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Histochemical and immunohistochemical study of the intrinsic innervation in colonic dysganglionosis

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Abstract Defective innervation of the neuromuscular junctions (NMJ) was recently described in intestinal neuronal dysplasia type B (IND B). The aim of the present study was to correlate the alterations in NMJs to other classically described parameters in dysganglionoses and to determine the relationship between NMJ abnormalities in IND B and clinical symptoms. The rectal biopsies and full-thickness colonic biopsy specimens of 17 patients were studied applying histochemical (acetylcholinesterase [AChE], lactic dehydrogenase [LDH], and succinic dehydrogenase [SDH] reactions) and immunohistochemical (neuronal-cell adhesion molecule [NCAM] and SY antibodies) methods. Thirteen patients had Hirschsprung’s disease (HD). IND B was diagnosed in 11 (associated with HD in 8 cases, isolated in 2, and associated with hypoganglionosis in 1). In the aganglionic segment of HD there was very intense AChE activity; in contrast, NCAM- and SY-immunoreactive nerve fibers were markedly decreased. A spectrum of abnormalities was observed in IND B, usually more severe in the most distal segments: giant and immature ganglia in the submucous plexus were observed in all cases; heterotopic myenteric ganglia were frequent (72.7%); hyperganglionosis was observed in 6 (54.5%) and was not related to the patients’ age; thick and tortuous NCAM- and SY-immunoreactive nerve fibers, irregularly distributed in the colonic wall, were observed in 81.8% of the cases. No relationship was observed between abnormalities of NCAM- and SY-immunoreactive nerve fibers and AChE activity, ganglion-cell

maturity, heterotopy, or the clinical symptoms presented by the patients with IND B. In hypoganglionism, low AChE activity and a slight decrease in NCAM- and SY-immunoreactive nerves were observed. Thick and tortuous, irregularly-distributed intrinsic NCAM- and SY-immunoreactive nerves were observed in every colon layer in IND B. Our results do not support IND B as a NMJ disorder.

Keywords Colonic dysganglionosis · Hirschsprung’s disease · Intestinal neuronal dysplasia · Neuromuscular junction

Introduction

Several malformations of the enteric nervous system (ENS) clinically resembling Hirschsprung’s disease (HD) are now recognized. These entities are grouped under the designation of colonic dysganglionoses and include HD, intestinal neuronal dysplasia (IND) types A and B, hypoganglionosis, immaturity of ganglion cells (GC), and a group of less well-characterized conditions such as heterotopic and hypogenetic GC [2, 5, 15]. Colonic dysganglionoses can be either isolated or associated, and may be related to a common pathogenesis [14]. It is widely accepted that the pathological diagnosis can only be made through special histochemical and/or immunohistochemical methods [15, 19].

IND type B of the submucous plexus (IND B) is the second most frequent form of colonic dysganglionosis after HD [28, 29]. It can occur as an isolated form [27], but an association with HD proximal to the aganglionic segment is reported in up to 75% of cases [1, 3, 15, 21, 24, 29]. This situation is frequently related to postoperative complications after definitive surgical treatment for HD [8, 21]. The diagnostic criteria of IND B have been a matter of controversy since the first description by Meier-Ruge [11, 13, 17, 18, 20, 21]. The main abnormalities include giant ganglia in the submucous plexus containing more than seven cells/ganglion,
hyperganglionosis, and high acetylcholinesterase (AChE) activity in the mucosa and the submucosal blood vessels [2, 10, 16]. Heterotopic and hypogenetic GC and immature ganglia are frequently observed, the latter found especially in young children [5, 16, 17]. The pathogenesis of IND B is unknown.

A decrease in the number of synapses was described in the aganglionic segment in HD despite proliferation of AChE-positive nerve fibers [9, 30–32], suggesting that these nerves have an extrinsic origin [9, 10]. In another immunohistochemical study applying markers of the synaptic and presynaptic regions, defective innervation of neuromuscular junctions (NMJ) was observed in IND B [12]. These abnormalities were not uniform in neither the bowel layers or in different cases [12]. Moreover, the authors found no correlation between the NMJ abnormalities and the severity of the patients’ symptoms [12]. Since a spectrum of alterations is described in IND B [11, 16, 22], different degrees of impairment of NMJ innervation may occur. A relationship between clinical obstructive symptoms and a high degree of histopathologic dysplasia of the ENS in a spectrum of dysplastic features was suggested [21]. However, other authors observed that the most severe symptoms in IND B were related to GC immaturity and heterotopic ganglia in the myenteric plexus [22, 26]. To the best of our knowledge, there are no studies correlating abnormalities of NMJs with other alterations such as abnormalities of the myenteric plexus, neuronal maturity, and AChE activity.

