MORPHOLOGICAL ASSESSMENT OF ENTERIC NEUROMUSCULAR DISEASE CAUSING INTESTINAL PSEUDO-OBSTRUCTION

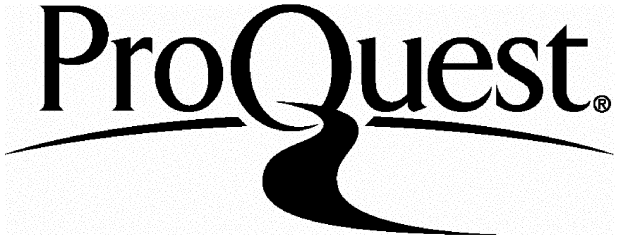
Virpi V Smith

A thesis submitted to the University of London for the degree of Doctor of Philosophy in the Faculty of Medicine.

July 1993

Department of Histopathology

Institute of Child Health



Abstract

Disorders of intestinal motility which cause functional obstruction (chronic idiopathic intestinal pseudo-obstruction; CIIP) can be divided into two broad categories (neuropathic or myopathic) distinguishable by electrophysiological studies. Hirschsprung's disease (HD), the commonest variety of CIIP, is associated with a distal aganglionic segment of intestine of variable length, but the morphological changes in other rarer forms of CIIP are less well defined. This thesis investigates currently available morphological methods and examines some immunohistochemical techniques in evaluating abnormalities in the smooth muscle and nerve cell cytoskeleton and the interface between these two elements as possible pathogenic factors in these puzzling conditions.

Morphometric studies of myenteric neuronal densities have established a baseline against which some neurogenic forms of CIIP, such as intestinal neuronal dysplasia (hyperganglionosis) and hypoganglionosis can be validated. A study of rectal suction biopsies, seeking changes reportedly indicative of intestinal neuronal dysplasia has suggested that some apparent abnormalities may merely be age-related variations. Similarly, previously reported abnormalities observed by silver staining of the myenteric plexus appear to be normal for the developing intestine.

The majority of patients with neurogenic CIIP, identified clinically by electrophysiology, have no morphological abnormality of enteric innervation recognisable by conventional histological techniques and immunohistochemical studies of neurofilaments (NF), neural cell adhesion molecule (N-CAM) or muscarinic

receptors were similarly unrewarding.

In contrast, in myopathic CIIP some important new ultrastructural observations have been made regarding fibrosis, atrophy and central vacuolation of intestinal muscle. Peripheral vacuolation and folding of the smooth muscle plasma membrane are considered to be artifacts. Immunohistochemical studies using NF antibodies, originally applied to detect neuropathic abnormalities, demonstrated a striking increase in nerves in the circular muscle in myopathic CIIP. Probably of greatest significance was the identification of contractile protein isoform abnormalities in intestinal myopathies. This new approach has demonstrated notable changes, unrecognised by conventional methods, that may affect enteric muscular function. It also opens the way to further studies at the molecular and genetic levels.

Acknowledgements

Without the involvement of numerous colleagues at the Hospital for Sick Children and the Institute of Child Health and those in other hospitals this study would not have been possible and I am truly grateful to them for referring samples from patients with CIIP for me to analyse. The help of few associates warrants a special mention. I am expressly indebted to Dr. Peter Milla, who first planted in me the seed of interest in CIIP almost two decades ago, for allowing me to study samples from his patients and for his long standing encouragement, never failing inspiration and friendship. I would especially like to thank my Departmental Head, Prof. Tony Risdon, for freely giving so much of his valuable time, his perceptive criticism, greatly appreciated guidance and intellectual support. I particularly wish to acknowledge friends and colleagues in the Departments of Histopathology, Paediatric Surgery and Gastroenterology, especially Glenn Anderson for his generous general encouragement, help and valued photographic advice; Ivan Brooke, Keith Lindley and James Moorecroft for improving my computer literacy and Dyanne Rampling for her compassion and histological support. I am also grateful to Drs Keith Lindley and Paul Sibbons for allowing me to examine animal tissues from their experiments.

This study was funded in part by the Joint Research Board of the Hospital For Sick Children and Institute of Child Health. The work reported in this thesis has been undertaken by me, with the exception of the electrophysiological analysis which was performed by Drs. Devane, Bisset, Ravelli and Milla, and some histological preparation of surgical material, carried out by Dyanne Rampling and her team, which I have analysed. I also thank Murrey Silverstone for some silver staining of sections I have assessed. Lastly I wish to thank my colleague of many years standing

Prof. Brian Lake, who has been my mentor in histochemistry and electron microscopy, for his supervision.

To Elina and Anna

'I hav finally kum tu the konklusion,
that a good reliable sett ov bowels
iz wurth more tu a man than enny
quantity ov brains'.

Josh Billings
(1818-1885)

Contents

List of Illustrations and Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

Chapter 8

Enclosures

Smith VV. Isolated Intestinal Neuronal Dysplasia: a descriptive histological pattern or a distinct clinicopathological entity. In: Inflammatory Bowel Diseases and Morbus Hirschsprung. Hadziselimovic FH and Herzog B (Eds), Kluwer Academic Publishers, Lancaster 1992, 203-214

Smith VV, Lake BD, Kamm MA and Nicholls JR. Intestinal Pseudo-obstruction with Deficient Smooth Muscle Alpha Actin. Histopathology 1992; 21:535-542

Smith VV. Intestinal Neuronal Density in Childhood: a baseline for the objective assessment of hypo- and hyperganglionosis. Pediatr. Pathol. 1993; 13:225-237

Devane SP, Coombes R, Smith VV, Bisset WM, Booth IW, Lake BD and Milla PJ. Persistent Gastrointestinal Symptoms after Correction of Malrotation. Arch. Dis.Child. 1992;67:218-221

Devane SP, Ravelli AM, Bisset WM, Smith VV, Lake BD and Milla PJ. Gastric Antral Dysrhythmias in Children with Chronic Intestinal Pseudo-obstruction (CIIP). Gut 1992;33:1477-1481

Chapter 1. General Introduction and Historical Appraisal

Intestinal obstruction is a common and sometimes life-threatening condition usually due to localised lesions in the intestinal wall, within its lumen or compressing it from the outside. Rarely, however, chronic or recurrent symptoms of intestinal obstruction may occur in the absence of any obvious mechanical cause, a situation described by the term chronic idiopathic intestinal pseudo-obstruction (CIIP). A heterogeneous spectrum of pathological changes in the intestinal wall may be associated with this functional disorder which affects intestinal motility and produces alterations in normal intestinal peristalsis. Normal gut motility is dependant on an intact neural circuitry comprising enteric plexuses communicating with each other and the rest of the bowel wall; it is also dependant on intact normal contractile tissue consisting of the circular and longitudinal muscle coats. Thus the abnormalities giving rise to CIIP can be subdivided broadly into two groups: neuropathic (primary neural defects) and myopathic (primary defects in the intestinal smooth muscle).

Defective intestinal motility is manifested clinically by gross abdominal distension and pain, bilious vomiting, food intolerance or difficulty in defaecation. The abdominal distension is a result of intestinal dilatation, often affecting the colon alone but sometimes involving other parts of the intestine.

For centuries these disorders were entirely unexplained or 'agnogenic' (Ravitch 1966), but recently, with the evolution of improved detection methods and better understanding of normal intestinal physiology, several distinct pathological entities have been identified, although many still remain unclarified. The diagnostic methods

used have evolved from gross anatomical examination, through histological, enzyme histochemical and ultrastructural to immunohistochemical techniques, each of which have contributed to the understanding and diagnosis of pseudo-obstructive disease.

1.1 Megacolon

The earliest surviving description of megacolon can be attributed to Frederici Ruyschii. In 1691 he described the death of a 5-year old girl with a grossly dilated colon, whose symptoms were not alleviated with the usual remedies employed in those times for deworming or to relieve flatulence.

Similar descriptions, dating two centuries later, of megacolon resulting in death failed to clarify the pathophysiology of this disease. The reports included a young doctor with long standing symptoms of 'dyspepsia, frequent acid vomitings and costiveness' (Parry 1825); a 17-year-old boy with 'acute intestinal obstruction, yet always constipated habit' (Ebers 1836); a 10-year-old boy, who from the 'earliest age never had spontaneous stools' (Barth 1870); a 4-year-old boy with 'difficulty on defaecation since the age of 3 months' (Gee 1884) and an 8-year-old girl, who had long been 'subject to constipation' (Bristowe 1885). 'An insufficient degree of intestinal muscular power and action as a physical cause for costiveness' was recognised by Jacobi in 1869. He urged examination of the 'locomotive power of the intestinal tract in every case of abnormal function where there is no local obstruction, no faulty secretion or apparently no improper food'. In particular he emphasised that 'in infants whose constipation dates from the first hour of life, an abnormal intestinal anatomy may be the cause for the costiveness'.

1.2 Hirschsprung's Disease

The first significant contribution to the unravelling of pseudo-obstructive disease was in 1886 when Harald Hirschsprung , a Danish professor of paediatrics, presented his detailed necropsy findings on two infants (aged 11 and 8 months) for the Gesellschaft fur Kinderheilkunde in Berlin. His paper was entitled "Stuhltragheit Neugeborener in Folge von Dilatation und Hypertrophie des Colons" and was published in the Jahrbuch fur Kinderheilkunde in 1888. In contrast to previous reports of megacolon, Hirschsprung demonstrated that the most striking dilatation was seen in the sigmoid and transverse colon and that the rectum was of normal calibre. Hirschsprung's invitation for reports of similar patients resulted in a literature review of 55 cases (Griffith 1899) and Hirschsprung himself reported four further patients in 1900.

Hirschsprung's disease (HD) was thus the first of the pseudo-obstructive disorders to be identified as a distinct clinical entity, although the pathology was far from clear for another 60 years.

1.2.1 Histological Observations in Hirschsprung's Disease

Suggestions of a neurogenic origin for HD were made first by Lennander (1900) and a number of authors subsequently noted that neurons in the myenteric plexus were very poorly developed (Bretano 1904) or completely absent (Dalla Valla 1920; Robertson and Kernohan 1938; Tiffin 1940; Bodian 1949). However, contradictory reports claiming the presence of myenteric neurons also appeared (Finney 1908; Schmidt 1909; Retzlaff 1920; Cameron 1928). Due to a lack of understanding that the abnormality responsible for pseudo-obstruction in HD was in the undilated segment of intestine, and to omissions in the recording the exact site of the specimens

analysed, no conclusions could be made until the late 1940's and early 1950's. HD is now understood to result from aganglionosis (i.e. an absence of neurons in the enteric plexuses) in the distal segment of the intestine; the dilated proximal intestine is usually morphologically and functionally normal. Since the absence of enteric neurons in HD is not always easy to demonstrate, other methods have been investigated to improve the diagnostic accuracy.

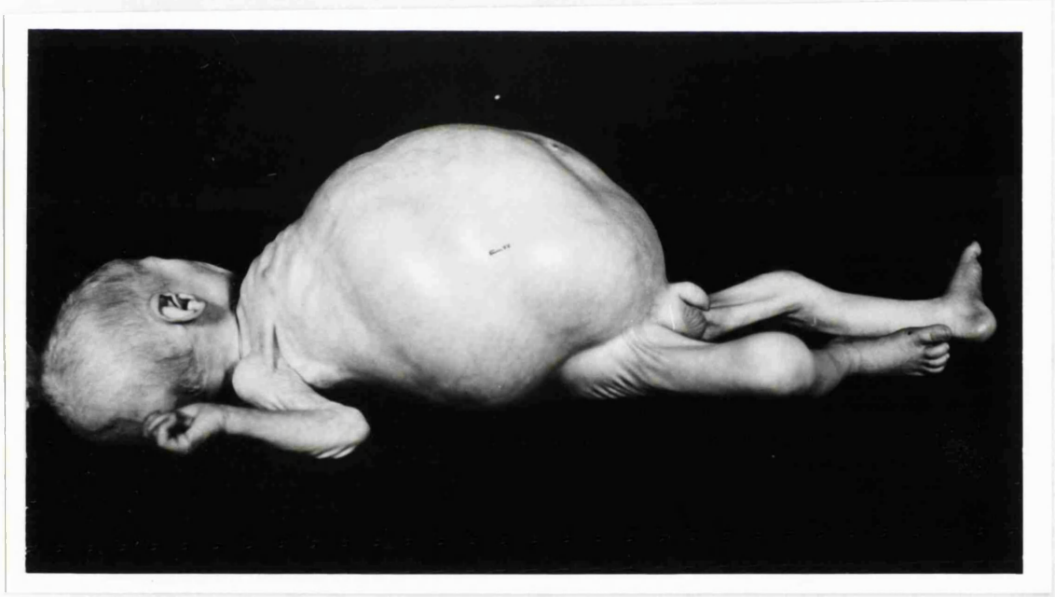


Figure 1. Hirschsprung's disease: Little Richard, one of the first patients shown to have aganglionosis of the distal intestine. He was successfully diagnosed and treated in January 1949 at The Hospital for Sick Children, Great Ormond Street. Note the enormously dilated intestine clearly visible through the abdomen.

1.2.2 Acetyl Cholinesterase Activity in Hirschsprung's Disease

Biochemically, high levels of cholinesterase activity were found in the aganglionic segments in HD compared with the ganglionic portions and normal controls (Kamijo et al. 1953). An overall increase in specific (acetyl) cholinesterase was more marked than that in non-specific (butyryl) cholinesterase. The increased acetyl cholinesterase (AChE) activity was shown histochemically to be associated with large bundles of non-myelinated nerve fibres whilst non-specific cholinesterase activity was associated mainly with smooth muscle fibres of the muscularis mucosae and the innermost layers of the circular muscle (Kamijo et al. 1953). An increase in non-specific cholinesterase activity was confirmed to be present in the muscularis mucosae but was noted also in the longitudinal muscle by Niemi et al. (1961). An increase in AChE-positive nerve fibres in the muscularis mucosae and the lamina propria of the aganglionic segment was reported by Meier-Ruge (1967, 1968, 1972) and by Elema et al. (1973) and the histochemical assessment of AChE-activity in mucosal biopsies emerged as the most reliable method for diagnosing HD (Meier-Ruge 1974; Lake et al 1978).

1.3 Pseudo-Hirschsprung's Disease

It soon became apparent that other pseudo-obstructive diseases clinically mimicking HD also existed in which either no aganglionosis could be detected (Ehrenpreis 1966; Bentley 1966; Nixon 1966; Spencer 1966; Lister 1966; Pages 1966; Duhamel 1966; Katz 1966), or 'aganglionosis was seen in one or the other of the enteric plexuses but not in both' (Pages & Duhamel 1966; Ikeda et al. 1988). The delay in identifying the pathology in HD can also be partly explained by the existence of these pseudo-Hirschsprung's diseases, which comprise a number of intestinal neuromuscular disorders.

1.3.1 Hyperganglionosis and Intestinal Neuronal Dysplasia (IND)

Further progress in the elucidation of pseudo-obstruction was made by the routine examination of the AChE staining pattern in biopsies from patients suspected clinically of having HD. In some patients a distribution pattern of AChE-positive nerve fibres emerged which was unlike that in controls or in patients with HD (Meier-Ruge 1971). This consisted of an increase in the number of nerve fibres, which were finer than those seen in HD and occurred in the lamina propria but not in the muscularis mucosae. Numerous neurons were present, and were found in unusual locations such as the lamina propria and within the muscularis propria (Meier-Ruge 1971,1974,1985; Puri et al. 1977a; Fadda et al. 1983; Scharli & Meier-Ruge 1981,1986). Giant ganglia with increased numbers of neurons in the enteric plexuses and variability in neuronal size, ranging from giant to minute, were also seen. This condition became known as hyperganglionosis (Garrett & Howard 1981) or intestinal neuronal dysplasia (IND), and two types (A and B) were described (Fadda et al. 1983; Scharli and Meier-Ruge 1981, 1986; Meier-Ruge 1985). Type A is reported to be associated with hypoplasia or aplasia of sympathetic nerves and mucosal inflammation, and to present clinically with bloody stools and symptoms of intestinal spasticity. Examination of the sympathetic, adrenergic innervation is claimed to be of value in diagnosing IND type A, but difficulties arise from the capricious nature of the formaldehyde- induced fluorescence methods used to identify adrenergic nerves in tissue sections. After exposure to formaldehyde vapour, catecholamine-fluorescent (extrinsic) nerve fibres are seen in the normal bowel around intestinal blood vessels and synapsing with enteric neurons. In IND type A the absence or a reduction in the number of these nerves have been reported (Fadda et al. 1983; Scharli and Meier-Ruge 1981, 1986; Meier-Ruge 1985).

Type B IND (Fadda et al. 1983; Scharli and Meier-Ruge 1981, 1986; Meier-Ruge 1985) lacks evidence of involvement of sympathetic nerves or mucosal inflammation, but exhibits hyperplasia of the sub-mucosal and myenteric plexuses and an increase in AChE-positive nerve fibres in the lamina propria (which has also been reported in type A) and around the adventitia of the submucosal vessels. These changes can occur in an isolated distal segment of the bowel or may involve the entire gastrointestinal tract. IND may also be seen proximal to the aganglionic segment in HD (Puri et al. 1977a; Meier-Ruge 1985; Fadda et al. 1987).

