Morphological and quantitative study of the myenteric plexus of the mouse colon

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Abstract

Morphological and morphometrical studies of the myenteric plexus of the mouse large intestine were performed using NADH diaphorase and acetylcholinesterase staining. Male albino mice (70-80 days old) were sacrificed after ether anaesthesia and the large intestine was immediately removed and submitted to the staining techniques cited above. Then, the colon was divided into three segments of the same length (first, second and last third) from the proximal to the distal end. The fragments were dissected, the mucosa and submucosa removed, lasting only the tunica muscularis. The membranes were mounted on slides and covered with coverglass for microscopic examination (4x and 10x objectives). At least 10 micrographs of each segment of the intestine were used for the point counting method for evaluation of the surface area of the plexus (4x micrographs). For neuron counts 10x micrographs were used. Nerve cell bodies volume and area were evaluated on 40x micrographs. The results showed: a) the myenteric plexus in the large intestine of the mouse is more dense in the proximal third of the colon than in the distal third; the number of neurons/cm² is significantly less in distal segment of the colon (65767 ± 10341 neurons/cm²) than in the proximal and intermediate segments (93242 ± 9185 neurons/cm² and 85188 ± 5154 neurons/cm², respectively). The mean volumes of the neurons were similar in the different segments of the colon (4549 ± 3493 μm³).

Keywords: myenteric plexus, large intestine, morphological study, mouse.

1 Introduction

Morphological studies and counts of the neurons in the plexus in the gastrointestinal tract have been done in different species using whole mount preparations or tangential sections (reviewed in Gabella, 1971). Vital staining with methylene blue was first used for studies in guinea pig, monkey, brown trout and rabbit. In fixed viscera, toluidine blue staining was used in whole mount preparations of the ileum, caecum and colon of the guinea pig and mice; thionine was used in sections of duodenum and ileum of cats and ileum of dogs while Bielschowski staining was used in sections of the cat duodenum (references for each species and thecnique in Gabella, 1971). Giemsa staining of whole mount of muscle layers of rat large intestine and of guinea pig gall bladder has also been used for neuronal counts in the myenteric plexus (BARBOSA, 1978; LIBERTI, QUEIROZ, POMPEU et al., 1994). In the recent years a histochemical thecnique do detect DPNH-tetrazolium reductase activity in the neurons of the myenteric plexus was introduced by Gabella (1969) and this technique has been used to quantitative analysis of the ganglia of small intestine of the rat, guinea pig, sheep and mouse (GABELLA, 1971; GABELLA and TRIGG, 1984; HANSMANN, HACKELOER and STAUDACH, 1986) and large intestine of the *Calomys callosus* (MAIFRINO, PRATES, De SOUZA et al., 1997).

There is only one quantitative study of the neurons in the myenteric plexus of the large intestine of mouse, and toluidine blue was the stain used in fixed tissue (TAFURI, and ALMEIDA CAMPOS, 1958). In this study the authors did not comment about the morphologic characteristics of the myenteric plexus in different areas of the large intestine because, although the toluidine blue staining is an excellent method for visualization of the pericarions, the nerve fibers are not stained as well. The acetylcholinesterase technique permits staining of both the nerve fibers and pericarions, allowing morphologic observations of the plexus as well as quantitative evaluations of the neurons in the ganglia.

The large intestine of the mouse has a thin wall that permits the staining by acetylcholinesterase solution and observation of the myenteric plexus in the integral preparations of the intestine wall after dissection of the mucosa and submucosal layers. This allows examination of the entire myenteric plexus along the large intestine, in the different areas of the organ with minimal stretching of the intestine wall.

In this communication we report some morphological and morphometrical observations of adult mouse large intestine myenteric plexus after staining with NADH diaphorase and acetylchlinesterase techniques.

2 Material and methods

The large intestine was removed after ether anaesthesia. After 25 minutes the intestine was rinsed and the lumen

perfused with warmed saline (37 °C) to eliminate the faecal content. The intestine was separated from the caecum and divided into three segments of the same length (proximal, intermediate and distal segments).

2.1 Histochemical dehydrogenase reaction (NADH)

Six male albino mice weighing 22 g (70-80 days old) were used for this technique. Each entire colon was initially washed in Krebs solution, ligated with cotton threads at its extremities and filled through a syringe needle in its lumen until slightly distended. After incubation in Krebs solution for 20-30 minutes, the pieces were transferred to a permeabilizing agent (0.3% Triton-X in Krebs solution) for 60 seconds and then submitted to three 10 minutes washings solution (GABELLA, 1969).

