of children have been published. The present work shows the occurence of high numbers of GLI cells (210–220 per 40,000 μ^2) and SRIF cells (40–50 per 40,000 μ^2) in the rectum, decreasing towards the descending colon with a GLI/SRIF cell ratio of about 5–6 in the rectum and 7–9 in the sigmoid. These data indicate a greater abundance of these cells in children in comparison with the data of Pearse et al. (1977), accompanied with a lower variation between GLI and SRIF cells. It is also possible that these differences are related to the quantitative method used. The data presented here, resulting from the average of several data per region, are truly representative of each region.

The changing distribution patterns of endocrine cells between the young and adult human colon as seen in electron microscopy are shown in Table 3. Type II cells, the second major type in adults (13 to 31%) are present in smaller percentages (3 to 11%) in children. This difference is greater in the rectum and sigmoid than in the descending colon. It may be that there is proliferation of this cell type during postnatal life, but their significance is not known. By contrast, type IV cells have a higher frequency in the rectum, sigmoid and descending colon of children than in adults. It is assumed that the ultrastructural L and D cells corresponding to GLI and SRIF cells, respectively, are included in this type because of their similar granule diameters (Cristina et al., in press). Moreover, the relatively widespread distribution of type IV cells is correlated with the numerous GLI and SRIF cells observed in the distal colon of children. Type III cells, which are thought to secrete vasointestinal polypeptide, are as rare in children as they are in adults.

The histogram (Fig. 2) shows that the 5th type of cell individualized has a granule diameter which is greater than 400 nm; it was observed only in children. Moxey and Trier (1977) reported 13 morphologically distinct endocrine cell types in the human fetal small intestine, some of which were putative "transitional" types; 6 of those types were not found in the normal adult. An identical process probably occurs in the colon and continues during the initial years of extraembryonic-life. We may hypothesize that the high number of GLI cells observed may result from the fact that some of these type V cells, still at an embryological stage, react to antiglucagon serum.

It must be pointed out that in the rectum and sigmoid there is not an exact correlation between the data on ultrastructural enterochromaffin cells and those concerning light microscopic argentaffin cells (Tables 2 and 3). Type I (EC) cells were the most frequent (50% of the total ultrastructural endocrine cell population), while the number of argentaffin cells per unit area was always lower than the number of GLI cells and a fortiori lower than the number of GLI + SRIF cells which are presumed to be included in type IV. The ultrastructural type IV cell was the second most frequent, following EC cells (20–30%). An undulating distribution of GLI and especially SRIF cells, along

the entire mucosal ring was noted; these cells were absent in some crypts. This distribution pattern probably also recurred in the proximo-distal axis and if this is so, the fact that adjacent mucosal rings were removed and one studied for argentaffinity, one for other methods, could explain certain differences observed. Nevertheless, it must be borne in mind that the percentages of EC cells per segment were nearly constant as was the argentaffin cell number per unit area, in both groups of children. Perhaps it is more plausible to suggest that not all ultrastructural EC cells are argentaffin and that a strict correspondance between both terms is not certain. Discrepancies between serotonin-containing cells and argentaffin cells (Tobe et al., 1966), between enterochromaffin, motilin and serotonin-producing cells (Forssmann et al., 1976; Polak et al., 1976) have already been described.

The cell which resembles the F cell described by Forssmann et al. (1977) because of the appearance of its granules, was localized near the extremity of the aganglionic sigmoid of a long case. Its mean granule diameter was similar to that of duodenal F cells but was larger than that of pancreatic F cells. The authors cited identified F cells as the secretory pancreatic-polypeptide (PP) cells. This polypeptide acts on smooth muscle of the gut including the colon (Lin and Chance, 1974). In Hirschsprung's cases colonic motility studies showed no peristalsis within the narrow aganglionic segment and relatively normal peristalsis within the other segments (Swensson et al., 1949). F cell distribution is presumed to be sparse in the human colonic mucosa and could be very scanty in segments with decreased peristalsis.

Our data indicate that gut endocrine cells are apparently not involved in Hirschsprung's disease. Although the present work furnishes no information concerning the origin of gut endocrine cells, it does not support the theory of Pearse. Although this theory has been verified for several types of APUD cells, such as the thyroid C cell, it has not been for gut endocrine cells. Le Douarin and Teillet (1973) and Andrew (1974) demonstrated that a category of gut endocrine cells, i.e. enterochromaffin cells, were not derived from neural crest. The former report did not exclude the possibility of an ectodermal origin from more cephalic regions of the neural embryonic axis. Pearse and Takor (1976) were themselves recently more cautious concerning the origin of gut endocrine cells. Taking account of the work of Okamoto and Ueda (1967) and the theories described above, our data suggest that endocrine cells either arise from endoderm or, if they arise from the ectoderm, the migration of neuroblasts and endocrine cells are independant.

Substance P, a local "hormone" for the regulation of intestinal motility, is found in enterochromaffin cells as well as in the myenteric plexus (Nilsson et al., 1975; Pearse and Polak, 1975; Heitz et al., 1976). It may be supposed that its decrease in the aganglionic segment (Ehrenpreis and Pernow, 1952; Tafuri et al., 1974) is more related to its decrease in the plexus than to the absence of a certain category of enterochromaffin cells. Since substance P antibodies were not utilized in the present study, we are not able to verify its presence in the observed enterochromaffin cells.

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