Interstitial Cells of Cajal and Intestinal Function in Trinidadian Children

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ABSTRACT

Objective: *This study was carried out to compare the density of the interstitial cells of Cajal (ICCs) in the bowel wall of children with Hirschsprung's disease (HD), anorectal malformations (ARM) and normal controls in Trinidad and Tobago.*

Subjects and Method: *Segments of bowel wall excised from eight children with HD, three controls and two children with ARM were immunostained with c-Kit primary antibody. Cells with features of ICCs were counted.*

Results: *All three controls and the two children with ARM had dense distribution of ICCs. Most children (6/8;75%) with HD had markedly reduced counts in aganglionic bowel. Two (25%) also had a decrease in ganglionic bowel. Possible influences were patient age and gender and the level of bowel sectioned.*

Conclusion: *Analysis of this sample suggests that immunostaining for c-Kit positive cells might be a useful screening test in the assessment of bowel motility disorders. The possible effects of age, gender and the level of bowel sampled await determination.*

Células Intersticiales de Cajal y Función Intestinal en Niños Trinitenses

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RESUMEN

Objetivo: *Este estudio se llevó a cabo con el propósito de comparar la densidad de las células intersticiales de Cajal (CIC) en las paredes intestinales de niños con la enfermedad de Hirschprung (EH), y malformaciones anorectales (MAR), frente a controles normales en Trinidad Tobago.*

Sujetos y Métodos: *Segmentos de las paredes intestinales les fueron extirpados a ocho niños con EH; tres controles y dos niños con MAR fueron inmunoteñidos con anticuerpo primario c-kit. Se contaron las células con características de CIC.*

Resultados*: Los tres controles y los dos niños con MAR presentaban una distribución densa de CICs. La mayor parte de los niños (6/8; 75%) con EH tuvieron conteos marcadamente reducidos de intestino agangliónico. Dos niños (25%) también tuvieron una disminución de intestino gangliónico. Entre las influencias posibles se cuentan la edad y el género del paciente así como el nivel de intestino seccionado.*

Conclusión: *El análisis de esta muestra sugiere que la inmunotinción para células c-kit positivas podría ser un útil test de pesquisaje a la hora de evaluar desórdenes en la motilidad intestinal. Los efectos posibles de la edad, el género y el nivel de intestino muestreado, están pendientes de determinación.*

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INTRODUCTION

The interstitial cells of Cajal (ICCs), first identified over a century ago (1), have gradually gained recognition, in conjunction with the enteric nervous system and muscle layers, as critical elements in the normal development and motility of the bowel (2). Attention has been attracted to these cells during investigations into disorders of bowel development and motility. Their recognition has been simplified by the discovery that the transmembrane tyrosine-kinase receptor c-

Kit is essential for their development and function (3). To counteract the difficulties associated with studying their distribution by light microscopy, c-Kit cells and synapses can now be visualized by immunohistochemical methods involving the use of commercially available monoclonal antibodies (4).

Variations in the distribution of the ICCs have been ascribed to variables such as patient age and the level of the bowel being assessed, as well as to a number of clinical conditions, which include Hirschsprung's disease (HD) and its variants and congenital anorectal anomalies (ARM). However, reported disparities have been inconsistent, making evaluation difficult.

This study investigates the distribution of these cells in a group of Trinidadian children affected by HD and ARM and compared the findings with those in normal children. This was effected by conducting immunohistochemical studies on specimens obtained by bowel biopsies and/or resections from children affected by these conditions and from normal controls.

SUBJECTS AND METHODS

Permission to conduct the study was obtained from the Ethics Committees of the University of the West Indies and the Eric Williams Medical Sciences Complex where treatment was carried out. During the period of interest, the years 2002 to 2004, 11 children had one or more stages of the surgical treatment of HD performed at the hospital. The diagnosis in eight of these cases had been suspected on initial clinical presentation and barium enema examination. Confirmation was obtained by the absence of ganglion cells on microscopic examination of a full thickness biopsy of the rectal wall. In the other three patients, a full thickness biopsy of the colon was obtained at the time of laparotomy for the relief of intestinal obstruction.

The records of the cases were retrieved and specimens suitable for the study were sought. The tissue blocks of three of the aganglionic biopsies either could not be located or had insufficient tissue available for further processing. Assessment of the corresponding specimens of resected ganglionic bowel was accordingly not pursued. Specimens from eight children remained available for examination. Four of these were male and four female. Their ages ranged from two months to 13 years at the time the specimens were obtained with a median of two years.

