J.Vet. Malaysia (2002)14 (1 &2): 15 - 18

PGP 9.5 IMMUNOREACTIVITY IN THE COLON OF MUTANT (LS/LS) MICE

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SUMMARY

The current work was conducted to map the innervation in the gastrointestinal tract of normal and lethal spotted mutant (ls/ls) adult mice. Protein gene product 9.5 (PGP 9.5), which is a ubiquitin c-terminal hydrolase, has been found in a wide range of tissues. Therefore, antibody against PGP 9.5 was used to map the entire innervation of the gut. Immunohistochemical studies indicate that density of PGP 9.5 immunoreactive neurons were highest in the most proximal and distal colon of normal mice, but in the mutant (ls/ls) mice, there were aganglionosis in the 10mm region of the colon from the rectum. The relative paucity of PGP 9.5 immunoreactive neurons in this segment might contribute to the lack of tone in the muscle and therefore leads to the development of the megacolon.

Keywords: Hirschsprung's disease, PGP 9.5, innervation, gastrointestinal tract

INTRODUCTION

The enteric neurons are derived from two regions: i. vagal region and ii. the sacral region (Kapur *et al.*, 1992; Sundgren *et al.*, 1998; Taraviras and Pachnis, 1999). Vagal crest cells give rise to majority of neurons and glia of the enteric ganglia. It first enters the foregut mesenchyme and migrates in a rostro-caudal direction to colonize the entire gut. For normal innervation of the gut to occur, neural crest cells (NCC) must be able to migrate, differentiate and survive (Gershon, 1997; Taraviras and Pachnis, 1999; Hearn and Newgreen, 2000). However, failure of NCC to colonise the hindgut can lead to Hirschsprung's disease or megacolon in human and animals. This is characterised by colonic obstruction, which leads to constipation, and there will be absence of enteric neurons at the terminal gut. This is characterized by a complete absence of ganglion cells at this region. However, it is still not clear if the other regions of the gut is also involved.

Protein gene product 9.5 (PGP 9.5) is a neuron specific protein, which belongs to the ubiquitin C-terminal hydroxylase family (Thompson et al., 1983). Standard immunohistochemical techniques have demonstrated the presence of PGP 9.5 in neurons and nerve fibres at all levels of the central and peripheral nervous system, in many neuroendocrine cells and in many other tissues. Therefore, using antibody against PGP 9.5 can determine the crest cells that had differentiated into neurons.

The objective of the current work is to study the innervation of the gut in normal adult and mutant mice.

MATERIALS AND METHODS

Ten normal adult mice (C57/ls) and 10 lethal spotted (ls/ls) mutant mice were used in the current study. For these studies, we utilise a mutant mouse, the lethal spotted (ls/ls), in which a naturally occurring single point mutation in the gene encoding endothelin-3 (ET-3), causes a spotted coat and aganglionosis of the terminal bowel (University of Liverpool). The mice were killed and the gut specimens (from duodenum until the distal colon) were removed. The specimens were fixed in 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS) overnight and then cryoprotected by immersion in 20% sucrose in 24 hours. A sample was taken every 5mm starting from the most caudal region of the colon until the most proximal duodenum. Transverse cryostat sections (8μm) were processed for localisation of PGP 9.5 using indirect immunofluorecence method. The sites of primary antibody binding were visualised with donkey anti-rabbit IgG coupled with Cy^3. At least 33 consecutive sections were examined from each tissue block. The number of immunoreactive nerve cell bodies was counted in 4-6 visual fields per section using a x25 objective. The mean number of immunoreactive nerve cell bodies per field was then determined for each region of the gut in individual mice. This value was used in determining the mean and standard error of cell numbers in the two groups of animals.

RESULTS

General

In the control mice (C57/ls) the distribution of PGP 9.5 immunoreactive (PGP IR) fibres was densed. In the myenteric plexus a large number of nerve cell bodies, nerve fibres and nerve bundles were observed (Fig. 1).