We studied the ENS in colonic dysganglionoses applying histochemical and immunohistochemical methods. Our aim was: (1) to correlate the alterations in NMJs to other classically-described parameters in dysganglionoses, especially IND B; and (2) to determine any relationship between NMJ abnormalities in IND B and clinical symptoms.

**Materials and methods**

Seventeen cases of neuronal intestinal malformations in which both rectal and full-thickness colonic biopsy specimens were available were selected from the files of the Institute of Molecular Pathology and Immunology, Porto. All patients were treated at the Pediatric Surgery Division of the Hospital São João, Porto. Twelve patients were male and 5 were female; their ages ranged from 1 month to 11 years. Forty-two cases were presented with obstructive symptoms and 3 had enterocolitis.

Rectal biopsies containing mucosa and submucosa were taken at 1, 3–4, and 5–8 cm above the pectinate line. Full-thickness colonic biopsies taken from different segments (rectum, sigmoid, descending and transverse colon, splenic and hepatic flexures) were performed when rectal biopsies were inconclusive and/or during pull-through surgery. The specimens were processed using Meier-Ruge method [18]. Briefly, each sample was mounted with objective compound tissue (OCT) vertical to the mucosal surface and cut at −15 °C stepwise in 15-µm serial sections, which were distributed on five slides. Two slides were used for AChE reaction [7], one counterstained with Mayer’s hematoxylin, one stained with hematoxylin-eosin, and the other two, in which the sections were disposed alternatively, were stained for lactate dehydrogenase (LDH) [4] and succinic dehydrogenase (SDH) [23] reactions.

The sections were fixed in 4% saline formalin and covered with Crystal Mount; the slides were mounted with Entellan.

NMJs and synapses were studied by immunohistochemistry applying neuronal-cell adhesion molecule (NCAM) and SY antibodies. Full-thickness samples were fixed in Zamboni’s fixative for 24 h at 4 °C, cryopreserved in graded solutions of sucrose (10%, 20%, and 30%), 24 h each), embedded in OCT, and frozen at −70 °C until they were processed. Frozen sections (−15 °C) of 10-µm were transferred to gelatin-coated slides and dried for 30 min. In some cases paraffin-embedded material was also available.

For immunohistochemistry a modification of the avidin-biotin-peroxidase method was applied with 3, 3’-diaminobenzidine as chromogen [6]. Sections were incubated overnight at 4 °C with monoclonal antibodies against NCAM (1B6 diluted 1:500 for frozen sections and NCAM diluted 1:200 for paraffin sections; Novocastra, Newcastle, UK) and SY (diluted 1:1000 and 1:100 for frozen and paraffin-embedded sections, respectively; Sigma-Aldrich, St. Louis). All sera were diluted in Tris-buffer saline, pH 7.3 containing 1% bovine serum albumin, and all incubation steps were performed in a humid chamber. Sections were counterstained with Mayer’s hematoxylin; negative controls were performed by substitution of the primary MoAbs with immobiloglobulins of the same class and concentration.

Neuronal intestinal malformations were classified according to Meier-Ruge [15]. The results obtained by histochemistry and immunohistochemistry in every segment of each case were correlated whenever possible. In 6 cases full-thickness samples of normal colon were analyzed.

**Results**

The main clinical data and histopathologic diagnoses of the 17 patients are shown in Table 1. Thirteen patients had HD, involving the rectum and sigmoid in 11 and the whole colon (total aganglionosis) in 2. IND was diagnosed in 11 patients, 8 of whom had an association with HD. Two of these patients developed enterocolitis after a pull-through operation and another had persistence of the symptoms after a colostomy performed in a dysplastic segment. The other 5 patients with IND B and HD became asymptomatic following definitive surgical treatment, although dysplastic lesions were present at the proximal resection border in 3 and GC immaturity was diagnosed in the proximal segments in 2. Two patients had isolated IND B and 1 had enterocolitis at the time of diagnosis. Two patients had hypoganglionosis extending to the sigmoid, and 1 of them had associated IND B. Neuronal immaturity and hypogenetic GCs in the myenteric and submucous plexuses were observed in the proximal segments in 6 cases. Other isolated minor abnormalities, which did not fulfill the criteria for diagnosis of IND B, were observed in the proximal segments of 4 patients.

**Histochemical and immunohistochemical observations**

**Normal colon**

The proximal segments of 6 patients showed normal AChE activity. Ganglion cells were stained by LDH reaction, and most of them showed SDH activity. The two NMJ markers presented identical immunoreactive