Full thickness biopsies taken from the sigmoid colon stoma were available from two children, aged 11 and 20 months, with ARM at the time of restoration of bowel continuity. Normal segments of colon and rectum were obtained from three children who died unexpectedly without any previous history or evidence of bowel disease. The autopsy diagnoses were: bronchopneumonia associated with Respiratory Distress Syndrome in a month old baby, extensive brain damage in a one-year old girl following a motor vehicle accident and multiorgan failure from systemic viraemia in a six-year old boy. Postmortem intervals for the autopsy cases were less than 24 hours.

For each of the specimens of bowel obtained, full thickness sections of the wall were cut from the aganglionic segment and, in the case of resected specimens, from at least one proximal ganglionic segment, when this was available. Sections were stained with haematoxylin and eosin and by immunohistochemistry for c-Kit (CD 117).

Paraffin sections were cut to a thickness of four μm, prepared and mounted on silanized glass slides. The cut sections were deparaffinized in xylene and rehydrated through alcohol. Target antigen retrieval was performed by microwave heating of tissue sections in citrate buffer at pH 6.0 for 20 minutes. After blocking endogenous peroxidase activity with 3% hydrogen peroxide, sections were incubated with c-Kit primary antibody (polyclonal rabbit anti-human antibody, DakoCytomation) diluted 1:400. This was followed by sequential incubation, washing and incubating with phosphate buffered saline (PBS) between steps. Counterstaining with Harris' haematoxylin completed the immunostaining process. The ICCs, thus stained, appeared as thin elongated cells with short processes. In the myenteric plexus, they were arranged around ganglion cells (Figs. 1, 2).

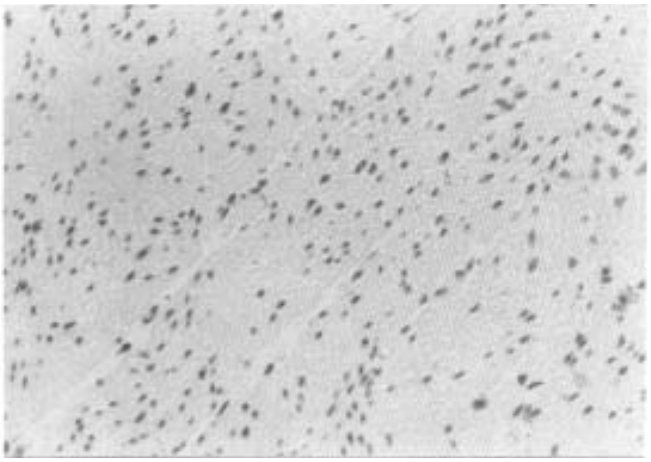

Fig. 1: Hirschsprung's disease (case 6) – a section of aganglionic descending colon, stained with c-Kit antibody (x 40). The myenteric plexus and muscular layers are devoid of c-Kit positive cells.

Cell counts were carried out in the submucosa, myenteric plexus and muscular layers of bowel wall by two independent observers both experienced histopathologists. Magnification of 40x was used over an area of $1mm^2$ per compartment. Counts of less than half the lowest control count were assumed to be abnormally diminished.

RESULTS

The correlation of ICC counts for the individual layers of the bowel wall showed some divergence between the two observers, and accordingly, comparison of cell counts for each bowel layer was not pursued. However, the total cell counts

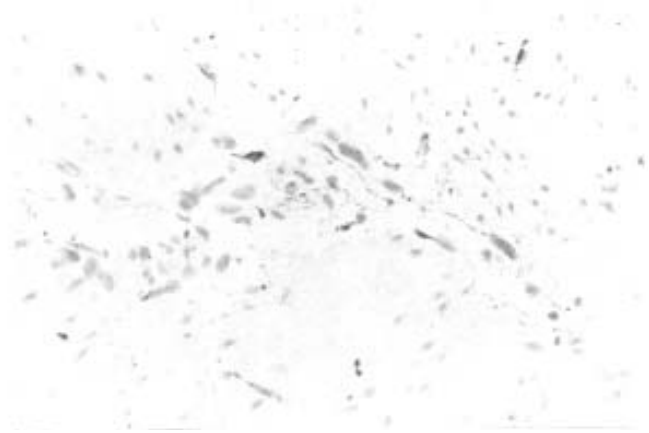

Fig. 2: Hirschsprung's disease (case 8) – a section of ganglionated descending colon, stained with c-Kit antibody (x 40). The myenteric plexus contains ganglion cells which are closely apposed to several elongated c-Kit positive cells with short processes, the features of interstitial cells of Cajal.

obtained coincided to a great extent and these were accordingly subjected to analysis.