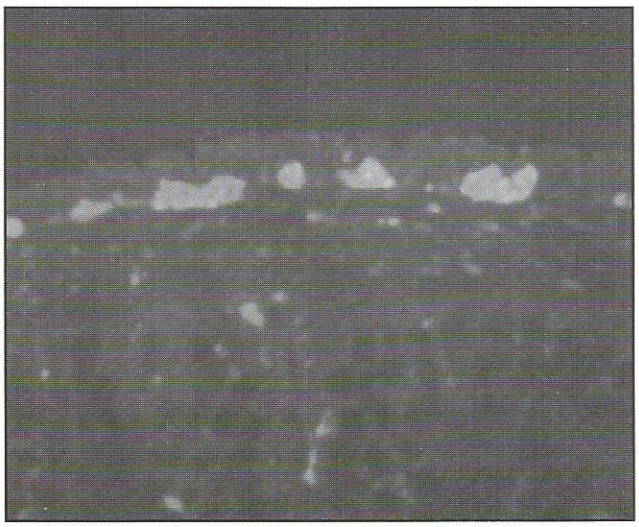

Fig. 1. PGP 9.5 IR nerve cell bodies in the myenteric plexus of control mouse.

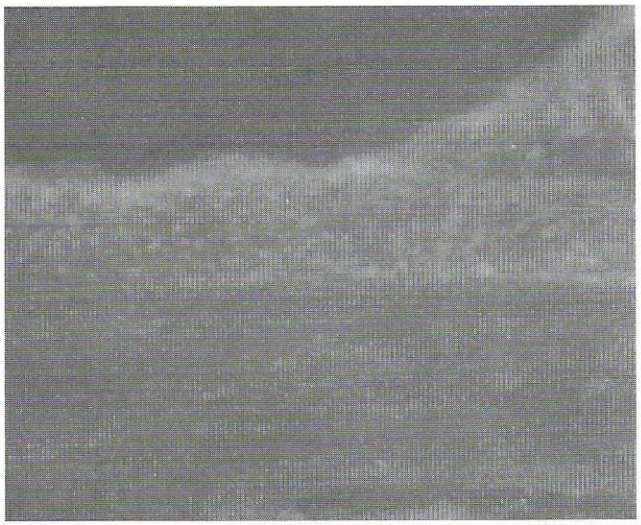

Fig. 2. Absence of PGP 9.5 IR nerve cell bodies in the myenteric plexus.

Their distribution density was similar from the duodenum until the caecum. However, highest density of innervation was found at the most proximal colon and most distal colon (5mm caudo-cranially) (Table 1).

Table 1. Density of PGP 9.5 IR fibres in control and ls/ls mice

Tissues	Control	ls/ls
Colon (caudo-cranially)		
5mm	++++	-
10mm	++++	-
20mm	+++	+
30-40mm	++	+
50-60mm	++++	+++
Caecum	+++	+++
Terminal ileum	+++	+++
Jujenum	+++	+++
Proximal duodenum	+++	+++

In the ls/ls mice, there was a total aganglionosis in the first 10mm of the distal colon (Fig. 2). Starting 20mm proximal from the distal colon, few nerve fibres were found and one or two nerve cell bodies were found at the myenteric plexus. The number of nerve cell bodies increased gradually and highest number of nerve cell bodies were found at the most proximal colon. The innervation started to look normal at 50-60mm colon (caudo-cranially).

PGP 9.5 immunoreactivity

In the ileum of ls/ls mice, the number of immunoreactive cell bodies per field was similar to that in controls (ls/ls, 0.48 ± 0.02; normal, 0. 56 ± 0.08; means ± SE, n=10) (Table 2). There were similar total number of myenteric neurones in the ileum of the two groups and so the proportion of PGP 9.5 neurones expressed as a percentage of the total was similar in the two groups (normal, 10.7 ± 0.5; ls/ls 9.4 ± 0.5; n= 10). In contrast, in the proximal colon of ls/ls mice the number of PGP 9.5 IR cells was significantly less than in controls (ls/ls, 0.39 ± 0.02; normal, 0.71 ± 0.02; $P< 0.05$; n=10). The difference was not simply a consequency of lower total numbers of proximal colon myenteric neurons in ls/ls mice, since the proportion of immunoreactive PGP 9.5 cells was expressed as a percentage of the total the difference was still significant (normal, 11.4 ± 0.3; ls/ls, 6.7 ± 0.6; $P<0.05$; n=10).