Disorders in which hyperplasia of the myenteric plexus is a cardinal feature range from hyperganglionosis (IND) through glial cell hyperplasia to frank neurofibromatosis (Feinstat et al. 1984; Navarro et al. 1990; Fuller and Williams 1991; D'Amore et al. 1991). Many of the changes seen in IND are present in the intestine from patients with neurofibromatosis (von Recklinghausen's disease) and intestinal involvement may be the first sign of this condition (Fuller & Williams 1991). Glial cell hyperplasia (Navarro disease) may be accompanied by hypoganglionosis (a reduced number of neurons) or the neuronal numbers may be 'normal'. Whilst a few reports of myenteric plexus neuronal numbers exist in experimental animals (Christensen et al. 1984; Gabella 1987c,1989b), in man only scanty information is available (Munakata et al. 1978; Schuffler et al. 1978; Schuffler and Jonak 1982; Feinstat et al 1984; Krishnamurthy et al. 1985) and there is even less data for children (Meier-Ruge et al. 1970; Ikeda et al. 1988). Thus, what constitutes normoganglionosis in different parts of the human intestine has not been satisfactorily defined.

1.3.2 Hypoganglionosis

Hypoganglionosis alone and without associated glial cell hyperplasia was recognised first by Karl Tittel (1901). Bentley (1966) also described pseudo-obstruction associated with smaller and more sparse ganglia and scanty neurons in the myenteric plexus. Hypoganglionosis may be a primary condition due to developmental mechanisms similar to those that cause HD (Munakata et al. 1978; Yanagihara et al. 1991) or be secondary to inflammatory processes specifically attacking the enteric plexuses (Houropian and Kim 1982; McDonald et al. 1985; Krishnamurthy et al. 1986; Burns et al. 1990; Lennon et al. 1991). An early illustration of the presence of a mononuclear inflammatory infiltrate in the myenteric plexus with scanty degenerate neurons in a 7-year-old boy was provided by Cameron (1928), who believed that the abnormality noted gave a clue to the pathology responsible for HD. Mechanisms postulated for secondary hypoganglionosis include auto-immune phenomena or the effect of toxins. In the early phase the plexuses are populated by chronic inflammatory cells which then disappear leaving denervated intestine (Horopian and Kim 1982; Krishnamurthy et al. 1986; Burns et al. 1990). Secondary hypoganglionosis or aganglionosis may also result from nerve damage following surgery (Dajani et al. 1986; Cohen et al. 1993). A hypoganglionic segment of varying length may also be seen in HD proximal to the aganglionic segment. In the absence of reliable baseline values for normal intestinal neuronal density, assessment of hypoganglionosis is subjective and requires clarification.

1.3.3 Immaturity of Enteric Neurons

The presence of immature neurons thought to be responsible for intestinal motility disturbances was described by Spencer in 1966 (who also referred to the unpublished

work by Smith Blanca), by Bughaighis and Emery (1971), and Erdohazi (1974). Intestinal neurons apparently at morphologically different stages of development were observed by Smith Blanca (1968). Using silver staining, a method commonly employed in neuropathology, Tanner et al. (1976) revealed the absence of enteric argyrophilic neurons in some patients and regarded this as a possible cause of pseudo-obstruction. Although silver staining has been studied in the experimental animal and control adult bowel (Smith Barbara 1967, 1968a,1968b; Schuffler 1989) very little is known of the normal appearances with this method in young children and newborn babies.

1.3.4 Other Neuropathic Diseases

Silver staining has been reported to reveal degenerative changes (loss of cell bodies, fragmented or swollen clubbed processes and axonal drop out) in the neurons of the myenteric plexus in pseudo-obstruction (Dyer et al. 1969; Schuffler et al 1978; Smith Barbara 1982; Schuffler and Jonak 1982; Krishnamurthy et al. 1985; Krishnamurthy and Schuffler 1987; Schuffler 1989). Abnormalities of argyrophobic cells have also been noted (Schuffler 1989; Navarro et al. 1990).

The adynamic bowel syndrome, characterised by abdominal distension with a total absence of bowel sounds, was described in three children by Nixon (1966). On conventional histology the ganglia in the myenteric plexus were smaller and less numerous than normal; similar findings were reported in a further four patients investigated by Kapila et al. (1975). Subsequently in a patient with clinical signs of adynamic bowel syndrome, the absence of AChE-positive nerve fibres in the lamina propria, submucosa and the circular and longitudinal muscle layers of the bowel wall

was observed by Puri et al. (1977b). The small, scanty ganglia showed normal AChE activity and on ultrastructural examination only the remnants of neural tissue elements were found in vacuolated, practically empty nerve tracts.

1.3.4.1. Neurofilament Studies

Despite the various changes in enteric innervation described in the preceeding sections, it is evident that in many patients with clear electrophysiological evidence of a neural-based pseudo-obstruction, no morphological abnormality can be detected by either light or electron microscopy.

Immunohistochemical studies in patients with pseudo-obstruction including HD, using antibodies to neurofilament proteins, revealed abnormalities not recognised by conventional morphological examination (Kluck et al. 1986; Tibboel et al. 1987). However these results were not reproducible (MacKenzie and Dixon 1987; Sams et al. 1992) and the discrepancies noted may be related to the fact that all these studies were performed in fixed, paraffin-wax embedded tissue, which may cause alterations in the epitopes recognised by the antibodies employed.

Neurofilament proteins in the normal central nervous system exist in weakly phosphorylated or dephosphorylated forms in the neuronal perikarya and dendrites. In contrast, the axonal neurofilament proteins are heavily phosphorylated. In neurons damaged by toxins, nerve section or crushing, and in neurodegenerative disease such as Alzheimer's disease, neurofilament phosphorylation increases in the neuronal cell bodies (Nixon and Sihag 1991; Leigh et al. 1989).

Eaker et al. (1990) compared myenteric plexus with cortical neurons in the rat by neurofilament immunostaining using several poly- and monoclonal antibodies on frozen tissues. They found absent or reduced immunoreactivity in the rat myenteric plexus neurons (perikarya and processes) with antibodies directed against phosphorylated neurofilaments compared with strong immunostaining in the axons of the brain. With antibodies recognising dephosphorylated neurofilaments strong immunostaining was seen in both the brain and myenteric plexus neurons. They concluded that neurofilament phosphorylation in the enteric neurons was low. No attempts to date have been made to assess the degree of neurofilament phosphorylation in pseudo-obstructive diseases.

1.3.5 Smooth Muscle Abnormalities

It has long been recognised that abnormalities could also be present in the contractile tissue comprising the smooth muscle of the gut wall in patients with CIIP. Absence of one of the intestinal smooth muscle coats (Humphry et al. 1980), absence of all intestinal muscle layers (Emanuel et al. 1967; Hussain et al. 1992) or the presence of an extra, oblique outer muscle layer (Yamagiwa et al. 1988) have been described. Abnormalities consisting of fibrosis and myocyte vacuolation were detected by light microscopy on conventionally stained histological sections in adults (Schuffler et al. 1977a,1977b; Faulk et al. 1978; Lewis et al. 1978; Jacobs et al. 1979; Smith JA et al. 1982; Smout et al. 1985; Anuras et al. 1983a, 1986a; Alstead et al. 1988; Rodrigues et al. 1989; Colemont & Camilleri 1989; Martin et al. 1990) and in children (Puri et al. 1983; Milla et al. 1983; Bagwell et al. 1984; Anuras et al. 1986b; Kaschula et al. 1987; Vargas et al. 1988; Schuffler 1988a; Nonaka et al. 1989). Smooth muscle in the urinary tract may also be affected resulting in

megacystis and/or megaureter. The almost universally fatal megacystis-microcolon-intestinal hypoperistalsis syndrome was regarded as the most severe form of intestinal myopathy (Puri et al. 1983), even though neural abnormalities were also observed in this disease by some authors (Berdon et al. 1976; Armoury et al. 1977; Wiswell et al. 1979; Kirtane et al. 1984; Bindl et al. 1989). The milder, adult forms of intestinal myopathy present during the second decade of life , although symptoms can often be traced to early childhood (Milla 1991).

Conventional histology in these disorders can identify only the grossest degrees of fibrosis and smooth muscle vacuolation, and more subtle morphological abnormalities may remain undetected. Milder degrees of fibrosis, central vacuolation and other changes in smooth muscle cells can, however, be detected ultrastructurally (Schuffler et al. 1977; Shaw et al. 1979; Smith JA et al. 1982; Puri et al. 1983; Milla et al. 1983; Krishnamurthy and Schuffler 1987; Lake 1988; Venizelos et al. 1988; Alstead et al. 1988; Martin et al. 1990). Nevertheless, as was noted with the neuropathic forms of CIIP, in biopsies from many patients with clear electrophysiological evidence of muscle disease no abnormality is found on light or electron microscopy.

It is imperative to appreciate the different ultrastructural appearances that can occur in normal intestinal smooth muscle in various normal physiological states (Gabella 1981, 1987a,1987b, 1988, 1989a, 1990a,1990b,1990c,1990d) to distinguish them from pathological changes suggestive of intestinal smooth muscle disease. Equally, the artefacts produced in both smooth muscle and nerves by surgical trauma (ischaemia, hypoxia) and any delay in fixation of surgically resected specimens of bowel need special consideration lest they be interpreted as being of pathological

significance.

1.4 Aims

The purpose of this thesis is to examine and evaluate current histopathological methods in the diagnosis and classification of non-Hirschsprung's pseudo-obstructive disease, particularly in childhood. The specimens examined were obtained from a variety of patients with neuropathic and myopathic forms of CIIP, in many of whom the diagnosis was supported not only by clinical observations but also by electrophysiological studies (see Chapter 2). The examination and evaluation of these samples has been accomplished as follows:

(a) Establishment of a baseline of neuronal density in the normal myenteric plexus in order to address the problems of hypo- and hyperganglionosis (see Chapter 4).

(b) Application of recently described criteria for the diagnosis of IND on rectal suction biopsies and a critical evaluation of their validity (see Chapter 3).

(c) Appraisal of silver staining in normal intestine during childhood to assess whether the appearances in the myenteric plexus differ between children and adults and which changes may be normal in maturing bowel (see Chapter 5).

(d) Analysis of neuromuscular ultrastructure in pseudo-obstruction and comparison of the changes encountered with variations seen under experimentally induced conditions to differentiate true pathological alterations from artifacts produced by fixation and surgical trauma (see Chapter 6).

(e) The use of immunohistochemical techniques in an attempt to identify new methods which will recognise specific entities within the broad classification of neuropathic/neurogenic and myopathic/myogenic motility disorders. The integrity of intestinal nerves, smooth muscle and some aspects of the interface of the intestinal neuro-muscular junction have been assessed in biopsy material from patients with pseudo-obstruction and controls. Neural markers (antibodies to neurofilament triplet proteins and their phosphorylation), smooth muscle markers (antibodies to contractile filaments), a marker for neural cell adhesion molecule, and an antibody to muscarinic receptors have been used for this purpose (see Chapters 7 and 8).

Chapter 2. Clinical Investigation of Pseudo-obstruction by Electrophysiological Techniques

2.1 Introduction

Chronic idiopathic intestinal pseudo-obstruction (CIIP) comprises a spectrum of disorders in which persistent or recurrent obstructive symptoms are associated with defective gut motility rather than anatomical blockage.

Efficient peristalsis and the propagation of ingested food along the length of the bowel is dependent on intact contractile smooth muscle forming the longitudinal and circular muscle coats of the bowel wall. Although smooth muscle is capable of independent contraction, a neural input is important in co-ordinating these contractions to produce orderly peristaltic activity. The enteric nervous system (ENS), sometimes termed the 'little brain of the intestine', serves this purpose. It comprises three enteric plexuses, the myenteric plexus of Auerbach (the motor division) and the two submucosal plexuses of Meissner and Henle (sensory and secretory division), which communicate with each other and with the smooth muscle of the gut wall through intrinsic nerve fibres.

The 'little brain' behaves like an intelligent computer terminal containing motor programs, which can be modulated by the 'main frame computer', the central nervous system (CNS). Communication with the CNS is through autonomic ganglia by the sympathetic and parasympathetic pathways and the enteric nervous system programs are selected on the basis of sensory input. The 'little brain' is ultrastructurally and physiologically more akin to the CNS than to autonomic ganglia in that there is a

paucity of extracellular spaces, so that glial elements and neurons are closely applied to one another without intervening connective tissue. There are also large areas of perikaryon in direct contact with the surrounding ganglionic basal laminae (Gabella 1987a). In addition a multiplicity of co-localised non-adrenergic non-cholinergic (NANC) nerves is seen both in the ENS and in the CNS (Furness and Costa 1987).

The ENS consists of sensory neurons, interneurons and both excitatory and inhibitory motor neurons. The messages received by sensory receptors are modulated by myenteric interneurons which co-ordinate switching on or off inhibitory or excitatory motor neurons in the myenteric plexus to control the contractile activity of the smooth muscle layers. There are also motor programs in the interneuronal synaptic circuits which are independent of sensory input and produce cyclical contractions which form the migrating motor complexes (MMCs) referred to later (see below).

Thus intestinal motor activity depends on the contractile function of the intestinal smooth muscle coats and the integrated actions of intrinsic and extrinsic neural and humoral influences which co-ordinate the segmental relaxation of the inherently contractile smooth muscle tissue (Wood 1988a,1988b; Wood 1989a,1989b). CIIP can therefore be classified as myopathic or neuropathic, depending on whether the primary defect is in the smooth muscle of the bowel wall or in the complex enteric neural circuitry. CIIP can also result from abnormal humoral metabolism, but for the purpose of this study only disorders intrinsic to the gut are considered.

Intestinal motor function can be studied by a variety of electrophysiological techniques. Small intestinal manometry using perfusion catheters (see Methods;

2.2.2.1) is one such method and considerable knowledge of the normal motor patterns exists including those seen in premature infants (Ruckebusch 1986; Bisset et al. 1988a,1988b,1989; Berseth 1989).

In the normal small intestine motor activity in the fasting state consists of a quiescent phase (phase I) followed by a phase of irregular contractions (phase II) and finally by bursts of rhythmic phasic contractile activity known as migrating motor complexes (MMCs; phase III; Fig.1). MMCs occur sequentially at different levels of the intestine at the maximum rate for smooth muscle contraction (12 cycles/min in small intestine) and at a pressure of 40-80 cm of water. Their pattern is cyclical, and at any given site in the small intestine an MMC is seen about every 90 minutes. Following a meal the cyclical fasting pattern is disrupted and replaced by continuous contractions. This continuous pattern lasts for a variable period (typically for 3-4 hours) depending on the volume and composition of the meal after which the cyclical fasting pattern reappears.

In disease various abnormal patterns are associated with disorders of the smooth muscle (Fig.2) and enteric nerves (Fig.3). In the former, there are weak contractions of low amplitude (Milla 1991; Devane et al. 1992a). In the later, the contractile activity is of normal or increased pressure, but MMCs are irregular and show abnormal propagation down the gut (Milla 1991; Devane et al. 1992a).

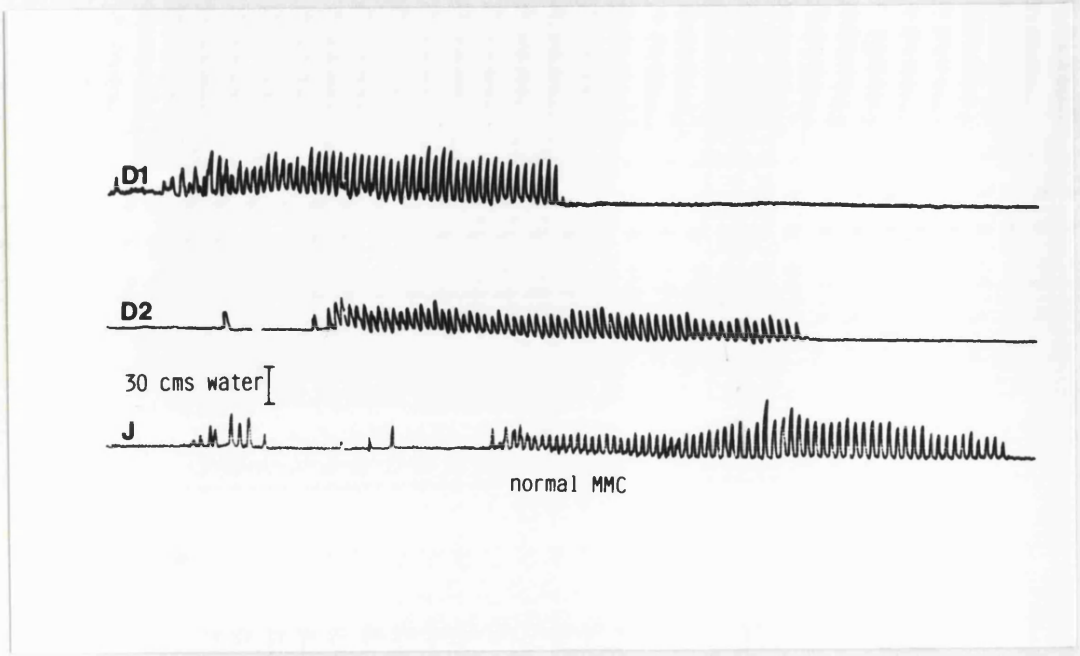


Figure 1. A recording of intraluminal pressure in the small intestine showing phase III of the migrating motor complex with normal amplitude, frequency and aborad propagation. The ports for channels D1 and D2 are positioned sequentially in the duodenum and the port for channel J is at the proximal jejunum; all ports are 5cm apart.

