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Plasticity of the enteric nervous system in patients with intestinal neuronal dysplasia associated with Hirschsprung's disease: a report of three patients

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Abstract Intestinal neuronal dysplasia is a controversial form of dysganglionosis that has been described both as an isolated disorder and in association with Hirschsprung's disease. It has been blamed for the bad outcome of bowel function in patients operated on for Hirschsprung's disease. According to various authors, intestinal neuronal dysplasia could be a primary disorder or secondary to other diseases of the bowel. The aim of this study was to assess the plasticity of the enteric nervous system in patients with Hirschsprung's disease-associated intestinal neuronal dysplasia and its ability to evolve spontaneously to normal innervation patterns. Since we prospectively introduced the assessment of the enteric nervous system of the ganglionated bowel in patients operated on for Hirschsprung's disease, 31 patients have been operated on for Hirschsprung's disease in our institution between 1995 and 2002. Among these patients, nine suffered postoperatively from severe constipation and five from bouts of entocolitis. IND was found in eight of them. We studied the evolution of the innervation in three of these patients by repeated serial full-thickness biopsies of the colon. All three patients underwent a colostomy before or after the pull-through procedure. Histopathological assessment of the enteric nervous system was made with conventional acetylcholinesterase histochemistry, rapid acetylcholinesterase histochemistry and immunohistochemistry for the Protein Gene Product 9.5 and the antigen CD56. This evolution was compared with the clinical outcome of bowel function. In our three patients with Hirschsprung's disease-associated intestinal neuronal dysplasia,

this form of dysganglionosis evolved to normal innervation patterns within a period ranging from 9 to 18 months. This evolution was accompanied by an improvement of bowel function in all. We conclude that Hirschsprung's disease-associated intestinal neuronal dysplasia can evolve to a normal innervation, at least under certain circumstances such as a colostomy. Specific histopathological techniques are required to assess accurately the enteric nervous system.

Keywords Intestinal neuronal dysplasia · Hirschsprung's disease · Enteric nervous system · Plasticity

Introduction

Several forms of histopathological abnormalities of the enteric nervous system (ENS) and particularly of parasympathetic innervation have been described. Among them, intestinal neuronal dysplasia type B (IND) and other forms of dysganglionosis such as hypoganglionosis and non-classifiable dysganglionosis (NCD) are still debated entities [1, 4, 9, 14, 20].

Some authors consider IND to be a primary disease [3, 10, 21], but it might likewise be secondary to obstruction, inflammation or other pathological conditions of the bowel [18, 19, 25]. It also has ill-defined clinical consequences. IND has been reported as an isolated lesion (i.e., not associated with other innervation disorders) [21, 26, 27] and has been found in patients with Hirschsprung's disease (HD), proximal to the aganglionosis. It also has been blamed for the bad outcome of bowel function (BF) after the pull-through procedure (PT) [6, 22]. In addition, hypoganglionosis has been found to be an isolated innervation disorder and to occur in association with aganglionosis in the transition zone [7, 14, 20]. It also has been mentioned as a cause of postoperative complications after surgery for HD. Milder forms of innervation changes of the ENS

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such as an increased number of ganglia and hypertrophied nerves in the submucosa (SM) have been found. The clinical significance of these forms of NCD is still puzzling as well [4].

Reports have described spontaneous favourable evolution of isolated IND [18, 26], confirmed by histopathological studies. These observations could explain reports of long-term improvement of BF in patients with a bad short-term outcome after surgery for HD [17, 24]. This clinical evolution has already been described in other reports [2, 13].

Our hypothesis was that HD-associated IND could evolve to normal innervation patterns. To our knowledge, this form of plasticity has not yet been confirmed by histopathological studies. We thus decided to compare the clinical follow-up of BF with the evolution of the histological patterns of the ENS in three patients who underwent surgery for HD. In order to avoid confusion and debate about IND [1], we believe that only clear criteria should be used for its diagnosis. Although other features have been described in association with IND (e.g., ectopic ganglion cells and marked acetylcholinesterase activity in the mucosa in the muscularis mucosae and around vessels [6]), our diagnosis was based on the presence of submucosal giant ganglia containing more than seven mature cells [15]. Criteria proposed by Meier-Ruge were used for the diagnosis of other types of dysganglionosis [8, 13].