Table 2. Number of PGP 9.5 immunoreactive cell bodies per field (Mean ± S.E.) in the gut of normal and ls/ls mice

Tissues	Normal	ls/ls	
Terminal ileum	0.56 ± 0.08	0.48 ± 0.02	NSD
Caecum	0.66 ± 0.07	0.67 ± 0.03	NSD
Proximal colon	0.71 ± 0.02	0.39 ± 0.02	$p<0.05$
Distal colon	0.69 ± 0.02	0.23 ± 0.04	$p<0.05$

DISCUSSION

In the present study, aganglionosis affects variables length of the colon in the mutant (ls/ls) mice and this was restricted to the terminal 10mm. There was a reduced innervation in the intervening segments. Many previous studies concentrated only on the most distal part of the colon (Bu'lock *et al.*, 1984; Koteeswaran *et al.*, 2000). This is the first reported study on the innervation of the gut in adult mutant mice starting from the duodenum. The affected mice of the strain ls/ls have shown to possess disproportionately reduced numbers of PGP 9.5-IR neurons at region that lies proximal to the terminal aganglionic segment. In most proximal regions e.g. ileum, the numbers of PGP 9.5- IR nerve cell bodies were similar to that of a normal mice.

It is established that in ls/ls mice, an aganglionic colon develops as a consequence of impaired migration of neuroblasts from the neural crest (Webster, 1973). The precise mechanisms involved remained unknown but could include (a) micro-environment factors that delay migration of the cells towards their final positions (Rothman *et al.*, 1993., Payette *et al.*, 1988) or (b) a defect in the vagal neural crest neuroblasts themselves (Payette *et al.*, 1988; Gershon, 1997; Erickson and Goins, 2000). But these two possibilities had been ruled out by a more detailed study conducted by Kapur *et al.* (1995) to investigate the cellular mechanism of the migration of the neuroblasts in ls/ls mice. In his study, a promoter DbH (a marker for enteric neuroblast) and n-lac Z reporter gene was introduced into NCC of aganglionic mice to study the fate of enteric neuroblasts. The study confirmed that the aganglionosis is due to failure of colonisation of distal gut by enteric neuroblasts and not a failure of neuroblast differentiation preceded by normal neuroblast migration.

It appears from the present study that some sub-populations of enteric neurons in ls/ls colon survive better than others. Thus myenteric plexus neurons survive less well than sub-mucous plexus neurons, since the latter occur in normal numbers for much of the region proximal to the terminal colon. In addition, within the myenteric plexus some sub-populations of cells seem especially vulnerable; in particular PGP 9.5-IR neurons occur in relatively lower numbers in the hypoganglionic segments compared with normal colon.

There are two possibilities for the selective loss of PGP 9.5-IR neurons at the myenteric plexus. First, it is possible that the migration of neuroblasts that give rise to these neurons is selectively affected in ls/ls mice. Secondly, the PGP 9.5 phenotype might be determined by local factors that exist for only a brief and critical period, and that in the case of ls/ls mice occur before the arrival of most myenteric neurons. Further work is needed to confirm these possibilities.

However, it is significant that the hypoganglionic segment of the bowel corresponds to the mega-colon. In ls/ls mice this is frequently seen as a continuous column of faeces rather than as a grossly distended segment. The relative paucity of PGP 9.5 IR neurones in this segment might contribute to the lack of tone in the muscle and therefore exacerbate the development of the mega-colon.

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RINGKASAN

IMMUNOREAKTIVITI PGP 9.5 PADA KOLON MANCIT MUTAN (LS/LS)

Penyelidikkan ini bertujuan untuk mengetahui penyarafan di dalam sistem gastro-usus pada mancit normal dan mancit mutan 'lethal spotted' (ls/ls) yang dewasa. Antibodi terhadap protin gene product 9.5 (PGP 9.5) telah digunakan untuk mengkaji keseluruhan penyarafan pada gastrousus. Kaedah immunokimia menunjukkan bahawa density neuron yang immunoreaktif terhadap PGP 9.5 adalah paling tinggi didapati di bahagian paling proksimal dan distal kolon pada mancit yang normal, tetapi pada mancit mutan (ls/ls) terdapat aganglionosiis di bahagian 10mm kolon dari rectum. Kekurangan neuron yang immunoreaktif pada PGP 9.5 pada segmen ini mungkin menyebabkan kekurangan tona otot dan ini akan menyebabkan megakolon.