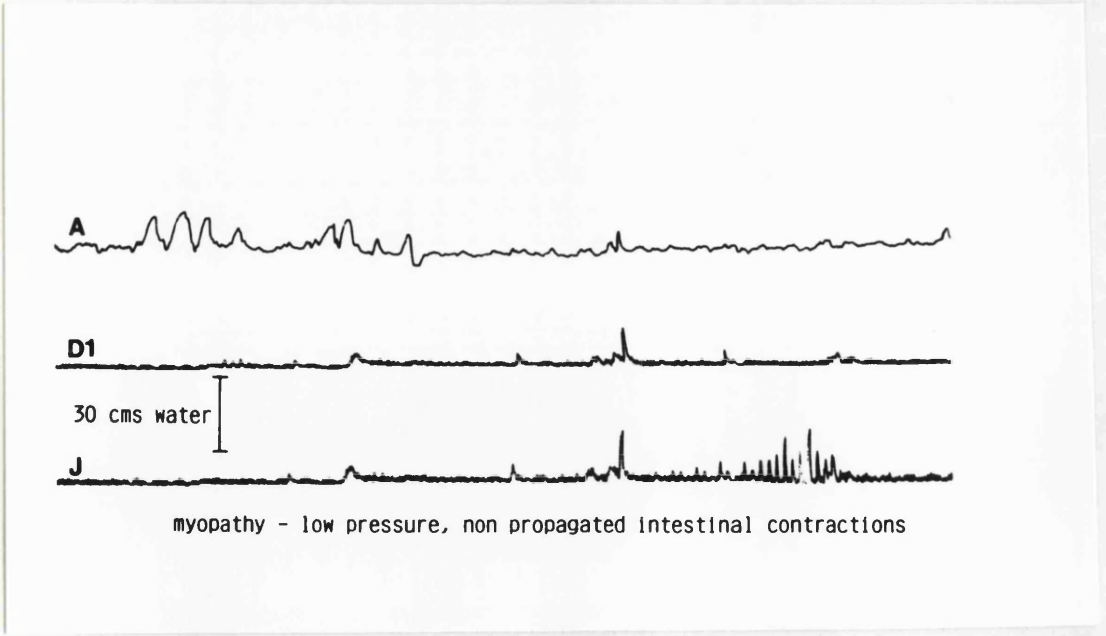


Figure 2. Phase III pressure activity in a baby with myopathic pseudo-obstruction. Note the low amplitude contractions. (A) gastric antrum, (D1) duodenum, (J) proximal jejunum.

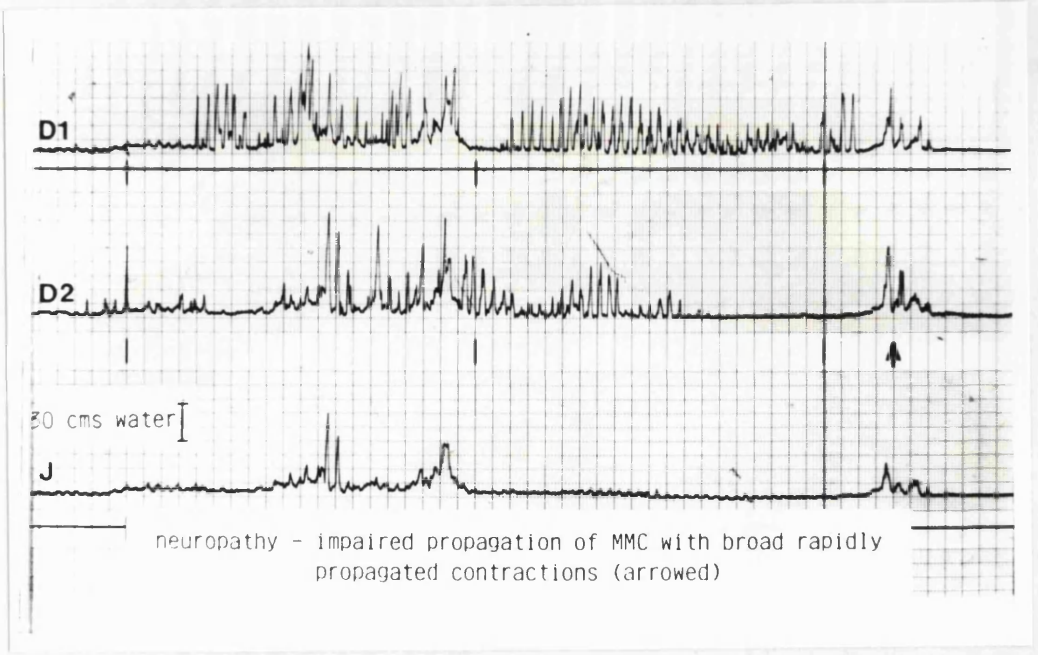


Figure 3. Phase III pressure activity in the small intestine of a child with neuropathic CIIP showing irregular contractions and poor propagation. (D1) and (D2) duodenum, (J) proximal jejunum.

Motor activity can also be studied by the less invasive technique of surface electrogastrography (EGG), which merely requires the positioning of electrodes on the skin overlying the gastric antrum. Studies using EGG have shown that antral dysrhythmias are seen both when disease is present throughout the gastrointestinal tract and sometimes when disease appears to be present only in regions of the gastrointestinal tract remote from the stomach. This method is therefore considered to be an ideal screening investigation in intestinal pseudo-obstruction (Devane et al. 1992b; Reddy et al, 1992), but a normal pattern does not necessarily exclude disease distal to the stomach. The intrinsic constant rhythmic polarisation and depolarisation of the plasmalemmal membranes of smooth muscle cells gives rise to electrical slow wave or electrical control activity (ECA). Gastric ECA can be detected by EGG and

the dominant frequency of the contractions determined. This dominant frequency is 3 cycles/min (cpm) in the normal stomach; see Fig.4 (Kelly 1981) and 12 cpm in the small intestine (Weisbrodt 1981).

A pattern of chaotic variable frequencies without a dominant frequency (Fig.4) is associated with myopathic disease (Devane et al. 1992b), and tachygastrias (of increased dominant frequency; Fig.4) or, less commonly, bradygastrias (of decreased dominant frequency), are indicative of a neuropathic process.

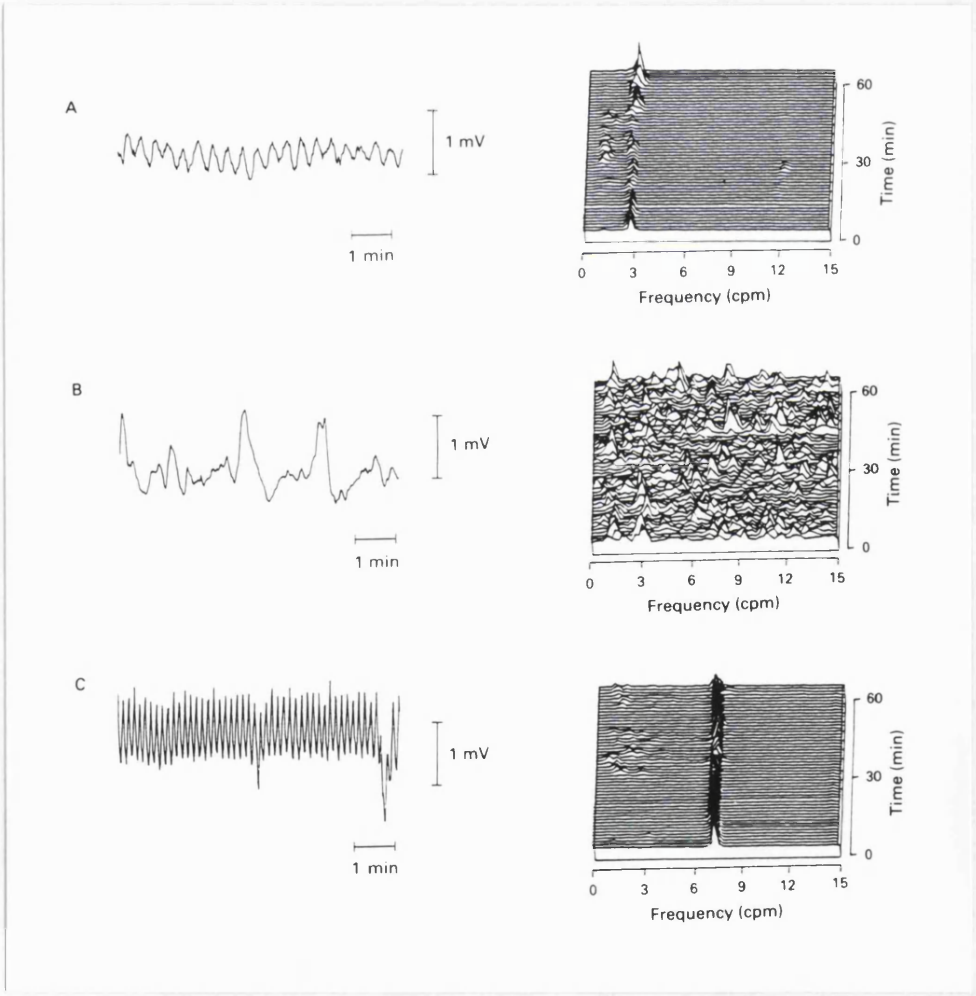


Figure 4. Surface electrogastrography: frequency displayed as a pseudo-3-dimentional representation of a running spectral analysis of muscle cell activity and a short extract from the real time signal. (A) Normal frequency of electrical control activity (ECA); (B) continuously irregular ECA (myopathic); (C) tachygastria (neuropathic).

Thus electrophysiological studies can be used to investigate patients with intestinal pseudo-obstruction to identify abnormalities in either the enteric innervation or in the intestinal smooth muscle. Some clinical features may also give a clue to the pathophysiology. Due to the rarity of these conditions, studies of significant numbers of patients are limited and few have been systematically investigated.

In this chapter the clinical symptoms and electrophysiological investigations are considered in patients with apparently disturbed intestinal motor activity in whom the diagnosis of HD had been excluded. The information gained was then used as a basis for investigations to define corresponding morphological abnormalities in intestinal samples from patients with CIIP which are described and discussed in the following chapters.

2.2 Materials and Methods

2.2.1 Patients Investigated by Electrophysiological Techniques

Electrophysiological investigation was performed in 26 subjects (14 female and 12 male) with clinical evidence of CIIP. Antroduodenal manometry alone was performed in 10 patients, electrogastrography alone in two, and both investigations in 14. The ages at the time of manometry ranged between 9 days and 48 years (median 9 months). These investigations were performed and analysed by Drs. Milla, Bisset, Devane and Ravelli.

2.2.2 Methods

2.2.2.1 Small Bowel Motility Studies

Motor function of the small intestine after an overnight fast (Fenton et al. 1983), was assessed manometrically in 24 patients using phase III activity to probe the integrity of the enteric nervous system and intestinal smooth muscle. The patients were sedated (chlorpromazine 2mg/kg intramuscularly) and a triple-lumen catheter with portholes 5 cm apart was positioned under fluoroscopic control so that the distal port lay in the proximal jejunum and the proximal two ports in the duodenum. The tube was perfused with 0.9% saline at a rate of 0.2 ml/min using a pneumohydraulic constant pressure perfusion pump (Arndorfer Medical Specialities, Winsconsin, USA). The intralumenal pressures were measured by three transducers (Series 3, Luerlock, Gaeltec, UK), the outputs of which were displayed on an oscillographic chart recorder (Washington MDU-4). Activity was recorded for a minimum of three cycles of migrating motor complexes (MMCs) where these were present, or for a minimum period of 4 hours if the MMCs were absent.

2.2.2.2 Surface Electrogastrography (EGG)

Fasting gastric electrical control activity was recorded and analysed in 14 patients using a modification of the method described by Van der Schee and Grashuis (1987). The potential differences across three pairs of silver/silver chloride electrodes, matched for impedance and placed on the skin over the epigastrium with ECG gel, were measured for a 1-hour period following an overnight fast (or a fast of at least 4 hours in the smallest infants). Skin impedance was reduced to less than 5 kOhms by light skin abrasion. The signals picked up by the electrodes were amplified and filtered using band-pass filters with cut-offs of 0.01 Hz and 0.25 Hz for the high and

low-pass filters, respectively, to remove frequencies originating from cardiac electrical activity. The signals were captured, digitalised and stored using an IBM-compatible microcomputer, as well as being displayed on a conventional Gould 2800 S/8 chart recorder for later off-line analysis. A running spectral analysis of the real-time signal captured on disk was performed. Modular computerised algorithms for data capture, digital conditioning of the captured signal, division into subsections, and frequency analysis (PC-Dats, Prosig Computer Consultants) were used. These modules were ordered and invoked by a Fortran code routine (RM-Fortran, Ryan McFarland). Each 1-hour recording was subdivided into 53 overlapping segments of 256 seconds (75% overlap); each segment was digitally filtered (band-pass Butterworth filter, 0.015-0.25 HZ, slope 24 Db/Octave) and the frequencies present were determined by Fast Fourier Transformation and autoregressive modelling. The peak frequency present in each segment was extracted and the dominant frequency of the 1-hour recording (that present in the greatest number of segments) was obtained.

2.3 Results

Electrophysiological abnormalities by antroduodenal manometry, EGG or both techniques were seen in 23 of the 26 patients studied. These were indicative of neuropathy in 11, myopathy in 11 and combined neuropathy/myopathy in one (see Table 1).

Table 1. Electrophysiological investigations

Abnormality detailed by:	Trace Indicative of: Neuropathy	Myopathy	Neuro/myo-pathy	Normal
Both ADM & EGG	4	6	0	0
ADM but not by EGG	1	1	0	0
ADM (EGG not performed)	6	3	1	0
EGG (ADM not performed	0	1	0	1
Neither by ADM nor EGG	0	0	0	2
Total	11	11	1	3

ADM antroduodenal manometry
EGG electrogastrography

Of the 11 'neuropathic' patients, antroduodenal manometry alone was performed in six, and both manometry and EGG in five. The recordings of fasting small bowel motor activity by antroduodenal manometry showed irregular, bizarre MMCs without cyclical propagating activity in all 11 patients. In five neuropathic patients also investigated by EGG, persistent tachygastria (7-9 cpm) of the gastric antrum was seen in four, but in the fifth the recording was entirely normal with a dominant frequency of 3 cpm.

Of the 11 'myopathic' patients, antroduodenal manometry was performed in 10 and

EGG in seven; one patient was investigated by EGG alone. Antroduodenal manometry showed a grossly reduced amplitude of contractions in all channels of the recording in all 10 patients. The one patient examined by EGG alone showed a chaotic pattern with no dominant frequency. Of the seven patients examined by antroduodenal manometry and EGG, both techniques showed changes of myopathic disease in six, but in one EGG was normal despite myopathic manometry.

In one other patient the recording of small bowel fasting motor activity showed small amplitude contractions as well as irregular, non-propagating MMCs, thus indicating abnormalities in both the smooth muscle as well as the neural elements.

In three patients no electrophysiological abnormality could be detected by small bowel manometry and/or EGG.

2.4 Clinical Presentation

All the 26 patients studied had symptoms of intestinal obstruction with vomiting, an inability to tolerate feeds, abdominal pain and distension with alternating constipation and diarrhoea. A diagnosis of HD had been excluded in all cases by histopathological examination of rectal suction biopsy samples. The severity of the symptoms varied; in some patients vomiting or abdominal pain and distension predominated, while in others constipation was the major problem. No obvious differences in these general symptoms were noted between patients with neuropathic or myopathic disease.

Of these 26 patients 10 presented soon after birth, 11 before the age of 2 years and five between 6 and 35 years. The median age at presentation was 4 weeks. A history

of constipation, vomiting and abdominal pain since childhood, although not severe enough to require investigation, was given by one patient aged 35 years at the time of presentation. A sudden onset of obstructive symptoms was observed in two patients (aged 10 and 17 years).

In some of the patients associated abnormalities were seen and their frequencies varied to some extent between those with primary myopathy compared to those with neuropathic disease (see Table 2).

Urinary tract abnormalities, ranging from recurrent urinary tract infections associated with mild dilatation of ureters or bladder to gross hydronephrosis, were seen in 14 patients. In three hydronephrosis was discovered by antenatal ultrasonography and in five others the initial presentation was with symptoms referrable to the urinary tract. Intestinal electrophysiology was myopathic in 11 patients, neuropathic in one whilst in one other patient the electrophysiological recording (trace) was normal. The patient with electrophysiological evidence of combined myopathic/neuropathic abnormalities also had a mildly dilated bladder. One patient with normal electrophysiology complained of urinary tract dysfunction, which had not been investigated clinically.

Table 2. Clinical details

Associated Abnormality:	Electrophysiology: Myopathy (n=11)	Neuropathy (n=11)	Neuro/Myopathy (n=1)	Normal (n=3)
Urinary tract abnormality (n=14)	11	1	1	1
Pyloric Stenosis (n=4)	0	4	0	0
Malrotation (n=11)	4	5	0	2
Family History (n=5)	1	4	0	0
Cardiac Anomaly (n=3)	2	1	0	0

Pyloric stenosis was seen in four patients, all with a neuropathic trace. Malrotation of the mid gut was present in 11 patients, one of whom also had gastroschisis. Electrophysiological studies showed neuropathic changes in five of these patients, myopathic changes in four and a normal trace in two. A short small intestine was noted in three patients, all of whom had a neuropathic trace.

A family history of an intestinal motor abnormality was present in five patients (four patients with a neuropathic and one a myopathic trace). These included a newborn

baby boy (neuropathic) who died in infancy, whose deceased maternal uncle had been similarly affected. There was also an affected stepbrother with a different father. All the three babies had short small intestine, malrotation and pyloric stenosis. The other four comprised an 8-month-old boy (neuropathic) with a family history of death in infancy and similar symptomatology affecting several other male family members; a newborn boy (myopathic) with an affected sister; and twin girls (both neuropathic) whose symptoms matched their now deceased elder brother.

Three patients had cardiac anomalies (a ventriculoseptal defect in one and patent ductus arteriosus in two). One of the patients with patent ductus arteriosus had a neuropathic trace and the other two had myopathic traces.