Materials and methods

Patients

Since 1995, we have prospectively introduced a systematic assessment of the ENS of the ganglionated bowel [16] in patients operated on for HD. Between 1995 and 2002, 31 patients were operated on for HD at our institution. In eight patients, IND was found in association with HD. Three of these patients were included in this study because of the severity of the outcome of their BF. In two of them (cases 1 and 2) a colostomy had to be performed after the PT because of a bad outcome of the BF, and the first diagnosis of IND was made at that time. Of note, colostomies had to be performed in these patients because a cautious follow-up could not be ensured in their native country. Therefore, creating a colostomy seemed to be the only safe method to avoid enterocolitis, which could have been potentially fatal. Biopsies were performed prior to PT in the last patient (case 3), who was included in a prospective study that comprised the assessment of innervation above the aganglionic zone (AZ) with serial full-thickness biopsies before proceeding to PT [16].

Histopathological evaluation

Intraoperative evaluation of the innervation was carried out either with conventional hematoxylin and eosin (HE) staining or with the rapid acetylcholinesterase (AChE) reaction [7]. All samples were thereafter assessed with conventional AChE histochemistry according to Karnovsky and Roots [5] and immunohistochemistry for protein gene product 9.5 (PGP 9.5), a cytoplasmic marker specific for the neural system [8] and for the antigen CD56 (CD56), a killer-cell associated antigen whose expression is comparable to that of NCAM [12].

Rapid AChE staining

Each specimen was snap-frozen in Tissue-Tek OCT compound (Sakura Finetek, Torrance, USA). Frozen sections (6 µm thick) were cut serially.

The incubation medium was 40 mg of acetylthiocholine iodide, 52 ml of 0.1 M sodium acetate buffer (pH 6.0, adjusted with 0.1 M acetic acid), 3.8 ml of 0.1 M tri-sodium citrate, 4 ml of 30 mM copper sulfate, 1.6 ml of 4 mM tetraisopropyl pyrophosphoramide (iso-OMPA), 8 ml of distilled water and 8 ml of 5 mM potassium hexacyanoferrate III. The sections were incubated at 37°C for 10 min in this medium and washed in distilled water. They were then treated by freshly prepared 3,3'-Diaminobenzidine-tetrahydrochloride dihydrate (DAB, Fluka Chemie, Buchs, Switzerland), 25 mg in 100 ml Tris buffered saline (TBS: 0.05 M Tris, 0.9% sodium chloride, pH 7.6) containing 0.015% hydrogen peroxide for 5 min, rinsed in tap water and counterstained in Mayer's acid-free hematoxylin, dehydrated and mounted in Eukitt.

Tissue preparation for immunohistochemistry

The tissue was fixed in buffered formalin (4% paraformaldehyde solution in phosphate buffered saline), dehydrated and embedded in paraffin wax. Paraffin sections (6 µm thick) were cut and mounted on 3-aminopropyl-triethoxysilane-coated glass slides, deparaffinized in xylol, immersed for 45 min in methanol with 0.3% hydrogen peroxide to block endogenous peroxidase activity and rehydrated through graded alcohols. Sections were then subjected to microwave pre-treatment in 10 mM citrate buffer (pH 6) for 20 min, according to the method of Shi et al. [23] and rinsed in TBS.

Immunohistochemistry antibodies

For the CD56 immunodetection, the first antibody was a mouse anti-CD56 monoclonal antibody (1:50, CD56, clone 1B6; Novocastra, Newcastle upon Tyne, UK), and the second antibody was a biotinylated horse antimouse IgG (1:200, Vector Laboratories, Burlingame, USA).

For the PGP9.5 immunodetection the first antibody was a rabbit anti-PGP 9.5 polyclonal antibody (1:800, PGP 9.5, Anawa, Wangen, Switzerland), and the second antibody was a biotinylated goat antirabbit IgG (1:400, Vector Laboratories).