The number of ICCs identified in the three controls ranged from 131 to 219 and counts of less than 65/mm^2 in the other specimens were taken to be severely reduced. Counts from the stomata of the two patients with ARM ranged from 114 to 130.

Eight segments of ganglionic bowel, which had been excised from six of the patients with HD (HD-G), had a range of ICCs of 10 to 214. This included three segments (derived from patients 1 and 7) with less than 65/mm^2. The corresponding range for the nine aganglionic segments taken from the eight cases was two to 161, seven (from six children) being less than 65/mm^2 (Table 1). Thus, six of the eight children with HD had marked diminution of ICCs in aganglionic bowel and two in ganglionic bowel. One of the latter continued to have a daily average of two normal bowel motions during a follow-up period of more than two years following completion of surgery. The other, however, had early and persistent problems with a poorly functioning stoma in the transverse colon. She required a second laparotomy two weeks after the first for the construction of a more proximal stoma.

Tabulation of the data according to age, gender and bowel level showed that the four youngest patients were evenly affected by a reduced density of ICCs in aganglionic bowel. Two of three had normal counts in ganglionic bowel. The four patients over two years of age, however, all had reduced counts in aganglionic bowel as did one of the three for whom ganglionic bowel was available for study (Table 2).

With respect to the effect of gender, all four girls had reduced counts in aganglionic bowel, and two of the three with available specimens were similarly affected in ganglionic bowel. The four males, on the other hand, were evenly split with respect to reduced and normal counts in aganglionic

Table 1: Relationship of number of ICCS to selected variables

Case No.	Age (yrs)	Gender	Segment	Level	No. ICCs/mm^2
C 1	1/12	M	G	DESC	219
C 2	6	M	G	RECT/SIG	131
C 3	1	F	G	RECT/SIG	180
ARM 1	11/12	M	G	SIG	130
ARM 2	20/12	M	G	SIG	114
HD 1	2	F	AG	RECT	22*
			G	SIG	44*
				TRANS	51
HD 2	13	F	AG	RECT	3**
HD 3	6	F	AG	RECT	44*
			G	DESC	154
HD 4	2/12	M	AG	SIG	161
	2/12		AG	DESC	131
	2		G	TRANS	117
HD 5	/9	M	AG	RECT	44*
			G	DESC	83
HD 6	2	M	AG	DESC	2**
HD 7	11	F	AG	RECT	15**
			G	SIG	10**
			G	DESC	45*
HD 8	1	M	AG	SIG	139
			G	DESC	214

Key: HD = Hirschsprung's Disease; ARM = Anorectal Malformation; C = Control.
AG = Aganglionic Bowel; G = Ganglionic Bowel;
ICC's = Interstitial cells of Cajal
DESC = Descending colon; RECT = Rectum;
SIG = Sigmoid colon; TRANS = Transverse colon
* = <65 ICC's/mm^2 (half of the lowest count found in the controls)

Table 2: Distribution of variables in patients with Hirschsprung's Disease

Variable	No preparations examined*	< 65 ICC's/mm^2 AG	G	> 65 ICC's/mm^2 AG	G
Age:*					
#2 yr	9	2/4**	1/3	2/4	2/3
>2 yr	8	4/4	1/3	0/4	2/3
Gender:*					
Male	8	2/4	0/3	2/4	3/3
Female	9	4/4	2/3	0/4	1/3
Bowel level:*					
Transverse	2	–	1	–	1
Descending	6	1	1	1	3
Sigmoid	4	1	1	1	1
Rectum	5	5	–	0	–
n specimens	17	7	3	2	5

AG = aganglionic segments; G = ganglionic segments; No. = number
* most patients had more than one level of bowel examined
** ratios are no. of results/no. of patients

onic bowel. All three with suitable samples had normal counts in ganglionic bowel.

With regards to the influence of the level of bowel sampled, all the aganglionic segments of rectum suitable for study had reduced cell density (< 65/mm^2) but all came from female patients. Samples from other levels of bowel gave less consistent results. These details are summarized in Table 2.

DISCUSSION

This investigation revealed a marked reduction in the density of interstitial cells of Cajal (ICCs) in seven of ten aganglionic as well as three of seven ganglionic areas of bowel taken from eight patients with Hirschsprung's disease (HD). The two children with normal counts in aganglionic bowel were both male at or under the median age of two years. All five rectal aganglionic segments had reduced counts unlike the variable counts obtained at other aganglionic bowel levels. Girls appeared to be more consistently affected by reduced ICC counts in aganglionic bowel but this observation is compromised by the fact that all of the rectal specimens were derived from female patients.