2.5 Clinical Outcome

The initial management of all 26 patients required a laparotomy and surgical decompression. Ileostomy was necessary in 24, either as the initial manoeuvre or after a trial colostomy. Colostomies (one an ascending and the other a transverse colostomy) were adequate to manage the obstruction in two patients. Total parental nutrition (TPN) was necessary either transiently in 19 patients or long-term in seven patients, six of whom continue on long-term home TPN. Three of these patients had myopathic traces and three neuropathic traces. A summary of the clinical outcomes is shown in Table 3.

Six of the 26 patients died between 15 days and 19 months of age. One of these had neuropathic, and the other five myopathic manometry. Four patients (two neuropathic, one myopathic and one normal manometry) are managed with an ileostomy and

tolerate oral feeds; two patients with a neuropathic trace are well after ileo-anal or ileo-rectal anastomoses and six patients are on oral intake without enterostomies. One (myopathic) patient in the last group appears well without gastroenterological medication, but suffers from a possible limb girdle myopathy. Another (neuropathic) patient was lost to follow-up, and a third (myopathic) patient is subject to subacute obstruction; her oral intake is limited to semi-solids. The patient with electrophysiological evidence of combined neuropathy and myopathy is well on a normal oral intake but dependent on the prokinetic drug, cisapride. Of the three patients with normal manometry, one still complains of constipation and urinary tract dysfunction but is on oral food intake. The second patient has a troublesome 'neurogenic bladder' but constipation is managed with antegrade continence enemas (ACE; Malone et al. 1990). In this procedure the appendix is brought to the surface to form a fistula through which the large bowel can be irrigated to liquify the solid colonic contents. The third patient has an ileostomy but is thriving on oral feeds.

One patient with myopathic electrophysiology has suffered pulmonary emboli, possibly related to long-term parental nutrition. Another patient with a myopathic trace had a cerebrovascular accident (probably a cerebral infarction). Both of these patients had been noted to have puffy hands and feet throughout the course of their intestinal disease. The twin sisters with neuropathic traces now show signs of spastic paraplegia. Their now deceased elder brother with a similar intestinal motor disorder also had spastic paraplegia. One further patient with neuropathic manometry also suffers from spastic paraplegia.

Table 3. Clinical outcome

	Electrophysiology			
	Myopathy (n=11)	Neuropathy (n=11)	Neuro/Myopathy (n=1)	Normal (n=3)
Death	5	1	0	0
TPN	3	3	0	0
Oral food/ ileostomy	1	2	0	1
Oral food/ colostomy	1	0	0	0
Oral food/ resection/ anastomosis	0	2	0	0
Oral food +/- prokinetics	1	3	1	1
Oral/ACE- irrigation	0	0	0	1

2.6 Discussion

The cyclical nature of smooth muscle contraction in the fasting upper gastrointestinal tract was already noted in the last century (Beaumont 1833; Schwartzenburg 1849; Busch 1858; Bayliss and Starling 1899). Gastric contractions at a frequency of 3 cycles per minute was documented by Morat in 1893 using small balloon to record pressures in dogs, rabbits and in one patient. The first definitive experimental demonstration of fasting migrating motor complexes in dogs can be credited to Boldyreff (1902,1911). With the development of manometric techniques the current concept of intestinal motor patterns emerged both in the experimental animal (Ruckebusch and Laplace 1967; Szurszewski 1969) and then in the human (Stanciu

and Bennett 1975).

Various in vitro and in vivo experiments have clarified the different motor patterns seen on electrophysiology (Weisbrodt 1981; Wood 1981, 1990). Smooth muscle cells are inherently contractile, and polarisation/depolarisation of the myocyte membrane has been shown to correspond to the electrical control activity (ECA) or slow wave activity seen by in vivo and in vitro electrophysiological methods (Furukawa et al. 1986; Bywater and Taylor 1986; Bywater et al.1987,1989;Taylor and Bywater 1988). The nerve blocking agent tetrodotoxin suppresses the inhibitory control and allows the smooth muscle cells to contract at the maximal frequency of the ECA (Rinecker 1969; Daniel and Sarna 1978). The spike potentials seen in MMCs can be shown by microelectrode stimulation of the myenteric neurons to be the result of neural control facilitating the opening of ion channels in the myocyte membrane; they are enhanced by cholinergic agents and inhibited by adrenergic amines (Wood 1972; Weisbrodt 1981,1987; Furukawa et al. 1986; Bywater and Taylor 1986; Bywater et al. 1987, 1989).

Small bowel manometry is a minimally invasive investigation and the information gained from it is valuable in the analysis of small bowel motor activity. Investigation of patients with intestinal pseudo-obstruction by this technique provides useful information about the nature of the abnormality and the extent of the intestinal involvement (Milla 1983; Summers et al. 1983; Wozniak et al. 1984; Stanguellini et al. 1987; Hyman et al. 1988; Anuras 1990). The less invasive technique of EGG is a useful screening procedure and also provides data in intestinal pseudo-obstruction (Devane et al. 1992), although abnormalities which are present distally in the intestine

may or may not be reflected in the stomach. The fact that in this series of 26 patients, only two who had abnormalities by antroduodenal manometry had normal EGG traces, gives some indication of the frequency that this occurs.

Abnormal electrophysiological data was seen in 23 of the 26 patients studied with clinical evidence of CIIP. Half of these patients had a neuropathic trace and the other half a myopathic trace. Certain generalisations in the clinical presentation could be observed in the light of the electrophysiological data. Most, but not all, of the patients with a myopathic trace, but only one of those with a neuropathic trace, had additional abnormalities in the urinary tract. The two patients with clinical evidence of vascular complications (multiple pulmonary emboli, cerebral infarction and oedema of the extremities), both of whom also had urinary tract abnormalities, had myopathic traces. These findings indicate that the smooth muscle abnormalities in these patients may be more generalised and not confined to the intestinal smooth muscle.

Malrotation of the small intestine was common, occurring in about half the patients, including those with neuropathy as well as those with myopathy, but pyloric stenosis and a short small intestine were seen only in patients with neuropathic disease.

Of the 26 patients studied a family history was seen in only five, three of whom had a neuropathic disease process and two had myopathic disease. The clinical features seen in other family members were invariably very similar to those seen in the propositi.

The main purpose of this thesis is to identify structural alterations in the intestine that

are the morphological counterpart of the functional changes identified electrophysiologically. Ideally, structural abnormalities in patients with a neuropathic trace would occur in the enteric innervation, whilst those with myopathic changes would have corresponding morphological abnormalities of smooth muscle.

However, as shown in Table 4, this simple correlation was not possible. In this table the preliminary histological and ultrastructural changes encountered in surgical biopsy material from the patients submitted to electrophysiological studies are compared with the results of the latter investigations. These biopsies were from various regions of the intestine and included full thickness rectal biopsies as well as resected segments of intestine obtained when the clinical management of individual patients required surgical intervention to relieve obstructive symptoms.

It can be seen from Table 4 that morphological abnormalities, usually only at the ultrastructural level, were seen in smooth muscle in nine of the 11 patients with myopathic traces. However, such changes were also seen in two of the neuropathic patients, in one of the patients with a normal trace and in the patient with combined neuropathy and myopathy. Smooth muscle changes are considered in detail in chapter 6; they are often subtle and are frequently identified only on electron microscopy. In addition, artifacts introduced by ischaemia and handling during surgery, as well as those inherent in the use of relatively large specimens for electron microscopy when fixation is not optimal, make interpretation of these changes difficult in terms of identifying those that are truly pathological. Indeed, the main objective of Chapter 6 is an attempt to discern which of the smooth muscle abnormalities crudely lumped together in Table 4 can be defined as meaningful in this respect.

Table 4. Correlation of electrophysiology and morphology

	Electrophysiology			
	Myopathy (n=11)	Neuropathy (n=11)	Neuro/Myopathy (n=1)	Normal (n=3)
Nerve abnormality on routine LM or EM	1	3	0	1
Smooth muscle abnormality on LM or EM	7	1	1	1
Changes in both nerves and muscle	2	1	0	0
Normal morphology	1	7	0	1

It is also to be noted that three of the myopathic patients had neural abnormalities detected histologically, and one myopathic patient had apparently normal morphology. The neural changes consisted of an increase in AChE-positive fibres in the lamina propria or muscularis propria; in two of the three patients smooth muscle abnormalities were also present. One of the problems in our present state of understanding is to know to what extent secondary structural changes might occur in either smooth muscle or the enteric innervation when there is a primary defect in the opposite compartment. It would certainly not be surprising for this to occur in view of the intimate functional relationships between them.

Correlation between the electrophysiological and morphological findings were even less striking in the neuropathic patients. Structural abnormalities of the enteric innervation were seen in only three of these 11 patients as well as in one patient with a normal trace. In addition, smooth muscle abnormalities were seen in two neuropathic patients whilst seven had apparently normal morphology. The conclusions from these preliminary findings are that functional abnormalities of enteric innervation often have no obvious morphological counterpart recognisable on routine light and electron microscopical techniques, or that in some of the patients examined they were absent from the particular segment of intestine examined. In either event, the apparently non-specific nature of the neural abnormalities is exemplified by the fact that they were equally prevalent in patients with myopathic electrophysiology.

Nevertheless, as detailed in Chapters 3,4 and 5, some apparently more specific neural abnormalities can be defined in certain varieties of CIIP, although they are probably overdiagnosed.

Thus the preliminary information gathered here indicates that there is no simple relationship between the functional and structural abnormalities in CIIP. However, in the following chapters, which use the tissue samples from the patients described here as well as samples of intestine from other patients with CIIP not subjected to manometric investigation, the morphological investigations are expanded in much more detail. Not only the inadequacies and inconsistencies apparent in structural analysis, but also the positive contributions that conventional histology, silver staining, enzyme- and immunohistochemistry and electron microscopy can make to the study of pseudo-obstruction are clarified.

Chapter 3. Intestinal Neuronal Dysplasia

3.1 Introduction

Intestinal neuronal dysplasia (IND), an abnormality of intestinal innervation associated with pseudo-obstructive dysmotility, was first described in 1971 by Meier-Ruge. He identified morphological abnormalities best demonstrated histochemically, which included hyperplasia of both the intermuscular and submucosal plexuses and an increase in acetyl cholinesterase (AChE)- positive fibres in the lamina propria and circular muscle. Hyperplasia of the plexuses was characterised by frequent, large ganglia in association with thickened nerves. He also described the presence of heterotopic neurons in the lamina propria and occasionally within intestinal muscle layers.

Two types of IND have been described (Scharli and Meier-Ruge 1981, 1986; Fadda et al. 1983; Meier-Ruge 1985, 1992). Type B is reported the commoner form, while type A is rare and is described in only 2.2% of all dysganglionoses (Meier-Ruge 1992). In two patients with clinical and histological suspicion of IND type A (bloody diarrhoea, mucosal inflammation and increased AChE-positive nerve fibres in the lamina propria) seen at the Hospital for Sick Children, Great Ormond Street (GOS), no aplasia or hypoplasia of the sympathetic innervation described to be diagnostic of the disorder (Fadda et al. 1983; Meier-Ruge 1992) could be proven. Moreover, one of these patients was subsequently at the age of 12 years shown to have Crohn's disease. Since there is uncertainty about the existence of IND type A and proven cases are not available for study, this disorder is not further considered in this chapter.

IND is reported to occur as an isolated abnormality or in association with Hirschsprung's disease (HD), involving a variable length of bowel proximal to the aganglionic segment. At GOS, IND has been diagnosed very infrequently and only in association with HD. In these patients the diagnosis was based on clear-cut morphological abnormalities enumerated below. Others (Meier-Ruge 1971,1985,1992; Scharli and Meier-Ruge 1981,1986; Fadda et al. 1983; Munakata et al. 1985; Borchard et al. 1991; Scharli 1992) have used a variety of more subtle criteria, not all of which are necessarily present in every case. These authors have varied in their diagnostic requirements and in the emphasis they have placed on particular features. It is in these series IND has been diagnosed as a separate entity as well as a concomitant with HD. However, the less distinct criteria employed, which might be difficult to confirm objectively, have not been evaluated against age-matched control material. In addition, any correlation of the changes identified with the clinical features or the eventual outcome are often impossible to deduce in the necessary detail from the published papers (Meier-Ruge 1971,1985,1992; Scharli and Meier-Ruge 1981,1986; Fadda et al. 1983; Munakata et al. 1985; Borchard et al. 1991; Scharli 1992). Thus confirmation that some of the appearances taken to indicate IND are true pathological abnormalities has been difficult or impossible to ascertain.

It is apparent that the widely varying frequency with which IND is reported in different series of patients whose criteria for inclusion are unclear reflects differences in interpretation of these changes. For example, at GOS only seven cases (0.3%) have been recognised amongst 2420 patients biopsied between 1975 and 1991, whilst Scharli (1992) reported 54 cases (62%) in a series of 115 patients; other authors (Scharli and Meier-Ruge 1981, 1986; Fadda et al. 1983; Meier-Ruge 1985) have

quoted incidences of 18-30%, which is similar to that of HD.

3.1.1 Full Thickness compared with Mucosal Biopsy Specimens

To date, a full thickness biopsy of the bowel wall has been regarded at GOS to be necessary for the confirmation of IND, since diagnostic features involve the mucosa, submucosa, myenteric plexus and the circular muscle coat. Furthermore, important criteria such as the increase in AChE-positive nerve fibres in the lamina propria can occur in other conditions (an increase can be seen, for example, in chronic inflammatory bowel disease; Lake 1990) and at GOS it has been felt necessary to evaluate all the changes before making a definitive diagnosis.

In most centres, however, the histological and histochemical investigation of gut motility disturbances is based first on tissue biopsies of the rectal mucosa and submucosa, and often on rectal suction biopsy specimens. There has been a tendency to diagnose IND in such small samples, and this has been a further factor in producing the markedly different incidence figures.

3.1.2 Investigations Covered in This Chapter

It is intended firstly to specify the diagnostic criteria used previously at GOS in diagnosing IND on full thickness specimens, and then to enumerate the criteria proposed by a working party in 1991 (Borchard et al.) to be applied to rectal suction biopsy specimens. This is followed by the results of a study made on rectal suction biopsy samples to see how far these latter criteria can be applied.

In addition there is a separate study to evaluate the density of AChE-positive nerve

fibres in the circular muscle coat as an aid to diagnosing IND, since it has been suggested that this may be relevant in adult patients (Meier-Ruge 1992).

In the following chapter the value of neuronal density counts in the myenteric plexus in the diagnosis of both IND and hypoganglionosis is considered.

3.1.3 Hospital for Sick Children, Great Ormond Street, Diagnostic Criteria for IND

(a) At GOS special emphasis has been placed on the increase in AChE-positive nerve fibres in the lamina propria (Fig.1 and 2a). These fibres run vertically from the bottom to the top of the lamina propria and are finer than the coarse fibres, which often run in both the horizontal and vertical planes, seen in HD (Fig.2b). No increase in AChE-positive fibres is seen in the muscularis mucosae in IND (Fig.1) in contrast to HD where a gross and diffuse increase in thick, knotted fibres in the muscularis mucosae is virtually diagnostic of the disease (Fig.2b).

(b) In the majority of cases considered to have IND several heterotopic neurons were noted in the lamina propria in addition to the increased AChE-positive fibres (Fig.3). Whilst an occasional neuron in the lamina propria may be seen it is not, on its own, diagnostic of IND.

(c) Hyperplasia of the submucosal and myenteric plexuses has been regarded an important diagnostic feature and it incorporates firstly an increased frequency of ganglion cell clusters and sometimes the presence of large ganglia defined by Scharli (1992) as containing in excess of seven ganglion cells (Fig.4). The frequent ganglion

cell clusters have been described as having a 'button-like' appearance (Fadda et al. 1983; Meier-Ruge 1985; Borchard et al. 1991) and are associated with thickened nerve processes (Fig.5).

An increase in AChE-positive nerve fibres around any of the submucosal blood vessels, whilst noted in some patients, has not been included as a significant discriminator for the diagnosis of IND in the material examined at GOS (see obligatory criteria by Borchard et al. 1991 below).

3.1.4 Diagnostic Criteria for Suction Rectal Biopsies (Borchard et al. 1991)

Two obligatory criteria, which must be present when making the diagnosis of IND were reported. These were: (a) hyperplasia of the submucosal plexus, and (b) increased AChE-positive nerve fibres around submucosal arteries. Hyperplasia was identified when submucosal ganglia were frequent and large containing in excess of seven neurons. Two other features, although not essential for the diagnosis were, (c) heterotopic ganglion cells in the lamina propria (neuronal heterotopia), and (d) increased AChE-positive nerve fibres in the lamina propria.

3.1.5 Comment on Diagnostic Criteria

Since 1975 only seven patients with IND, all of whom had concomitant HD with IND affecting bowel proximal to the aganglionic segment have been identified at GOS. IND was diagnosed after symptoms of disturbed intestinal motility continued following a corrective pull-through operation for aganglionosis. Increases in AChE-positive nerve fibres in the lamina propria is said not to be present in older children with IND (Borchard et al. 1991). However, biopsies from each of the patients

considered to have IND exhibited an unquestionable increase in these fibres in biopsies taken between 2 and 10 years of age. Although some increase in AChE-positive nerve fibres had been noted in a small number of suction rectal biopsies from other patients, the degree of this abnormality was insufficiently convincing to allow a diagnosis of IND in the absence of other diagnostic features. However, the absence of patients with isolated IND in the GOS series, and the overall much lower frequency of this diagnosis at GOS compared with other reports is striking. Nevertheless, the conservative approach adopted at GOS to the diagnosis of IND is shared by at least some other authors.