The segments of the colon were then incubated for 60 minutes at 20 °C in 20 mL of a medium containing 0.5 mg.mL^{-1} nitro blue tetrazolium (Sigma) in distilled water (5 mL), 0.1 mol.L^{-1} sodium phosphate buffer (5 mL, pH 7.3), distilled water (10 mL) and 0.5 mg.mL^{-1} β-nicotinamide adenine dinucleotide (reduced form). The reaction was stopped by immersion in 10% buffered formalin solution in which the specimens were fixed (24 hours minimum). The pieces were next longitudinally opened and, the mucosa and submucosa layers of the segments were removed and the specimens were thoroughly washed in distilled water. Then, whole-mount preparations were laid in glycerol on a microscope slide and sealed with Entellan (Merck, Germany).

2.2 Demonstration of acetylcholinesterase activity (AChE)

In six pieces AChE activity was demonstrated with its direct coloring method as described elsewhere (KARNOVSKY, and ROOTS, 1964; SANTER and BAKER, 1988). The specimens were filled with 4% paraformaldehyde in phosphate buffer (pH 7.4), ligated at their oral and aboral limits and immersed in the same fixative solution for 2 hours at 4 °C. After this period the pieces were opened and were maintained overnight at 4 °C in a solution with hialuronidase (Hialosime, Apsen), Krebs solution and tetraisopropylpirophosphoramide (isoOMPA; Sigma). The segments were then removed, washed in Krebs solution and once again incubated overnight at 4 °C in a second solution containing 50% of the cited solution plus 0.17 mol.L^{-1} acetilthiocholine iodide, 0.1 mol.L^{-1} phosphate buffer (pH 7.1), 100 mmol.L^{-1} sodium citrate, 30 mmol.L^{-1} cupric sulfate, 5 mmol.L^{-1} kalium ferricyanatum and 0.3% Triton-X 100. The mucosal and submucosal layers were removed and the segments dehydrated in an increasing alcohol series (70-100 degrees), immersed in benzene for 20 minutes and mounted on microscope slides with Entellan (Merck, Germany).

2.3 Morphometry

Each segment was mounted on a slide, covered with a coverglass and gently pressed (with a wood block weighing 50 g) before microscopic examination. A standard microscope, with a 25 watts 12 volts halogen lamp, equipped with an automated microphotography apparatus was used. The micrographs were taken on 35 mm film with objectives of 40x or 10x and were printed in an automated system with the same final magnification (150 x 100 mm). The micrographs taken from whole-mounts stained with acetylcholinesterase with low magnification objective (4x), were used for calculations of the surface area occupied by the plexus.

The micrographs obtained with the 10x objective, taken from whole-mounts stained with NADH diaphorase were used for counting the neurons in the ganglia. The neurons were counted on the micrographs obtained with the 10x objectives and the results transformed in number of neurons/cm^2. For evaluation of the surface area occupied by the plexus in relationship to the serosal surface, a counting point system was used. A transparent grid was placed over the micrographs and the crossing points of the grid over the ganglia and nerve fibers were counted and the radio of the plexus surface to the serosal surface was obtained and expressed in percentage. In an attempt to document the measurement of the area covered by each photo, a 1 mm scale divided in 10 μm intervals was photographed with the same objectives used to take the micrographs of the plexus.

The micrographs taken with the 40x objective were used to evaluate the greater orthogonal diameters of the neurons. The cell body of the neurons appeared as ellipsoidal structures and the two orthogonal diameters were measured on the micrographs obtained with the high power objective (in 100 neurons of each segment, 400 cells, total). The volume of each neuron and the area of the maximal profile of each neuron (nerve cell body) as seen in the whole-mount preparations, were estimated using the following formulas: for the volume: $4/3\pi\ (r1)^2.\ (r2)$; and for the area: $\pi.(r1).(r2)$.R1 and r2 are the lesser and the greater radius (diameters/2), respectively (HANSMANN, HACKELOER and STAUDACH, 1986). When necessary, statistical analysis was performed using the software GB-STAT Professional Statistic & Graphics, version 4.0, MD, USA).

3 Results

The NADH diaphorase and the acetylcholinesterase staining gave good results for the mouse large intestine: the myenteric plexus was fully stained. In the NADH stained whole-mounts the ganglia were easily observed because the dark blue color of the neurons against the light blue background (Figure 1a). Sometimes the nerve cell bodies appeared unstained, as negative images in a blue background (Figure 1b) but the majority of the pericarions appeared dark blue, being possible the counting of the cells. The examination with high power objective showed the neurons packed in a tight fashion, although there was only a small degree of overlap between adjacent neurons (Figure 1b). Sometimes the exact border of the ganglia within the plexus was difficult to define and sometimes isolated neurons were observed in the connecting strands. The neurons were frequently elongated, but the long axis was less then two times the short axis.