Immunohistochemical procedures

To reduce non-specific antibody binding, the sections were incubated in normal horse serum for CD56 and normal goat serum for PGP 9.5 (1:30 in TBS) for 10 min, then with the first antibody mixed in TBS containing 5% non-fat dried milk for 30 min at room temperature. They were then washed in TBS and incubated with the second antibody for 30min at room temperature. After washing in TBS, sections were incubated in avidin-biotin-horseradish peroxidase complex according to the manufacturer's indications (Vectastain ABC kit, Vector Laboratories). Peroxidase was visualised by freshly prepared DAB, 50 mg in 100 ml TBS containing 0.01% hydrogen peroxide and counterstained in Mayer's acid-free hematoxylin, dehydrated and mounted in Eukitt. Negative controls were performed using the procedure described above omitting the first antibody.

Results

All three patients had a neo-natal onset of symptoms. IND was found in serial biopsies harvested above the AZ. In all sections, diagnosis of IND was made on the

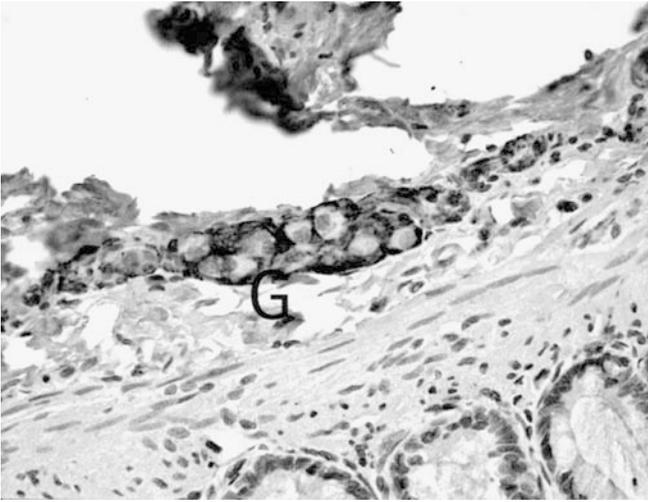


Fig. 1 Case 1. Transverse colon. Submucosal giant ganglion (*G*) (CD 56×200)

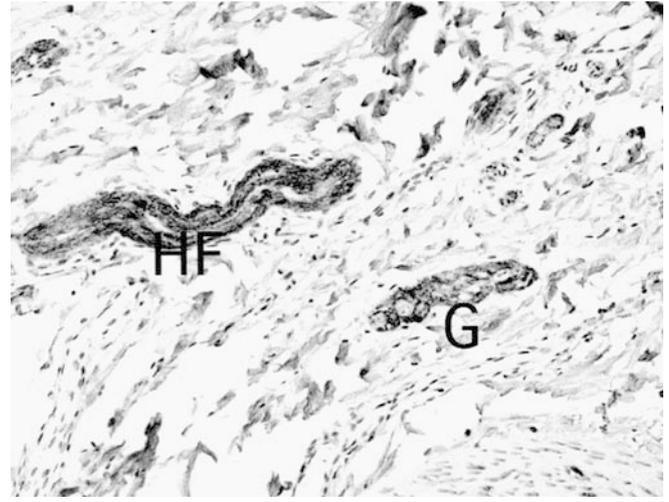


Fig. 3 Case 1. Transverse colon. Submucosal ganglion (*G*) and markedly hypertrophied nerve fiber (*HF*) (PGP 9.5×100)

basis of the presence of submucosal giant ganglia containing more than seven mature cells. Accompanying submucosal marked nerve hypertrophy was seen in all instances.