Schofield and Yunis (1991) investigated 456 children in whom they identified 38 (8.3%) with IND by the criteria of increased AChE-positive nerve fibres in the lamina propria and more than five ganglia per high power field in the submucosa. All but one of these children improved clinically during follow-up and they concluded that the changes they identified as indicating IND represented a histological pattern of little clinical relevance.

Whilst few would doubt the existence of a disorder with some of the diagnostic features described in IND as a cause of intestinal pseudo-obstruction in children, it is clear that the diagnostic criteria, particularly when applied to rectal suction biopsy specimens, differ in different centres and their significance in terms of clinical outcome is not well founded. The results of Schofield and Yunis (1991) conflict with much of the other data reported in the literature (Scharli and Meier-Ruge 1981,1986; Fadda et al. 1983; Meier-Ruge 1985) and for this reason a similar study of the material received at GOS was embarked on.

3.2 Study of Suction Rectal Biopsy Specimens

This investigation was planned in two parts. The first, discussed here, has involved the reassessment of a series of suction rectal biopsies taken from patients admitted to GOS, during the investigation of apparent intestinal motility disturbances, applying with minor modifications, the diagnostic criteria used by Borchard et al (1991). The second part will comprise a clinical follow-up of these patients and is not covered in this thesis.

3.2.1 Materials and Methods

Of a consecutive series of 548 infants and young children submitted to suction rectal biopsy during the investigation of suspected intestinal dysmotility during 1989-1991 at GOS, 137 (25%) were diagnosed as HD. Of the remaining biopsies from 411 patients, 326 (59%) were excluded from the study because the amount of submucosa included in the biopsy, whilst adequate for confirming or excluding HD, was insufficient for assessing changes that might be indicative of IND. Thus biopsies from 85 children aged 2 days to 10 years (median 11 weeks) remained. Of these children 16 (20%) were aged 1 week or less. All of these specimens had been regarded as 'within normal limits' when originally reported but were reassessed for the present study noting the following criteria:

(a). Any apparent increase in AChE-activity in the lamina propria, including minor and focal increases (Fig.6).

(b). Neuronal heterotopia.

(c). Hyperplasia of the submucosal plexus in terms of the frequency and size of the ganglia and the association of ganglia with thickened nerves (budding). Ganglia were classified as 'infrequent' when examination of several sections was necessary to identify a single submucosal neuron or cluster. If ganglia were readily found without an excessive search in most sections their frequency was termed 'average'. Ganglia were classified as 'frequent' when all the sections examined showed several neurons or clusters (Figs.7 and 8) in every field examined with a x10 objective. The sizes of the ganglia were assessed by counting neurons in a number of ganglia and the term 'giant' ganglion was applied if more than seven neurons were identified in a cluster.

(d). Increased number of AChE-positive nerve fibres around submucosal blood vessels (Fig.9).

Figure 1. IND (GOS criteria). Section of mucosa and submucosa showing marked increase in fine, vertical AChE-positive nerve fibres in the lamina propria. Note no increase in these fibres is seen in the muscularis mucosae. Magnification x160

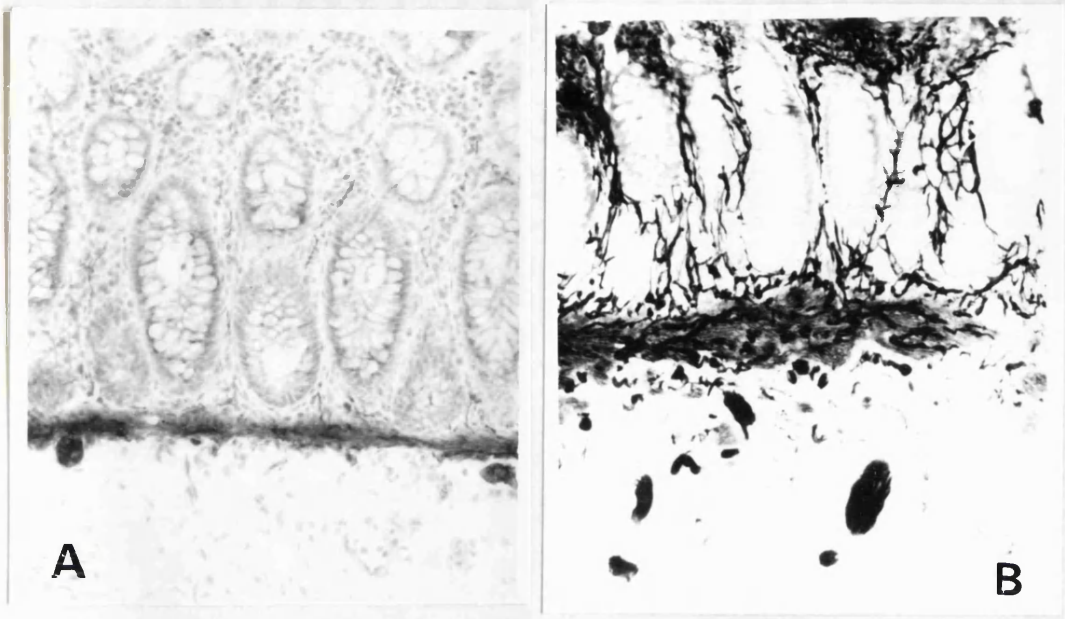


Figure 2. (A) Normal AChE-activity. (B) HD. Prominant increase in thick knotted AChE-positive nerves is seen in the muscularis mucosae together with an increase in coarse nerves which run both in vertical and horizontal planes in the lamina propria (compare with Fig.1). Magnification x120

Figure 3. IND (GOS criteria). Three heterotopic neurons in the lamina propria in association with an increase in AChE-positive nerve fibres. Magnification x120

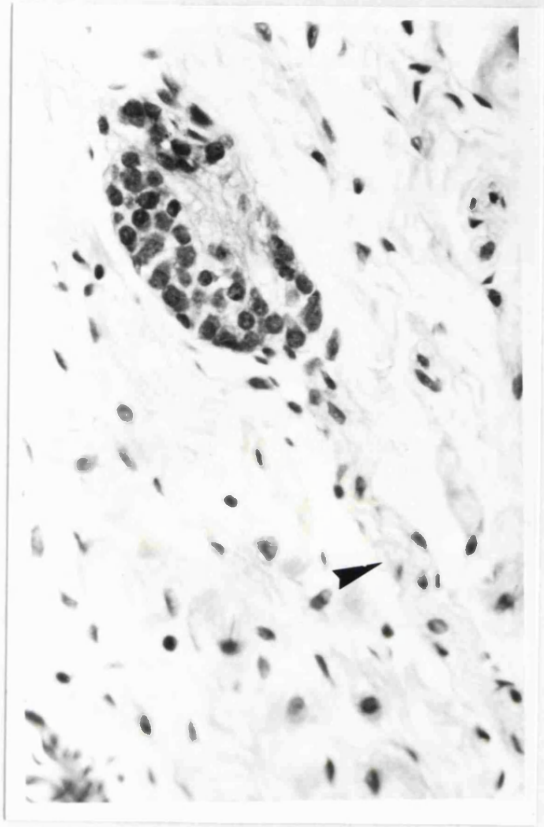


Figure 4. A 'giant' submucosal ganglion associated with a thickened nerve (arrowhead) from a patient with IND (GOS criteria). H&E. Magnification x480

Figure 5.'Button-like'ganglion in the submucosa from a patient with IND (GOS criteria). Note increased AChE-positive nerves in the lamina propria but not in the muscularis mucosae. Magnification x120

Figure 6. Focal increase in AChE-positive nerves in the lamina propria. Magnification x120

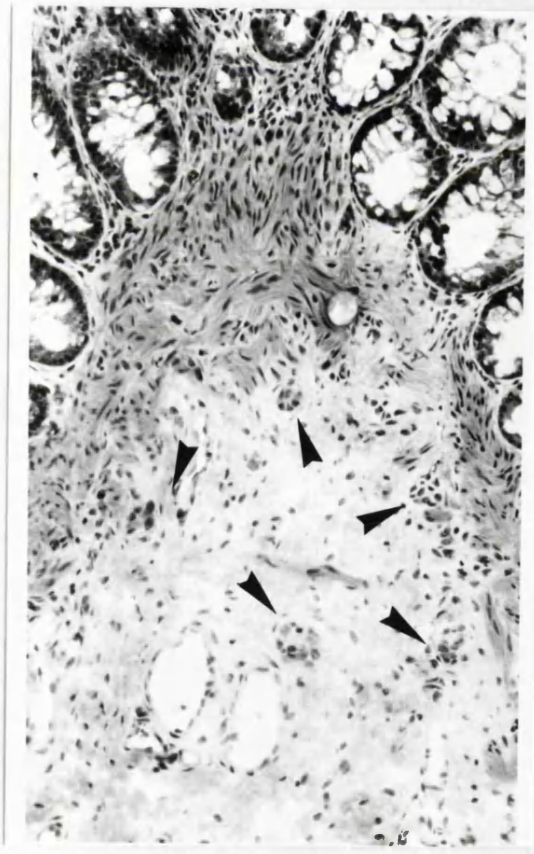


Figure 7. H&E stained section of rectal suction biopsy from a neonate showing frequent submucosal ganglia (arrowheads). Magnification x160

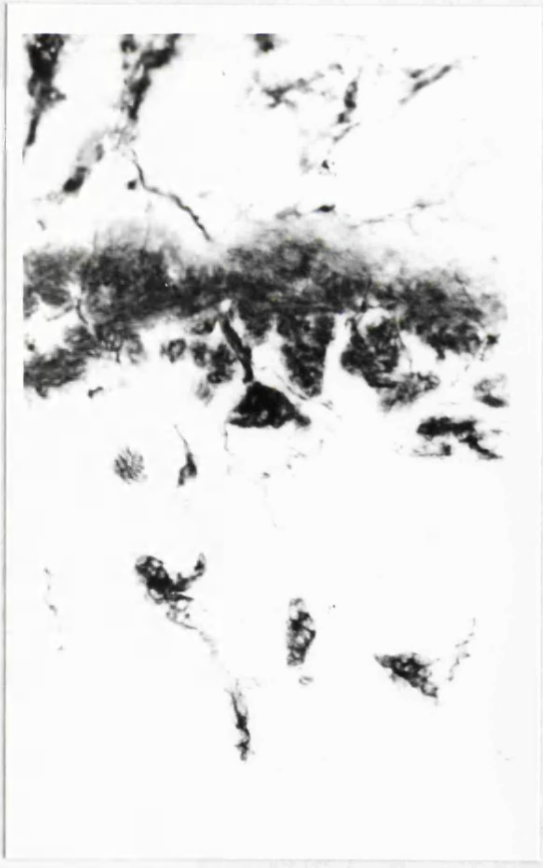


Figure 8. AChE-activity in a suction rectal biopsy from a neonate showing frequent submucosal ganglia in 'button-like' association with nerves. Note also a mild increase in lamina proprial AChE-positive fibres. Magnification x200

Figure 9. AChE-positive nerves around submucosal blood vessels appear more pronounced than in most samples and thus considered to be increased. Magnification x160

3.2.2 Results

In none of the biopsies were changes seen sufficient to diagnose IND by the GOS criteria, and in particular, the marked diffuse increase in AChE-positive fibres in the lamina propria, which were previously regarded as a key factor, was lacking. In this study an increase was recorded when it was of much lesser degree and often focal in distribution. By the reduced diagnostic thresholds described above, 60 patients (71%) were identified in whose biopsies some features of IND were detected.

In biopsies from 15 patients a mild and often focal increase in AChE-positive nerve fibres in the lamina propria was noted.

Neuronal heterotopia was not a feature in the biopsies examined, occurring in only one specimen in which it was not accompanied by any increase in AChE-positive nerve fibres in the lamina propria.

Ganglia in the submucosa were frequent in biopsies from 38 patients (45%), average in 31 (36%) and infrequent in 16 (19%); giant ganglia were present in only four patients (5%). As shown in Fig.10 a relationship existed between the frequency of submucosal ganglia and the patient's age at biopsy. Frequent ganglia occurred in 22 (73%) of the 30 patients biopsied under the age of 4 weeks, but in only 15 (29%) of the 52 patients biopsied after the age of 4 weeks. On the other hand, all 15 patients with infrequent ganglia were biopsied after 4 weeks of age. Comparing younger (aged less than 4 weeks) with older children (aged more than 4 weeks) significant differences between those with 'frequent', 'average' and 'infrequent' ganglia were established (chi sq = 18.28; $p < 0.01$).

In most biopsies only occasional isolated AChE-positive nerve fibres around submucosal arteries or veins were seen, but biopsies from 20 patients showed several prominent positive fibres.

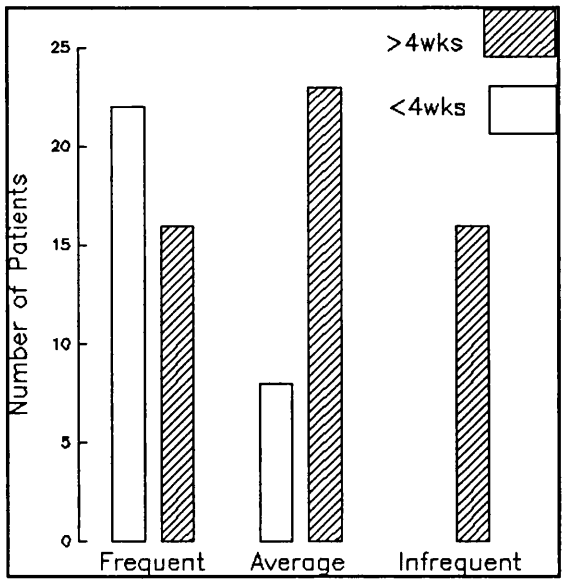


Figure 10. Relationship between frequency of submucosal ganglia (frequent, average or infrequent) and age (more or less than 4 weeks) at biopsy.

Frequent ganglia (38)	AChE around SM vessels (20)	AChE in LP (15)	Giant ganglia (4)	Number of patients
+	+	+	-	6
+	+	-	-	3
+	-	+	+	2
+	-	-	-	28
-	-	+	+	1
-	-	-	+	1
-	+	-	-	11
-	-	+	-	8
			Total	60 (71%)

Figure 11. One or more of the various histological features reported in IND (Borchard et al.1991) were encountered in 60 (71%) of the 85 patients studied. In only nine (11%) were both obligatory criteria for IND of Borchard et al. met (see box).

All four criteria were not seen in any one patient (Fig.11) but frequent ganglia and increased nerve fibres around the submucosal blood vessels coexisted in 9 patients, six of whom also had some increases in lamina proprial nerve fibres. It is of interest that of these nine patients with biopsies showing both obligatory criteria for the diagnosis of IND required by Borchard et al (1991) eight were aged more than 4 weeks, and five were aged more than 2 years at biopsy. An increase in AChE-positive nerve fibres around submucosal blood vessels were also seen in biopsies with average frequency of ganglia (9 patients) three of whom also showed an increase in positive nerve fibres in the lamina propria. Prominent nerve fibres were also seen around submucosal blood vessels in two patients with infrequent ganglia. Giant ganglia coexisted with increased fibres around submucosal blood vessels in two patients.

3.2.3 Discussion and Conclusions

This study confirms that at least some of the histological criteria other authors have used to diagnose IND can be identified in suction rectal biopsy specimens previously at GOS regarded as 'normal' from children with suspected pseudo-obstruction. One or more of these changes were seen in 60 (71%) of our 85 patients. However, the two obligatory criteria of Borchard et al (1991) were satisfied in only 9 (11%). In addition, neurons in the lamina propria (neuronal heterotopia), a feature which, together with an obvious increase in vertical AChE-positive fibres in the lamina propria, we have hitherto regarded as important for the diagnosis of IND, were absent from all the biopsies studied.

In the previous experience at GOS, IND has been diagnosed in only seven patients,

all whom had concomitant HD. Whilst these patients had undoubted persistent intestinal motor dysfunction even after surgical resection of the aganglionic segment, the histochemical abnormalities identified as IND were considerably more marked than those accepted by Borchard et al (1991) and in particular they had a marked diffuse increase in AChE-positive fibres in the lamina propria. The association between IND and aganglionosis is well recognised (Briner et al. 1986; Fadda et al. 1987) and IND has also been identified as an isolated entity independent of HD (Meier-Ruge 1971,1985; Scharli and Meier-Ruge 1981,1986; Fadda et al. 1983; Borchard et al. 1991; Scharli 1992). Whilst in many centres the reported incidence of IND is much higher than at GOS, rising to as high as 80% in association with aganglionosis (Briner et al. 1986) it is agreed that most patients improve with conservative management (Scharli and Meier-Ruge 1981; Fadda et al. 1983,1987; Meier-Ruge 1985; Munakata et al. 1985; Rintala et al. 1989; Pistor 1989; Schofield and Yunis 1991; Sacher et al. 1991; Simpser et al. 1991; Scharli 1992) and that this improvement may be accompanied by reversion of the histochemical changes to normality (Munakata et al. 1985; Simpser et al. 1991).

The question therefore arises whether the changes identified as IND really depict a clinico-pathological entity or whether they merely form part of the spectrum of normality. This problem is complicated by the complete lack, for obvious ethical reasons, of good, age-matched control biopsy material from children with normal intestinal function. It is of great interest, however, with regard to one important criterion for IND, namely hyperplasia of the submucosal plexus, that in this series 'frequent' ganglia were seen significantly more often in younger children aged less than 4 weeks at biopsy and so could merely represent an age-related phenomenon.