Recent experiments involving surgical or chemical ablation of ICC containing layers of bowel have clarified the possible physiologic roles of these cells. These include acting as the source of slow electrical waves, participating in the conduction of electric currents and being mediators of neural signals between enteric nerves and muscles (5). Motor neurotransmission in the gut has been shown to occur *via* synaptic-like contacts between varicose nerve terminals and intramuscular ICCs (ICC-M's). These ICC-Ms have been shown to be coupled to smooth muscle cells *via* gap junctions which allow them to function as electrical conductors (6).

The distribution of these cells is difficult to study with light microscopy and a strong case was originally made for the use of transmission electron microscopy for their definitive identification (4). However, this technology is not available at all centres. Recognition that the transmembrane tyrosine-kinase receptor c-Kit is essential for the development and function of the ICC was therefore timely and immunohistochemical staining with antibodies for the human c-Kit receptor has evolved into a valuable adjunct to the study of the distribution of ICCs by light microscopy (3).

In the normal bowel, ICCs form a dense network surrounding the myenteric plexus and cluster at the innermost part of the circular muscle. Variations in their structure and organization have been shown to occur depending on the layer and level of the bowel under scrutiny. In the normal human colon, the ICC network is prominent throughout the thickness of the musculature. The distribution was originally described as being similar in different parts of the colon and at different ages including the neonatal period (7). However, regional differences in the distribution of these cells have been described by some investigators (8, 9) and there is also conflicting evidence that ICC networks continue to develop well into postnatal life (9). The study being presented, though involving a small number of patients, supports the possibility of an effect of age, gender and the level of bowel studied on the density of these cells.

Variations in the density and distribution of ICCs have been demonstrated in several clinical conditions and this investigation has revealed a reduced density of these cells in the aganglionic bowel of the majority of patients affected by Hirschsprung's disease (HD). This is in accord with previous studies where myenteric ICCs were shown to be greatly reduced or absent in aganglionic bowel and sparse in the transitional zone. Muscular ICC density has also been shown to be reduced in the normally innervated bowel of patients with HD, an observation which has been proposed as an explanation for the persistence of problems with motility after definitive surgery (10, 11). However, this observation has not been unanimous and most investigators, while confirming a reduced density in aganglionic bowel, have found the distribution of these cells in the proximal ganglionated bowel of patients with HD to be identical to that of normal colon (7, 12). One of our patients with a diminished count in ganglionic bowel did not display any postoperative problems with motility during prolonged follow-up, but, in a retrospective study, it is difficult to be sure of the relationship of the bowel that was actually pulled through to the originally sampled site. The other girl with ganglionic cells and reduced ICC numbers at or just proximal to the stomal site had persistent problems with stomal function in the early postoperative period and required relocation of the stoma to a more proximal level in the bowel.

A reduction in ICCs has also been described in the associated conditions of hypoganglionosis (13) and other allied HD disorders (14) including achalasia of the internal sphincter (15). These discoveries have been the subject of two alternative interpretations: either that the enteric nervous system is required for full differentiation of the ICC network or that a common mechanism acting on the mesenchyme affects both the migration of neural crest derivatives and the differentiation of ICCs (7), each to a varying degree. The latter theory is favoured by the occasional finding of normal ICC counts in aganglionic bowel as occurred in two of our patients.

One study of 12 patients with anorectal malformations (ARM) undergoing stoma formation or closure showed a complete absence of ICCs in two patients and a markedly reduced density in circular muscle from the stomal site in five others. This was proposed as an explanation for a high incidence of postoperative constipation in these patients (16). Our two patients with ARM had normal density of these cells as has been more commonly reported (7, 14).

Thus ICCs have been implicated in several types of bowel dysmotility but reported findings have been inconsistent and a clear pattern is yet to emerge. This may be due to the co-existence of other influences on the aetiology of the

various clinical conditions involved. Nevertheless, analysis of this small series supports the suggestion that a diminution in ICC density is a valuable pointer to the existence of neuronal bowel abnormalities.

The practical application of this observation to specific diagnoses requires further clarification of the factors involved. In the meantime, the relatively low expense and ease of application of their study which requires only immunohistochemical staining and pathologic assessment to give rapid, quantitative values, are attractive. There is support for an argument for assessment of the density of ICCs as a screening test prior to more intensive investigation of these clinical problems when formal biopsy is clinically indicated. This may be feasible even in developing countries where chronic disorders of bowel motility, resistant to conventional management, are a frequent basis for referral to specialist clinics.

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