Case 1

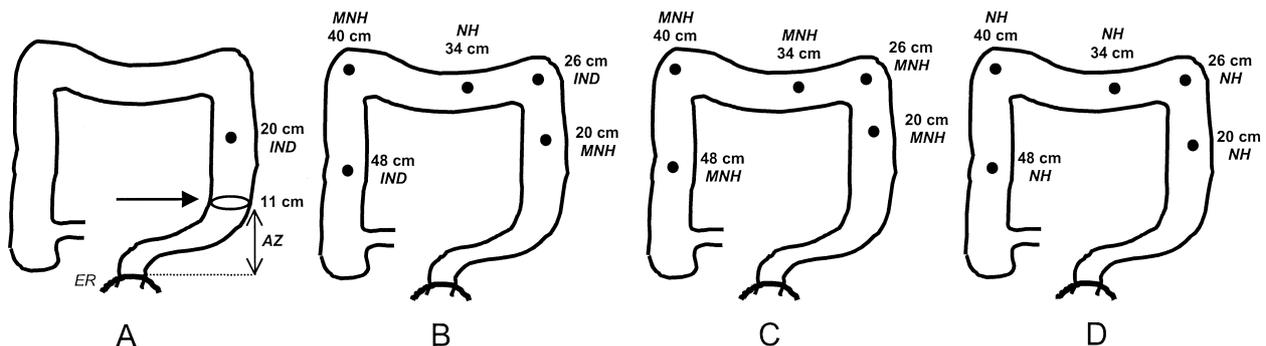
The patient was referred to our institution with a colostomy that had been created on the descending colon by 2 months of age. He underwent PT 6 months later. Aganglionosis was 11 cm long, and intraoperative HE staining showed a normal innervation above the AZ, but postoperative AChE and immunohistochemistry made on the resected colon displayed IND up to 10 cm above the AZ (Figs. 1, 2A). Postoperatively, the patient had severe constipation and multiple episodes of enterocolitis that mandated a colostomy 1 month later. During this procedure, five full-thickness colon biopsies

5×10 mm each were harvested up to the caecum. They displayed mosaic-like innervation changes with IND in two biopsies (Fig. 2B) and marked nerve hypertrophy in the SM in three. Six months later, biopsies were repeated at the same levels as before. At that time, a marked nerve hypertrophy was seen in the SM without criteria for IND (Figs. 2C, 3). The colostomy was then closed, but once again the patient had severe constipation and multiple bouts of enterocolitis. It was decided to place a new colostomy and to keep it for a longer period of time. Biopsies were repeated after 18 months. At that time, only a moderate nerve hypertrophy was seen and the colostomy was closed (Figs. 2D, 4). The patient had normal bowel movements and good continence with follow-up of 4 years.

Case 2

The patient was referred to our institution with a colostomy that had been created by 10 months of age on the mid-descending colon. PT was performed 4 months later. The colon was massively dilated above the colostomy. The AZ was 35 cm long, and intraoperative biopsies assessed with rapid AChE staining showed that colostomy had been made in the AZ. Serial full-thickness biopsies also displayed IND 10 and 24 cm above

Fig. 2 Case 1. Histopathology of biopsies: (A) at time of pull-through (PT), (B) 1 month after PT, (C) 7 months after PT and (D) 18 months after PT. Measurements taken above the excavatio rectovesicalis. *ER* Excavatio rectovesicalis, *AZ* aganglionic zone, *IND* intestinal neuronal dysplasia, *MNH* marked nerve hypertrophy, *NH* moderate nerve hypertrophy. *Arrow* indicates level of resection, *oval* represents colostomy



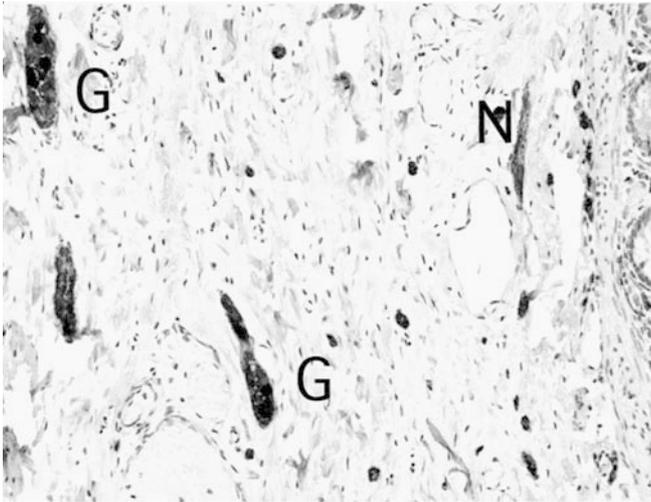


Fig. 4 Case 1. Transverse colon. Normal submucosal ganglia (*G*) and moderately hypertrophied nerve fiber (*N*) (PGP 9.5×100)

the AZ (Fig. 5A). Because of multiple episodes of enterocolitis and severe constipation in spite of conservative treatment for 1 month, we decided in favour of a colostomy. During this procedure, three biopsies were harvested along the colon. They all confirmed the presence of IND (Fig. 5B). The patient kept the colostomy for 1 year. At this time, renewed biopsies were made at the same levels as before. They all showed a normal innervation (Fig. 5C). The colostomy was consequently closed. With a follow-up of 4 years, the patient had good continence and daily bowel movements.