It is true, nevertheless, that our previous dismissal of minor differences in the histochemical pattern observed in rectal suction biopsies may relate to the tertiary referral practice at our hospital. Detailed follow-up of our patients, except those with established HD, tends to be performed at the referring hospitals which might blunt our awareness of residual problems of intestinal motility in those patients in whom aganglionosis was excluded. It is for this reason that we are conducting a careful follow-up of all the patients described here to establish any clinical significance of these findings.

3.3 Study on Increased AChE-positive Nerves in the Circular Muscle in IND

The increase in AChE-positive nerves in the circular muscle coat noted by Meier-Ruge (1971) could be relevant as an increase of such nerves in the circular muscle is claimed to be an important and sometimes the only feature of IND in adults (Meier-Ruge 1992). Neural density in the circular muscle was investigated in colonic samples of the patients in whom the diagnosis of IND was made together with control samples and intestine from patients with HD.

3.3.1 Materials and Methods

AChE-positive nerve density was analysed in 14 snap-frozen, full thickness specimens of colon. These samples comprised control specimens obtained at necropsy from six patients (aged 8 weeks- 1 year; median 3 months) who died of non-gastroenterological diseases (sudden infant death syndrome, Pena Shokeir syndrome, encephalopathic disease and 3 with congenital heart disease), and eight 'test' specimens. The latter were proximal resection margins of two pull-through specimens from patients (aged 6 and 7 months) with HD and six colonic resections from patients (aged 6 months -

12 years; median 4 years) with IND (GOS criteria).

The density of nerves, identified by their AChE activity (see Appendix), in the circular muscle was analysed. AChE-activity was demonstrated on cryostat sections of snap frozen blocks of bowel taken longitudinally to the long axis of the intestine thus sectioning the circular muscle coat transversely. A representative area of the circular muscle in the stained sections was photographed using a x10 objective and a green filter on ILFORD PAN F film and printed under standard conditions. The final print magnification was x212.

The nerve density was analysed using a simplification of the morphometric method of Aherne (1967; Aherne and Dunnill 1982) by assessing the number of nerves intercepting three parallel lines (measuring 17.7 cm in length; the distance between the lines being 0.8 cm). The lines were drawn on an acetate sheet which was placed over the circular muscle on the print. These lines were orientated parallel to the longitudinal muscle coat and at right angles to the direction of the smooth muscle cells in the circular muscle. The area analysed, when converted to the actual area of intestine, measured 544 μm^2. The number of intercepted nerves was recorded. The density was expressed as the number of nerves per 100 μm^2 of circular muscle.

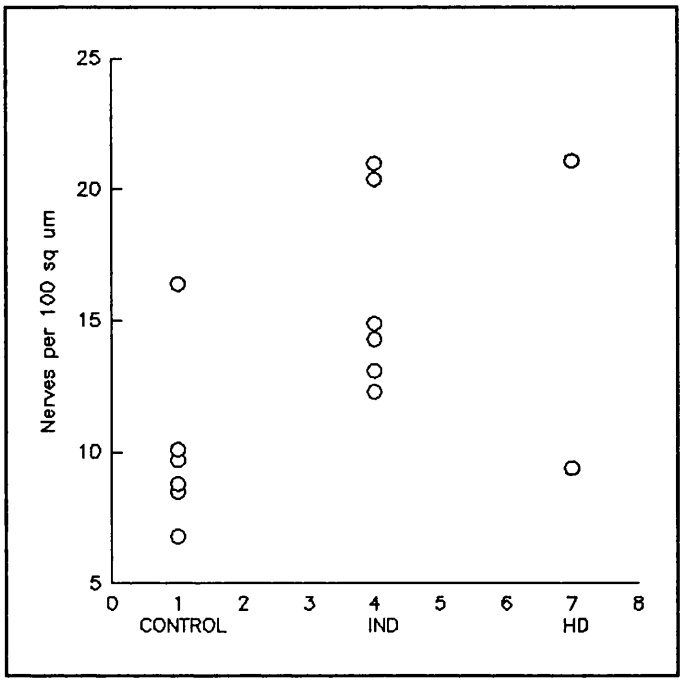


Figure 12. Density of AChE-positive nerves in the circular muscle in HD, IND and controls.

3.3.2 Results

The values obtained for AChE-positive nerve fibres in the circular muscle coat from the control samples of colon (n=6) ranged between 6.8-16.4 (mean 10) nerves/100 μm^2.

In contrast, those from the patients diagnosed as having IND (n=6) ranged from 12.3-21 (mean 16) nerves/100 μm^2. Using Student's t-test this increase was significant ($0.01 > p > 0.001$). Results in the proximal resection margins of the colonic pulled-through specimens from the two patients with HD were 9.4 nerves per 100 μm^2 in one (falling within the control range) and 21.1 nerves/100 μm^2 in the other (falling within the IND range).

3.3.3 Discussion and Conclusions

Despite the small number of samples analysed there was a statistically significant increase in the circular muscle nerve density in specimens of colon from patients diagnosed as having IND by other criteria.

In colon from one of the control patients (aged 8 weeks) an apparently elevated density (16.4 nerves/100 μm^2) of AChE-positive nerve fibres in the circular muscle was found. Although no gastrointestinal symptoms had been noted in this patient, other changes consistent with diagnostic features for IND in the colon could be detected. These included a mild focal increase in AChE-positive nerve fibres in the lamina propria. In the submucosa ganglia were frequent and showed 'button'-like association with thickened nerves and AChE-positive nerves around submucosal blood vessels were relatively prominent. However, no heterotopia or giant ganglia were identified. The myenteric neuronal density (see Chapter 4) was also slightly elevated (9.4/mm).

The proximal block from one of the patients with HD also showed an elevated circular muscle nerve density (21.1 nerves/100 μm^2). In this specimen there was a mild focal increase in AChE-positive nerve fibres in the lamina propria and the myenteric neuronal density (see Chapter 4) was also elevated (11.4/mm). No other features associated with IND were detected in this sample. The clinical significance of these findings cannot be assessed since the patient was lost to follow-up.

Although the sample size is small and the morphometric technique employed is simple, there appears to be an increase in AChE-positive nerves in the circular muscle

in specimens from patients with an intestinal motility disturbance and other morphological changes suggestive of IND. The practical value of this type of assessment in terms of aiding the diagnosis of IND is debatable but it may be useful as an additional diagnostic parameter, particularly in adult patients in whom other morphological markers of IND may be lacking (Meier-Ruge 1992).

Chapter 4. Neuron Density in Normal Intestine in Childhood as a Basis for the Assessment of Hypo- and Hyperganglionosis

4.1 Introduction

Whilst HD (aganglionosis) is the commonest and most easily identified neurogenic intestinal motility disorder, other rarer neuropathic disturbances are also recognised. These are intestinal neuronal dysplasia (see Chapter 3) in which an excess of intestinal neurons (hyperganglionosis) is one morphological feature, and hypoganglionosis which is characterised by a paucity of myenteric neurons (Garrett and Howard 1981).

Both hyper- and hypoganglionosis may occur as isolated abnormalities, or affect a portion of intestine immediately proximal to the aganglionic segment in patients with HD (Briner et al. 1986; Fadda et al. 1987; Borchard et al. 1991). In either event, their recognition is of clinical importance since complete surgical excision of the abnormally innervated bowel results in normal peristalsis (Fadda et al. 1987; Borchard et al. 1991). However, conservative treatment of patients with hyperganglionosis may be advocated initially since symptoms can improve spontaneously (see Chapter 3).

Patients, particularly neonates, presenting with signs of pseudo-obstruction are generally first investigated by taking a rectal suction biopsy specimen which contains both mucosa and submucosa. Such biopsies, examined histochemically for acetyl cholinesterase (AChE) activity and serially sectioned to identify neurons in the submucosal plexus, are excellent for confirming or refuting a diagnosis of HD, based on the characteristic AChE-staining pattern in the muscularis mucosae and lamina

propria (Lake et al. 1978) and the absence of submucosal ganglia . However, these specimens are of little value in defining hyper- and hypoganglionosis. The diagnosis of neuronal dysplasia on a suction rectal biopsy is a matter of continuing controversy (see Chapter 3) while that of hypoganglionosis is unreliable since the reduction in ganglia is usually confined to the myenteric plexus (Ariel et al. 1985; Fadda et al. 1987; Meier-Ruge 1992). A further point is that the distal rectum just above the dentate line (the mucocutaneous junction or anal verge) has a short hypoganglionic segment in normal subjects (Aldridge and Campbell 1968). Since the precise level at which a diagnostic suction rectal biopsy is taken is difficult to assess clinically, particularly in neonates, this hypoganglionic zone may be sampled, providing a further source of potential diagnostic error.

From a practical view point, specimens suitable for the assessment of hypo- and hyperganglionosis would include formal full thickness biopsies of rectum, pieces of large intestine removed during the fashioning of a colostomy for decompression or, in patients suspected of having hypo- or hyperganglionosis accompanying HD, pull-through specimens of colon removed during excision of the aganglionic distal segment. The purpose of this chapter is to analyse the extent to which routinely prepared, haematoxylin and eosin (H&E) stained sections of formalin-fixed paraffin embedded tissue can be utilised to recognise neuronal disorders of the intestine other than HD in these specimens. Using this technique alone, the distinction of these entities is quantitative, and relates to counts of neuronal densities in the myenteric plexus rather than the submucosal plexus. This is because submucosal counts are much more time consuming and difficult to standardise, and because hypoganglionosis appears to be confined to the myenteric plexus (Borchard et al. 1991; Scharli 1992).

Thus, the first requirement is to establish control data for myenteric neuronal density in normal intestine, so that deviations below (hypoganglionosis) and above (hyperganglionosis) the normal can be assessed objectively; since the majority of patients with pseudo-obstruction present in infancy (Milla 1991) there is a clear need for reliable baseline data in the paediatric age group.

This chapter describes the method used for intestinal neuron density counts and its validation, and then documents neuron counts of both transverse and longitudinal sections of full thickness specimens taken at post-mortem examination at defined points in the jejunum, ileum and colon from 21 children (aged 4 weeks to 10 years) as well as from the colon of 8 adults (16-83 years). This material provides normal control data concentrating on the paediatric age group.

Selected colonic resection specimens from six children with HD and one with suspected isolated hypoganglionosis are also examined. The HD patients included two with persistent impairment of intestinal motility thought to have concomitant intestinal neuronal dysplasia, and one considered to have hypoganglionosis accompanying HD. In each case neuronal densities are compared with controls and any differences noted are related to the clinical data available.

4.2. Materials and Methods

a) Control specimens

Sleeves of colon approximately 10cm long, the distal margins of which were 2cm proximal to the recto-sigmoid junction were removed at necropsy from patients dying of non-gastrointestinal diseases (congenital heart disease, sudden infant death

syndrome, pneumonia, metabolic liver disease, status asthmaticus, myocardial infarction and road traffic accidents). These patients comprised 21 children ranging in age between 4 weeks and 10 years (group 1) and 8 adults aged between 16 and 83 years (group 2). Delay between death and necropsy ranged between 6 and 48 hours (median 24 hours).

Sleeves of small intestine also approximately 10 cm long, one taken from the jejunum with an upper limit 2cm distal to the duodenojejunal flexure, and one from the ileum with a lower limit 2cm proximal to the ileocecal valve were also taken from all the children in group 1 and two of the adults in group 2.

For each specimen the bowel was opened, washed with saline and fixed in 10% formalin in phosphate buffer. The circumference was measured and both longitudinal and transverse blocks measuring 4.6-33 mm were taken from the proximal jejunum, distal ileum and distal descending colon and were processed routinely and embedded in paraffin wax. Sections (3μm) were cut and stained by haematoxylin and eosin (H&E) omitting the differentiation step of acid alcohol after Harris' haematoxylin.

b) Surgical specimens

Neurons were counted in longitudinal blocks (measuring 16.8-29.4mm; median 22 mm) from the proximal resection margins in pull-through specimens from four patients with HD. Two further patients with HD had persistent pseudo-obstruction following pull-through operations. Second colonic resections showed evidence of intestinal neuronal dysplasia proximal to the aganglionic segments diagnosed on adjacent blocks of frozen tissue which showed increased AChE-positive nerve fibres

and heterotopic neurons in the lamina propria with frequent and often large enteric ganglia in both myenteric and submucosal plexuses. Sections (12mm and 31.3mm in length) of the paraffin wax embedded tissue from the proximal resection margin of the second resections were analysed. Neuron density was also assessed in sections (27.5mm in length) of colon from a patient in whom isolated hypoganglionosis (small, sparse ganglia in the myenteric plexus) had been observed. All the above patients were children aged between 8 months and 10 years.

c) Methods.

In both longitudinal and transverse sections of each specimen, the total number of neurons in every tenth section at six consecutive levels was counted, and the neuron density expressed as the mean number of neurons per mm. The section length was measured with a calibrated eyepiece graticule. In a preliminary study on two tissue blocks counts of neurons identified by the H&E stain were compared with counts in the immediately subsequent serial sections, in which neurons were demonstrated by immunohistochemical methods using polyclonal antibodies to protein gene product 9.5 (Ultraclone Ltd, Cambridge, UK), neuron specific enolase and S100-protein (Dako Ltd.,High Wycombe, UK). The standard avidin-biotin-peroxidase complex (ABC) method with diaminobenzidine (DAB) was employed (Hsu et al. 1981). Biotinylated anti mouse and anti rabbit antibodies and the ABC complex were obtained from Dako.

The maximum diameter of neurons was also measured using a calibrated eyepiece graticule.

4.3 Validation

The numeric estimation of myenteric neurons has a number of potential problems. These are identification of neurons, counting the same neuron more than once, and how many and how long sections are needed for adequate volume of myenteric plexus to be analysed. These points were addressed and the investigations are reported below.

The reliability of the haematoxylin and eosin (H&E) stain for identifying neurons was tested in paraffin sections of two samples of post-mortem intestine by comparing density values obtained using immunohistochemical methods to aid recognition of nerve cells. The issue of how many sections (3μm thick) were required to record the accurate myenteric neuronal density was investigated by counts in 32 samples of post-mortem intestine. These counts were made in serial sections (n=24) and sections chosen at sufficiently separated levels to count each neuron only once, taking account of the measured neuronal size. The length of section required to represent the myenteric neuronal population was also considered.

4.3.1 Identification of Neurons

In order to establish the reliability of the H&E stain in neuronal identification, a comparison was made of neuronal densities (number of neurons per mm) obtained by immunohistochemical methods and the H&E stain. Neurons were readily identified in the H&E stained sections by their amphoteric, granular cytoplasm and open vesicular nuclei with prominent nucleoli. The supporting cells in the ganglia caused no problems in identification since the sparse cytoplasm of these cells was never granular or amphoteric and the compact nuclei were not confused with open vesicular

neuronal nuclei. The cytoplasmic appearance of the neuron sufficed to be included in the count (Fig.1).

Figure 1. A section of jejunum showing the myenteric plexus containing one neuron with a nucleus and nucleolus (thin arrow) and a second neuron in which only cytoplasm is seen (thick arrow). Both appearances are included in the assessment of neuron density. The remaining cells comprise supporting cells easily identified by the absence of granular, amphoteric cytoplasm and/or the presence of compact nuclei. H&E Magnification x480

Using the antibody to S100-protein, negative neuronal perikarya was clearly visible against the strong glial immunoreactivity in the ganglia. All neurons showed strong immunostaining with the antibody to neuron specific enolase (NSE) on the

background of positive nerve fibres. The antibody to protein gene product 9.5 (PGP 9.5) showed some variability in cytoplasmic reactivity, although most neurons were positively stained.

The mean density obtained in sections stained with H&E was almost identical to the density in sections where neurons were identified immunohistochemically demonstrating the reliability of the H&E stain in identification of intestinal neurons.

4.3.2 Neuronal Size

In children most neurons were 20-23μm in diameter with very occasional neurons measuring 30μm. Most neurons in the neonates were small (8-15μm) and only occasional neurons were larger. In adults, however, neurons measuring 30μm were common and occasional larger neurons of 40μm in diameter were also seen.

Thus the largest neuron seen in the intestine from children was 30μm in diameter and many measured less , especially in the very young. To avoid counting each neuron more than once, every 10th section of a series of serial (3μm) sections was chosen.

4.3.3 How Many Sections are Required for Analysis?

Some intersectional variation in neuronal density was seen among the 24 serial sections from each of 32 specimens of intestine analysed. Whether or not this is important was tested by comparing the mean density in all 24 sections with the mean density in sections spaced at levels so that individual neurons were counted only once.

This was done firstly by comparing the density value in the first of the serial sections

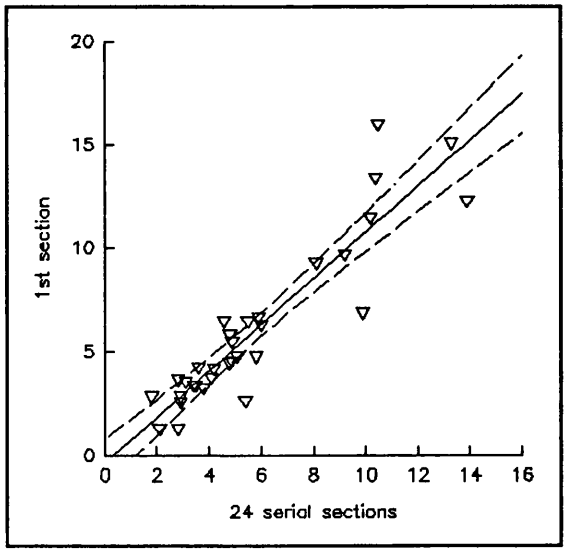


Figure 2 Comparison of neuron density between the first section and 24 serial sections.

Values on the axes represent neuron density per mm.