Observation of the acetylcholinesterase stained whole-mounts, showed that the plexus had different morphological patterns in the three segments of the colon. The proximal portion of the colon (ascending colon) showed large, polygonal, ring or like ganglia, often merged with one another. Although the large axis of each ganglion was parallel to the circumference, several ganglia did not have a distinct polarization with respect to the axis of the colon: the connecting strands were short, running along the length of the organ, forming a less geometric pattern (Figure 2a). In the interme-

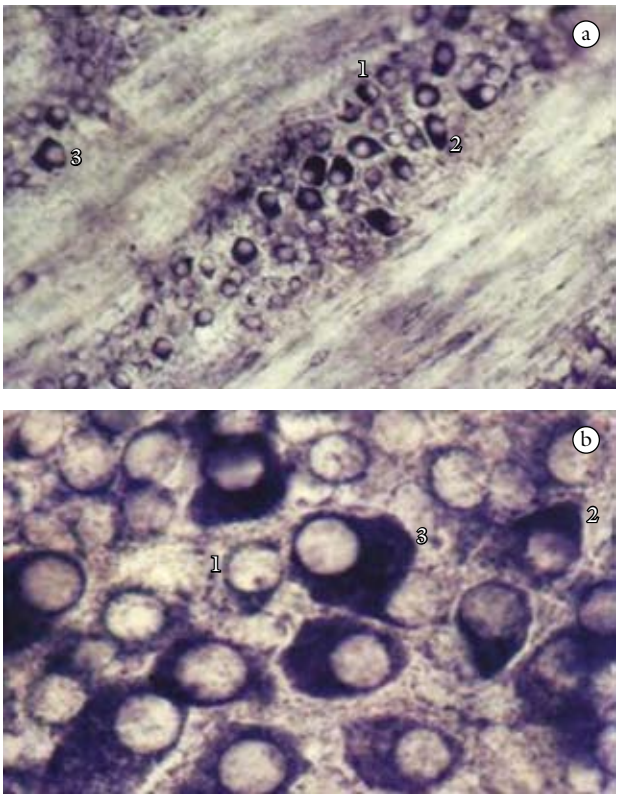

Figure 1. Myenteric neurons stained for NADH diaphorase taken from the proximal part of the colon of mice. Small (1), medium sized (2) and large (3) neurons are evident. Large neurons (3) are intensely stained. Observe that the neurons are packed in a tight fashion and there is only a small degree of overlap between adjacent neurons (b); a) 400x; and b) 700x.

diate portion, the ganglia were progressively elongated and parallel to the circumference of the organ (Figure 2b). Thin connecting strands, running in longitudinal direction, links these ganglia, forming a network with meshes more geometric and greater than in the proximal segment. In the distal third of the colon, the ganglia were frequently elongated and thin, but sometimes small and polygonal fibers were parallel to the circumference of the colon; the connecting fibers were parallel to the length of the organ, forming a more loose network, with large spaces between the ganglia (Figure 2c).

The stereological evaluation of the surface area of the plexus, in relationship to the serosal surface, confirmed the observations described above. The relative surface area of the plexus was greater in the proximal and intermediate segments as compared to the distal segment (Figure 2).

The neuron counts are in the Table 1. The differences observed between the mean numbers of neurons in the segments of the colon were significant, i.e. the difference observed between the mean number of the neurons in the distal area of the colon (65767 ± 10041) and in the proximal (93242 ± 5100) and in the intermediate part of the colon (85188 ± 5114) was significant.

The estimated volume of the pericarion of the neurons showed great variations (mean 4549 ± 3493 μm^3, and range from 504 to 22047 μm^3) as did the area of the pericarions (means: 336.3 ± 165.7 μm^2, and range from 76 to 1014 μm^2).