Case 3

This patient was referred to our institution as a neonate. After confirmation of HD with rectal suction biopsies, she was first managed with rectal washouts. By 2.5 months of age, we first assessed the colon innervation with five full-thickness colon biopsies that showed a 15-

cm-long aganglionosis and IND 10 and 15 cm above the AZ (Fig. 6A). A colostomy was then created. After 9 months, serial biopsies were repeated at the same levels as before. They all showed a normal innervation with only moderate nerve hypertrophy in two. PT was thus undertaken. Immunohistopathological examination of the resected colon displayed the presence of IND limited to 3 cm above the AZ and to the proximal stoma of the colostomy (Fig. 6B). The patient has normal BF with 6 years of follow-up.

Discussion

Dysganglionosis consists of a broad spectrum of innervation disorders that may be associated in the same patient. On one side of the spectrum, hypoganglionosis might represent the transition between normal innervation and aganglionosis. When ignored, it can be the cause of complications after surgery for HD [7, 14, 20].

On the other side of the spectrum, IND type B is characterised by a hyperganglionosis and hypertrophied nerves in the SM, as well as ectopic nerve cells in the LP [8, 13, 15]. It has been described as an isolated disorder [10, 21, 26, 27] and reported in association with other pathological conditions of the bowel like necrotising enterocolitis, mucoviscidosis, bowel obstruction in neonates and anorectal malformations with secondary megacolon or constipation [18, 19, 25]. IND could therefore be considered either as a cause or a conse-

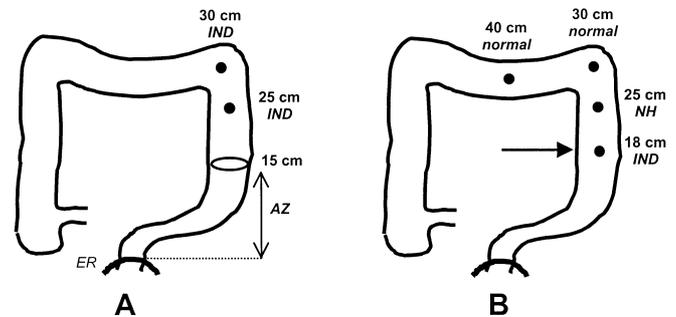


Fig. 6 Case 3. Histopathology of biopsies: (A) at time of biopsies and colostomy and (B) after 9 months at the time of pull-through. Measurements taken above the excavatio rectovaginalis. *ER* Excavatio rectovaginalis, *AZ* aganglionic zone, *IND* intestinal neuronal dysplasia, *NH* moderate nerve hypertrophy. *Oval* represents colostomy, *arrow* indicates level of resection

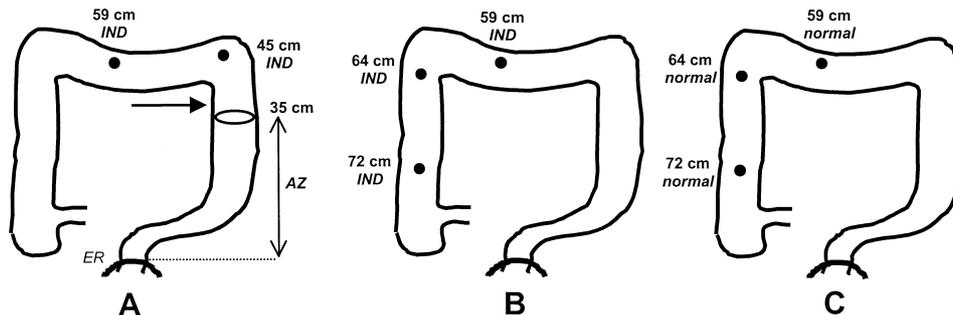


Fig. 5 Case 2. Histopathology of biopsies: (A) at time of pull-through (*PT*), (B) 1 month after *PT* and (C) 1 year after *PT*. Measurements taken above the excavatio rectovesicalis. *ER* Excavatio rectovesicalis, *AZ* aganglionic zone, *IND* intestinal neuronal dysplasia. *Arrow* indicates level of resection, *oval* represents colostomy