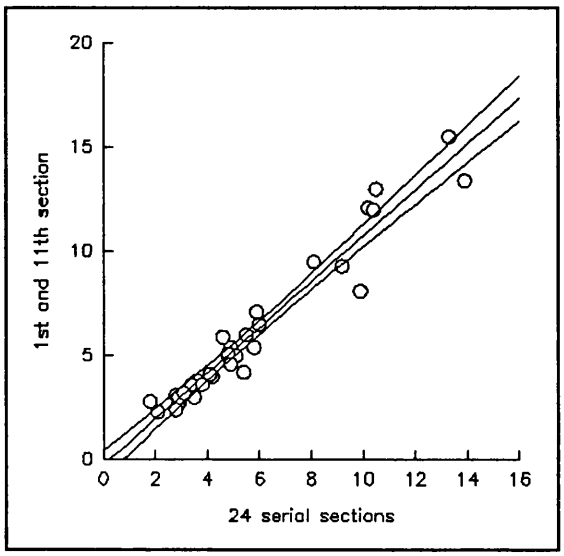


Figure 3 Comparison between neuron density in 1st and 11th sections and 24 serial sections. Values on the axes represent neuron density per mm.

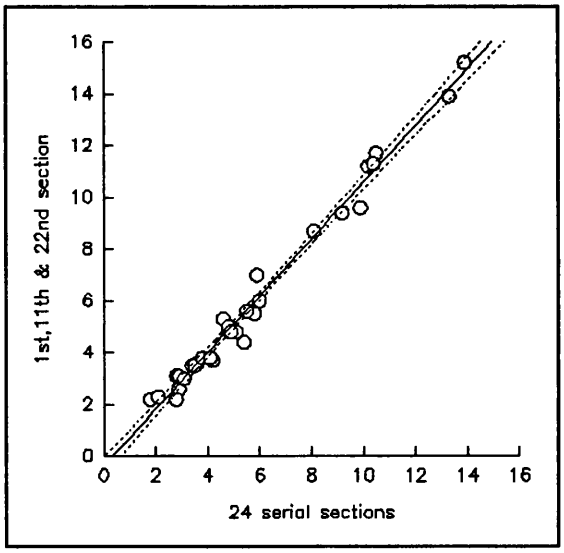


Figure 4. Comparison of neuron density between the 1st, 11th and 22nd sections and 24 serial sections. Values on the axes represent neuron density per mm.

with the mean of all 24 sections (Fig.2). Next the mean of the first and 11th sections (Fig. 3) and then the mean of the first, 11th and 22nd sections (Fig. 4) were compared with the mean of all 24 serial sections. A close linear relationship was seen for all three comparisons and no statistically significant difference could be shown between them. The correlations were: for the single section ($y=1.12x-0.428$, $r=0.919$, $p<0.001$), for two spaced sections ($y=1.104x-0.304$, $r=0.969$, $p<0.001$) and for the three spaced sections ($y=1.099x-0.435$, $r=0.99$, $p<0.001$).

This result indicates that a single section may provide a satisfactory and representative neuronal density value. However, the sample size present in one section obviously depends on the section length as well. Amongst the 32 specimens of intestine examined for the validation study, the section lengths ranged for the jejunum 6.8-15 mm (median 11.2 mm), for the ileum 4.6-19.2 mm (median 9 mm) and for the colon 5.2-18.6 mm (median 12.5 mm).

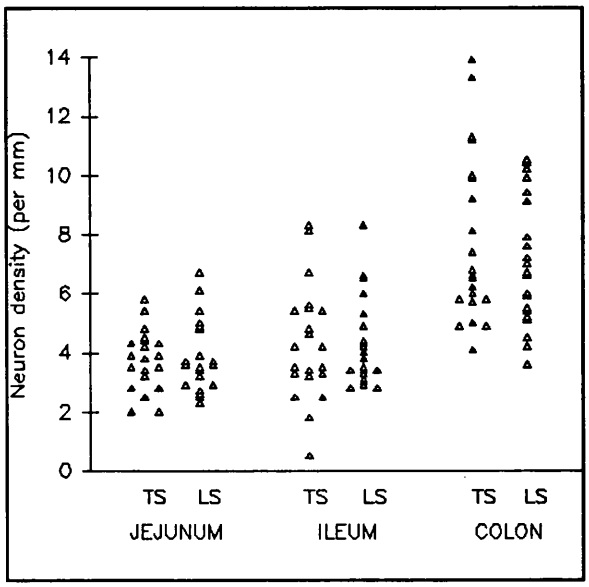
For the neuron density counts used for the baseline studies, a total of six sections spaced 30 μm apart (ie. every 10th section of the serial 3 μm-thick sections) was examined at each site. These samples were of length 13-24 mm (median 20 mm), for the jejunum, 10.4-26 mm (median 19 mm) for the ileum and 10-33 mm (median 22.2 mm) for the colon. These sample sizes are well in excess of the minimum required according to the validation studies but really reliable figures were considered to be necessary in view of the extraordinary variations in counts obtained by previous studies (Meier-Ruge et al. 1970; Schuffler et al. 1978; Schuffler and Jonak 1982; Ikeda et al. 1988).

4.4 Baseline Neuronal Density and Comparison of Density in Surgical Resections

a). Baseline Results

The neuron density in post-mortem tissue (Fig.5) is lowest in the jejunum and highest in the colon. Similar density values in both longitudinal and transverse sections in an individual bowel were seen and Fig.6. shows that there is a close correlation between LS and TS values in the same specimen ($y = 0.25 + 1.04x$; $r = 0.8$; $p < 0.001$). For children the mean neuron density in the colon was 7.7/mm (TS), 7/mm (LS) and in the small intestine about 4/mm (ileum 4.3/mm in both TS and LS ; jejunum 3.6/mm in TS and 3.7/mm in LS). In this group no significant variation could be shown in neuronal density with age (Fig.7). For adults the mean colonic neuronal density was 5.1/mm, perhaps indicating a slight fall in old age, although the numbers of patients were small and the result was not statistically significant (see Fig.8).

Figure 5.
Neuron density in post-mortem jejunum, ileum and colon expressed as the mean number of neurons per mm counted at six levels 30μm apart in a particular tissue block. Each symbol represents one patient. TS, transverse sections; LS, longitudinal sections.



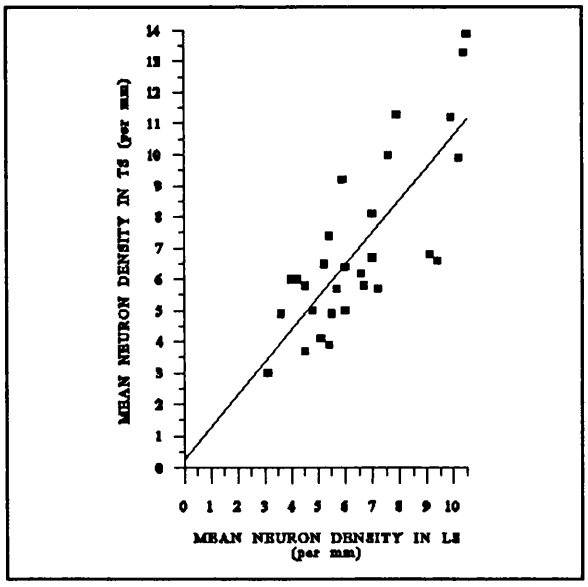


Figure 6. Neuron density (number of neurons per mm) compared in transverse sections and longitudinal sections from the same specimen of normal post mortem intestine. There is a close linear relationship and significant correlation between TS and LS counts. $y = 0.25 + 1.04x$; $r=0.8$; $p < 0.001$.

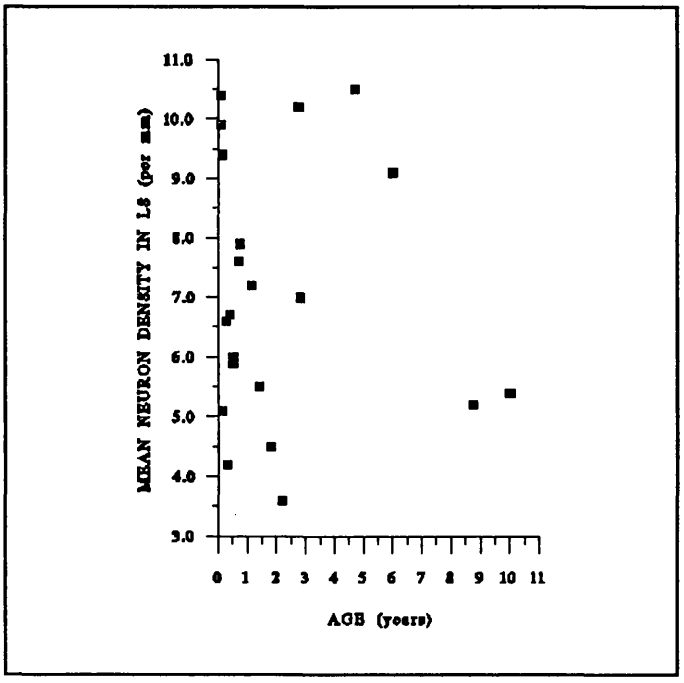


Figure 7. Neuron density in LS of post-mortem colon plotted against age (4weeks to 10 years).

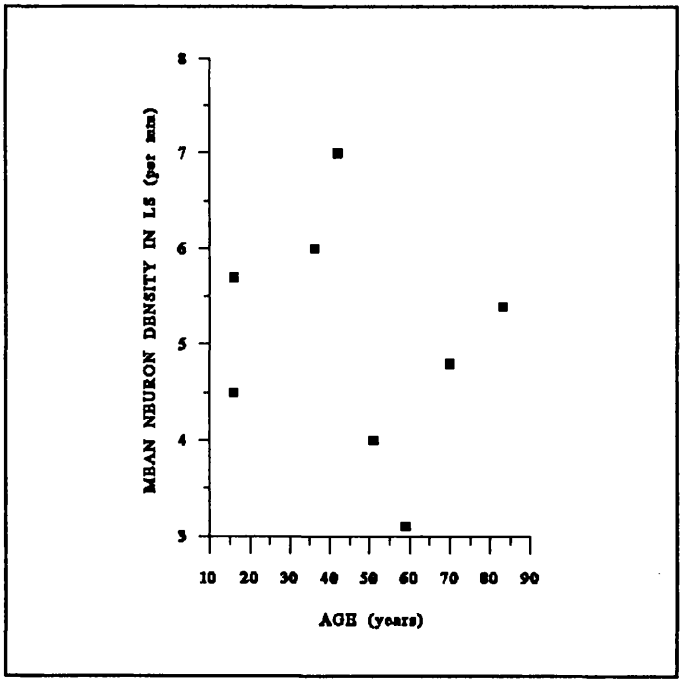


Figure 8. Neuron density in LS of normal post-mortem colon plotted against age (16-83 years).

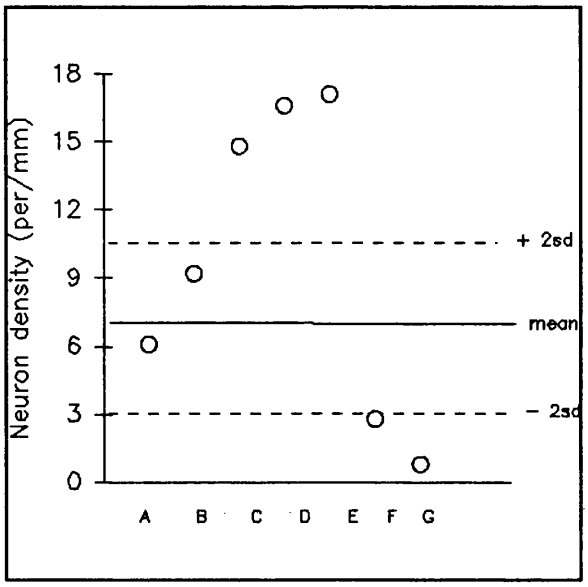


Figure 9. Neuron density in the most proximal block of surgically resected colon from seven patients. Patients A, B, C and G had Hirschsprung's disease and counts were made on the initial pull-through specimen. Patient C was later shown to have concomitant intestinal neuronal dysplasia. Patient G was regarded as having an unusually long hypoganglionic segment without hypertrophied nerve trunks. Patients D and E had persistent pseudo-obstructive symptoms following an initial pull-through operation for Hirschsprung's disease. Neuron density is that in a second pull-through intestine. On adjacent blocks of frozen tissue the diagnosis of intestinal neuronal dysplasia was made. Patient F had intestinal pseudo-obstruction and was observed to have microscopic features of isolated hypoganglionosis. Horizontal lines represent the mean control neuron density for colon in longitudinal section in children: 7/mm + or - 2SD.

b). Results in the Surgically Resected Colon

The neuron density in the surgically resected colons is shown in Fig.9. Neuron counts in the proximal blocks from two patients with Hirschsprung's disease (A and B) fell within two standard deviations from the control mean, but two others with HD were outside this range, one with elevated density (C) and the other below this range (G) indicating evidence of hyper- and hypoganglionosis respectively in these two patients. Similarly values just at the level of two standard deviations below the control mean

in patient F with a subjective impression of hypoganglionosis and of well above two standard deviations in the two patients (D and E) in whom a diagnosis of intestinal neuronal dysplasia was established by histochemical criteria.

In children the circumferences of colon at post mortem ranged from 2-6cm (mean 3.5cm), for ileum 1-4cm (mean 2cm) and for jejunum 1.5-5cm (mean 2.5cm). In adult colon the circumference was 3.5-7cm (mean 5.5cm), measured in fixed tissue. The circumference of surgically resected specimens measured from 3.5 to 5.5 cm.

4.5 Discussion

Previous assessments of neuron densities in the myenteric plexus, mainly on adult intestine using various methods, have resulted in a wide range of normal values (Table 1). No consistent study using a standardised procedure has been employed in children. The results presented here are 20 times higher than those of Schuffler and colleagues (1978, 1982). In both their studies and in this one a seemingly similar approach was employed. Each neuron was counted only once using sections at appropriately spaced levels in paraffin blocks. Schuffler et al. (1978) and Schuffler and Jonak (1982) counted only nucleated neurons whereas in this study all neurons were counted regardless of the presence or absence of nuclei. Those without nuclei were recognised by their characteristic cytoplasmic appearances alone. Different criteria for identifying which neurons are included may in part explain the discrepancies. However, it is unlikely that these considerations would entirely explain such large differences. The intestinal wall in children is thin and in order to obtain better orientation of sections in this study blocks of colon mostly included the taenia. This may have also contributed to the higher density values seen here, since neurons

are more numerous in the myenteric plexus adjacent to taenia than elsewhere (Schuffler 1988, personal communication).

Table 1. Comparison of Neuron Density in Different Studies of Normal Human Colon.

Study	Density	Number of Controls
Schuffler et al.,1978	27.8/100mm	7
Schuffler and Jonak,1982	42.9/100mm	20
Meier-Ruge et al., 1970	756/10mm	?
Ikeda et al.,1988	>30/10mm	?
Smith 1993 (present study)	7/mm	29

At the other end of the scale, Meier-Ruge and colleagues (1970) recorded neuronal densities ten times those encountered in this study and approximately 200 times those recorded by Schuffler et al. (1978) and Schuffler and Jonak (1982). The greater density recorded by Meier-Ruge et al. (1970) is partly the result of using sections three times thicker than in this study. However, it is unlikely that this could explain a ten-fold difference. Meier-Ruge studied exclusively bowel resected for Hirschsprung's disease (Meier-Ruge 1988, personal communication) and some patients may have had intestinal neuronal dysplasia (hyperganglionosis) proximal to the aganglionic segment, inflating the density recorded.

In the present study neuron densities have been systematically investigated in specified sites of the normal intestine in children and in adults by methods accessible to any histopathology laboratory. The mean density values were in keeping with observations of neural tissue volumes (Wells et al. 1987). In contrast, others workers have analysed adult intestinal neuron density alone (Schuffler et al.1978; Schuffler and Jonak 1982), or intestine from children with Hirschsprung's disease (Meier-Ruge et al. 1970).

The concept of Dirichlet domains (Loeb 1976; Wells et al. 1987) can be applied to describe the myenteric plexus, which is a three dimensional network of organized spatial structures. This network (see Fig.10) consists of a set of polygons outlined by nerve trunks with ganglia at the apices of the polygons. Ganglia are spaced 1-5mm apart in the colon (Bodian et al. 1949) and further apart in the small intestine (Wells et al. 1987). Any one section, particularly of a short length of intestine, may miss some or all ganglia (see Fig.10). By analysis of sections in longitudinal or transverse blocks of intestine at levels designed to include each neuron only once, the inclusion of a representative population of neurons is ensured, resulting in a reliable and reproducible values of neuronal density.