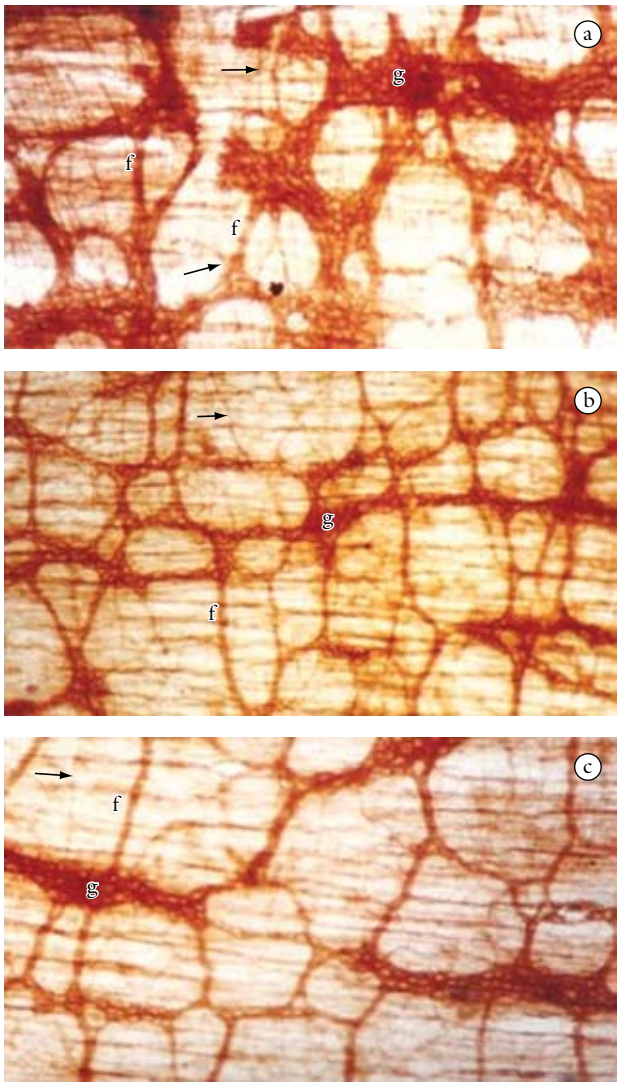

Figure 2. a) Myenteric plexus from the proximal; b) Intermediate; and c) distal portion of the mouse colon stained with AChE technique. In the proximal portion of the colon, the ganglia (g) are large, polygonal, ring or like ganglia; a) The connecting strands (f) were short, running along the length of the organ, forming a less geometric pattern; b) In the intermediate portion, the ganglia were progressively elongated and parallel to the circumference of the organ; and c) Thin connecting strands, running in longitudinal direction, links these ganglia. In the distal third of the colon, the ganglia were frequently elongated and thin. The connecting fibers were parallel to the length of the organ; 120x.

Table 1. Neuron number (per square centimeter) in Auerbach's plexus of the mouse colon. The results were obtained after counts in three different segments of large intestine. The differences observed between the proximal and intermediate segments of the colon (Man-Whitney test) are not significant (p = 0.754) but they are between the proximal or intermediate segments and distal segment (p = 0.0071).

Segment	Mean	SD	Range
Proximal	93242	5100	81357-103243
Intermediate	85188	5114	80540-98378
Distal	65767	10041	58430-74864

4 Conclusion

Our results showed that it is possible to do stereological and morphometrical observations of the myenteric plexus of the large intestine of the mouse using the NADH diaphorase and acetylcholinesterase staining dissecting the mucosa or muscle layers for microscopic observations. These histochemical methods are easier to execute than that used by other authors. A review of the micrographs presented by authors that used histochemical methods for staining the myenteric plexus in guinea pig and rats showed similar results, and these methods showed clearly the nerve fibers connecting the ganglia.

The morphological patterns of the myenteric plexus of the mouse colon have the same characteristics of the myenteric plexus observed in other species, in particular, the guinea pig and rats. Interestingly the surface density of the plexus decrease in the colon from the proximal to the distal segments, as Gabella (1971) showed in the drawing of the myenteric plexus of the colon and rectum of guinea pigs.

The advantage in examination of the intestinal segments in a whole preparation, is reduction of time for sectioning and staining the pieces. In the present experiment the fragments were put on the slide, covered with a coverglass to which constant and gentle pressure was applied. Using this technique, the spatial density of the plexus among different preparations (of different animals), would be reduced.

The neuron counts showed the staining methods used are also good for quantitative neuronal observations. Our results also showed that the number of neurons was significantly lower in the distal third of the colon, a difference not reported by Tafuri and Campos (1958) that referred to the number of neurons in the colon, without specification of the segment of colon being evaluated.

The mean volume observed for the pericarions of the neurons showed great variation as it has been demonstrated for different species in which morphometrical evaluation of neuron size has been reported (GABELLA, 1971; LIBERTI, QUEIROZ, POMPEU et al., 1994) in the large intestine of the rat.

We concluded that NADH diaphorase and acetylcholinesterase staining are excellent methods to study the morphology and quantitative aspects of the myenteric plexus of the colon.

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