quence of these diseases. Also, it has been encountered in as many as 40% of patients with HD, proximal to the AZ and blamed for the bad postoperative outcome of BF in these patients [6, 22].

These innervation changes remain controversial [1, 9]. This in part could be due to the sophisticated methods required to assess them, such as the acetyl-cholinesterase histochemistry and immunohistochemistry techniques, but also to the lack of agreement in the criteria used to classify these changes. Of note, our experience corroborates the reports of overlooked dysganglionosis when no more than "conventional" staining techniques like HE were used [6, 7]. Rapid AChE permits an intraoperative exploration of the innervation, but the final diagnosis should be confirmed with specific histopathological techniques like conventional AChE after Karnovsky and Roots and immunohistochemistry for PGP 9.5 and CD56. We would therefore suggest applying these diagnostic methods on full-thickness biopsies as soon as innervation disorders are suspected.

Simpser et al. [26] have reported the spontaneous regression of isolated IND in one patient. Same reports were made by Rintala and al. in infants with bowel obstruction [18]. Since 1995, we prospectively assessed the ENS of the ganglionated bowel above the AZ at the time of PT. From 1995 to 2002, 31 patients were operated on for HD in our institution. Nine had severe constipation after PT, and IND was found in eight of them. In all of these patients, BF improved in a period of time ranging from 1 to 5 years. In three patients who were repeatedly histologically reassessed because of the severity of their complications, this particular dysganglionosis evolved to normal innervation within a period ranging from 9 to 18 months. In one patient (case 1), this evolution occurred stepwise. In the biopsies repeated after 6 months, criteria for IND were not met, but changes of the innervation such as a marked nerve hypertrophy in the SM were still present. Considering that they were not found in the biopsies repeated after 18 months, these changes may represent a milder form of hyperplasia of the parasympathic nervous system and be intermediate innervation patterns between IND and normal innervation. The moderate nerve hypertrophy that was encountered in two patients did not have any clinical implication when seen as an isolated finding. Nevertheless, when found in a patient with disorders of the BF, it should raise the suspicion of the presence of more severe types of dysganglionosis coexisting in the same region of the bowel, and this should be searched for.

These observations have led us to the hypothesis that IND could be responsible for the bad postoperative short-term outcome of BF in some patients with HD and that the long-term improvement of BF reported by some authors [17, 24] might be explained by the normalisation of innervation patterns of the colon proximal to the aganglionosis, at least in some of these patients.

Distension or inflammation of the bowel wall, as well as stool retention, can explain the development or the persistence of IND. Colostomies that had been placed to avoid stool retention and consequent dilatation of the bowel may therefore have contributed to the plasticity encountered in our patients. Marty et al. [11] have successfully proposed postoperative repeated irrigations in all patients operated on for HD in order to reduce postoperative complications. This method may have had the same result as a colostomy on the bowel wall and thus on ENS.

Although some authors have considered IND as a primary disease and proposed resection of the involved bowel segments [2, 27], others have suggested conservative treatment with saline washouts [21, 26]. Our study shows that IND might evolve to normal innervation. Also, postnatal changes in the neuronal density of the myenteric plexus have been described [28], and IND changes might be part of this overall evolution. Dealing with this innervation disorder therefore requires being aware of its ability to have a certain degree of plasticity in order to avoid preventable major surgical procedures. The finding of IND is an indication for daily bowel washouts, or for a colostomy in selected cases. According to the outcome of BF, biopsies should thereafter be repeated after a delay of about 12 months in order to assess the evolution of innervation patterns.

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