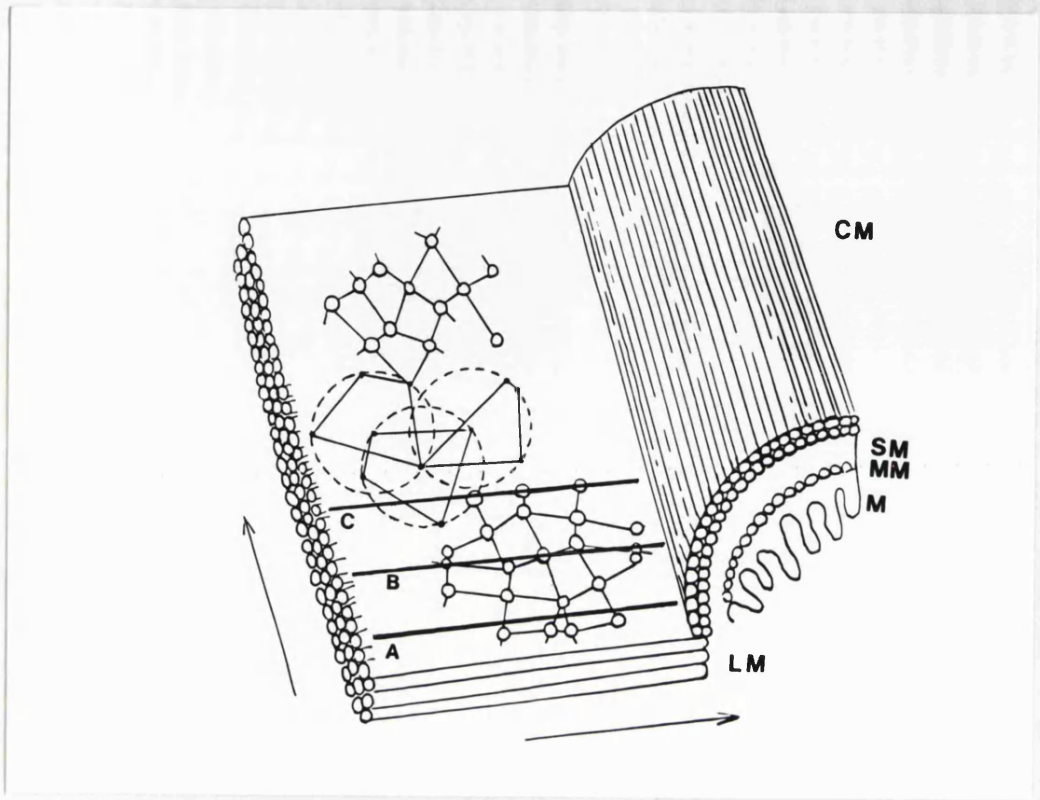


Figure 10. Diagrammatic representation of the myenteric plexus showing the concept of Dirichlet domains (circles). Random sections in planes indicated by lines A-C will miss some of the ganglia (B), all of the ganglia (A), or none of the ganglia (C) if short lengths of bowel are analysed. LM, longitudinal muscle; CM, circular muscle; SM, submucosa; MM, muscularis mucosae; M, mucosa.

Provided that a sufficient length is analysed, counts in a single section as shown by Fig.10 may also be valid. However, short lengths (less than 10 mm), particularly in the small intestine, may give false results in single sections due to the insufficient number of neurons in the more widely spaced ganglia.

Comparison of neuron densities in TS and LS from the same specimens of post-mortem intestine are shown in Fig.6. A close linear relationship is obtained indicating that either LS or TS are suitable for neuronal counting. The specimens were not

distended and their circumferences did not vary greatly. Clearly intestinal dilatation would produce an increase in circumference and some increase in the length. This could effect neuronal counts and could be a consideration in megacolon when the intestine is hugely dilated. However, in most cases of pseudo-obstruction surgical decompression of the bowel is achieved by colostomy before bowel resection so that intestinal dilatation is rarely a significant problem.

The largest neuron seen in the intestine from children was 30μm in diameter and many measured less, especially in the very young. In the adults studied here a greater proportion of large neurons was seen, occasionally measuring 40μm in diameter. No reports, other than in the fetus (Vaos 1989), exist about neuronal size in the human gut in relation to age, although growth in neuronal volume has been shown in the monkey brain from infancy to adulthood (Headon et al. 1985). Observations by Gabella (1987a) in experimental animals also confirm that the average neuron size is significantly smaller in the newborn intestine than in the adult bowel, the difference being more marked in larger animals such as sheep and cattle, than in the smaller species such as the mouse and shrew.

Deviations in neuronal density from normal baseline data in surgically resected intestine correlated well with persistence of clinical symptoms. Proximal blocks from resected intestine from two patients with HD (Fig 9; A,B) showed density values within two standard deviations from the established post-mortem mean, both of whom appeared to be cured by the bowel resections. In contrast, of the patients with residual problems of intestinal dysmotility after pull-through operations for HD, all were found to have density values outside two standard deviations from the control mean.

Two of these patients (Fig 9;D,E) had intestinal neuronal dysplasia (hyperganglionosis) diagnosed on the basis of other criteria (see Chapter 3). Moreover, patient C with a high density value was later shown by examining frozen tissue to have intestinal neuronal dysplasia (hyperganglionosis) proximal to Hirschsprung's disease. Thus neuron counts are helpful in confirming or refuting a subjective impression of hyperganglionosis when only paraffin wax embedded tissue is available for analysis. The diagnosis of hypoganglionosis is based on the presence of sparse small ganglia with scanty neurons. In patient F with symptoms of intestinal dysmotility and microscopical features of hypoganglionosis without concomitant Hirschsprung's disease, the neuron density was found to be just at two standard deviations below the mean, so that the subjective impression of a relative paucity of neurons was supported, but could not be diagnosed objectively as hypoganglionosis. In patient G with Hirschsprung's disease, who appeared to have an extensive transitional zone with small sparse ganglia and without hypertrophied nerve trunks proximal to the aganglionic segment, however, a grossly reduced density value well below two standard deviations from the mean was seen.

The length of the intestine increases with increasing body length and, since it is assumed that neurons do not proliferate after birth, a reduction in neuron density would be expected with increasing age. However, this has been challenged by the following observations in animals. In the rat and chick there is an increase in neurons in the myenteric plexus during post natal life, and tritiated thymidine uptake by neurons in the mouse duodenum can be seen at least 2 weeks after birth (Gabella 1987a). At birth the human gut measures on average about 4 meters (Bryant 1924) and in the adult about 7.5 meters (Bryant 1924). The growth of the intestine is most

rapid in the early years of childhood, (Bryant 1924) and by 10 years of age both the small gut and colon have reached the adult lengths. Despite expectations, based on the reported higher neuronal packing density in immature animals compared with adult animals (Gabella 1987), no obvious reduction in neuron density was encountered in this study in infants and children up to the age of 10 years when the length of the child's intestine has reached the adult dimensions. In addition there was no obvious reduction in neuron density seen in the young adults, although in old age a slightly lower density was encountered (Fig. 8).

To explain this apparent discrepancy, it is possible that not all neurons were identified in the very young due to the presence of small nerve cells, whereas in the older children and adults these cells were more readily recognised. As the adult neurons are larger it may also be possible that the same cell could have been counted twice. However, this would not be true in the intestine of the older children where the largest neuron measured 30μm. The small number of patients examined may also influence the results, as in all ages there are length variations in the gut (Bryant 1924). However, the possibility of neuronal proliferation in childhood cannot be ruled out and warrants more extensive study.

In conclusion this study has established a baseline of myenteric neuronal density in childhood. The method can be applied to routinely processed archival tissue sections and neuronal density analysis may be of value even in single sections, although this obviously depends on an adequate section length. The longer the intestinal length examined the more representative is the density value in a single section of that myenteric plexus. The section length needs to be in excess of 5 mm, but in the small

intestine where neuronal density is less than in the colon, it may be preferable to examine section lengths in excess of 10mm. This baseline data was shown to be clinically useful in identifying hyperganglionic segments of bowel where subjective impressions can be misleading. However, the diagnosis of marginal hypoganglionosis was not improved by this method in the single case examined. In one other patient hypoganglionosis was obvious by subjective examination and neuronal counts merely confirmed this observation.

Chapter 5. Assessment of Argyrophilia in the Developing Myenteric Plexus During Childhood

5.1 Introduction

In the previous two chapters the contribution of conventional histology and histochemical techniques to the study of neurogenic forms of pseudo-obstruction have been discussed. However, many patients with clinical signs of intestinal motor dysfunction and electrophysiological evidence of a neurogenic defect show no abnormalities by these methods. In addition, where abnormalities exist there may be poor correlation between their severity and the degree of functional obstruction noted clinically. The failure to identify neural abnormalities in the myenteric plexus might reflect either insensitivity of the staining methods in current use or the study of an insufficient volume of the plexus.

In 1967, Barbara Smith reintroduced a silver impregnation method used previously in neuropathology to the study of the intestinal myenteric plexus. This technique employed thick (50 μm) sections of the bowel cut tangentially, parallel to the mucosal and serosal surfaces at the level of the myenteric plexus. This increased the sample of neurons seen in any one section and allowed examination of their morphology in greater detail, as well as their spatial distribution and intercellular connections.

The silver staining method identifies two main types of neurons, those that take up silver (argyrophils) and those that do not (argyrophobes), together with intermediate types showing varying lesser degrees of argyrophilia. There is some regional variability in the proportions of argyrophilic and argyrophobic nerve cells, but both

types are normally present. The relative number of the two types of neurons also varies in different patients (Schuffler 1989).

In patients with pseudo-obstruction, the method has been applied mainly to intestine from adult subjects (Dyer et al. 1969; Schuffler et al. 1978,1985; Schuffler and Jonak 1982; Smith Barbara 1982; Schuffler 1989). The abnormalities identified by this technique include changes in the neuronal perikarya, such as blurring of cell margins and cytoplasmic swelling, fragmentation or 'vacuolation', with argyrophilia confined to a peripheral rim (Schuffler 1989). Changes described in the processes include swelling, beading or fragmentation of axons, clubbing of dendrites and axonal drop-out (Schuffler 1988b,1989). Some of these changes are seen in intestine from patients with other neurological abnormalities involving the peripheral and/or central nervous system. They can also be produced by inflammatory processes of viral, parasitic, neoplastic or toxic (purgatives) aetiology (Smith Barbara 1968b; Krishnamurthy and Schuffler 1987; Schuffler 1989). In the patients with pseudo-obstruction, the possibility that some of these changes may be secondary to neuronal degeneration in long-term obstipation or following the use of anthraquinone cathartics cannot be excluded.

Similar studies in childhood pseudo-obstruction are sparse (Tanner et al. 1976; Navarro et al. 1990) but complete absence of argyrophilic neurons has been proposed as the cause of intestinal motor dysfunction in three neonates (two boys and a girl) with short small bowel, malrotation and pyloric stenosis (Tanner et al. 1976). Two of these infants were born at 36 weeks gestation the third was delivered at full term. The intestinal samples were obtained at the age of 13 days, 24 hours and 15 days

respectively.

As with any technique, there is a need to establish base-line data in unaffected subjects to indicate the range of normal appearances against which any variations relating to disturbed function can be assessed. Since some of these variations may be age-related and since it has been noted that at birth not all enteric neurons have reached full morphological maturation (Smith Blanca 1968), a process that continues for at least the first 2 years of life (Bughaighis and Emery 1971), it is important to establish control data in the paediatric age range. In childhood, and particularly in the neonatal period, no systematic control data is available.

For this reason a number of colonic specimens obtained at necropsy from preterm babies, neonates, infants and young children dying from non-gastrointestinal diseases were studied by the silver staining method. The appearances of the myenteric plexus were examined by noting neuronal morphology, and the size and appearance of neuronal processes. The cellularity of the ganglia together with the presence or absence of argyrophilic neurons and the ratios of argyrophobic to argyrophilic cells were all assessed in relation to the age of the child.

Surgical specimens of terminal ileum from patients with pseudo-obstruction were also investigated. The ileal samples were obtained at decompression ileostomy when this procedure became necessary to relieve their obstruction. Thus the region of intestine examined (small rather than large intestine) was dictated by clinical considerations.

5.2 Materials and Methods

5.2.1 Control Intestine

Sleeves of sigmoid colon measuring 3 cm in length were obtained at post-mortem from 48 infants and children (including two preterm and one term stillborn infants) who lacked anatomical abnormalities and died of non-gastrointestinal diseases (congenital heart disease, encephalopathy, hydrocephalus, cerebro-spinal deformities, glioma, lactic acidosis, sudden infant death syndrome, haemorrhagic shock, diaphragmatic hernia, persistent fetal circulation, cardiomyopathy, pneumothorax, hydronephrosis). Of these patients 26 were male and 22 female. The age of the patients ranged from preterm infants to 14 years of age, comprising 2 fetuses (33 weeks gestation each) and one stillborn infant delivered at term, 18 neonates (8 hrs-1 month), 16 infants (2-6 months), 3 babies (7-12 months) and 8 children (18 months to 14 years). The delay between death and autopsy ranged between 6 and 48 hours (median 24).

5.2.2 Intestine from Patients with Pseudo-obstruction

Terminal ileum from 16 patients with pseudo-obstruction were also studied by the silver staining method. These patients ranged in age from 1 month to 17 years (median 20 months).

5.2.3 Methods

The intestine was opened and pinned out without stretching on cork with the mucosa towards the cork. The bowel was then fixed in a large volume of unbuffered 10% formol saline in excess of 14 days without changing the fixative. Blocks measuring 2x2cm were frozen mucosa down flat on a freezing microtome chuck. Frozen sections

(50 μm thick) were taken parallel to and including the myenteric plexus and collected in distilled water. In general in young children the myenteric plexus was contained in six to nine sections. Sections including the greatest area of the myenteric plexus were selected and post-fixed in Cajal's formol ammonium bromide (see Appendix) for 24 hours. After two rinses with distilled water, they were transferred to 50% alcohol containing 10% pyridine for 1 hour at 37°C. After two distilled water rinses sections were impregnated in 20% silver nitrate at 37°C for 1 hour, and without washing taken through three changes of 10% formalin, one change of 2% formalin and two changes of distilled water. They were then treated in ammonical silver nitrate (see Appendix) with constant agitation for 1 minute and then taken through three changes of 1% formalin with rapid agitation, and finally collected in distilled water. Sections were treated with 5% sodium thiosulphate for 5 mins, washed thoroughly in water, mounted on glass slides after teasing out the creases, dehydrated in alcohol, cleared first in carbol xylene and then in xylene before mounting in natural resin mountant.

The staining of these samples was performed in batches of six. At least one specimen known to contain strongly argyrophilic neurons was always included.

The myenteric plexus was studied noting the number of argyrophilic and argyrophobic neurons in at least four ganglia. Argyrophobic neurons were distinguished by their ample cytoplasm from smaller supporting cells with less cytoplasm. The morphology of processes and neuronal cell bodies was also examined. The cellularity of the myenteric plexus was assessed by calculating the mean number of neurons per ganglion and the ratios of the mean number of silver-negative to silver-positive cells

per ganglion were also calculated. Finally the appearances, the cellularity, the argyrophobe/argyrophil ratios and the presence or absence of argyrophilic neurons were correlated with the age of the child.

5.3 Results of the Base-line Study

5.3.1 Morphological Appearance and Uptake of Silver

Neurons in the 48 control specimens studied by the silver staining method clearly demonstrated variation in the nerve cell number, size and structure with a particular type (small, large, condensed or fuzzy argyrophils) predominating in a given patient. The axons and dendrites also varied from fine and thin to short and stubby processes. The outlines of neurons in some instances were fuzzy and indistinct but in others were well defined. The neuronal cell processes also exhibited variability in morphology ranging from fine, long and straight to nobbily and twisted structures with a tangled appearance. A variability in the uptake of silver by the neurons was also noted, with the neurons in some specimens showing only faint argyrophilia while their processes were stained jet black. Occasionally the perikarya appeared impregnated in a striped fashion reminiscent of crumpled tissue paper, and infrequently they were speckled and had numerous short brush-like processes. In some samples the argyrophilic neurons were predominantly small, spidery and strongly impregnated. Neurons in a few samples appeared bloated and swollen with bulbous processes while in others they seemed condensed and small. These varied silver staining appearances are illustrated by figures 1-14.

Figure 1. Silver staining in the myenteric plexus of a baby aged 2 months showing ganglia containing both argyrophilic and argyrophobic neurons and connecting nerve tracts. Magnification x130.

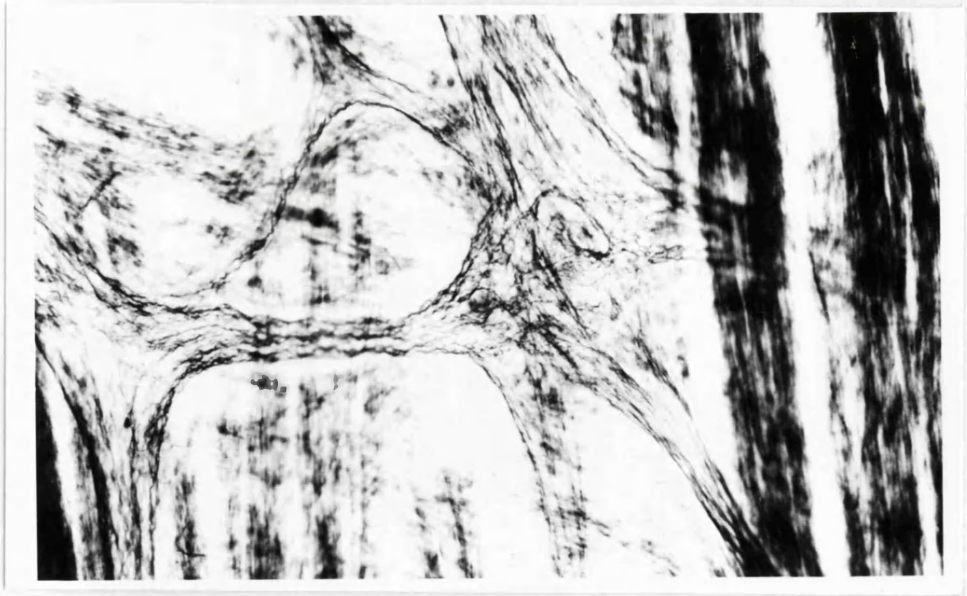


Figure 2. Silver staining in the myenteric plexus of a neonate aged 8 days showing strongly positive axons in the connecting tracts but only argyrophobes in the ganglia. Magnification x130.

The following illustrations show the variability of the neurons. The magnification of each x340.

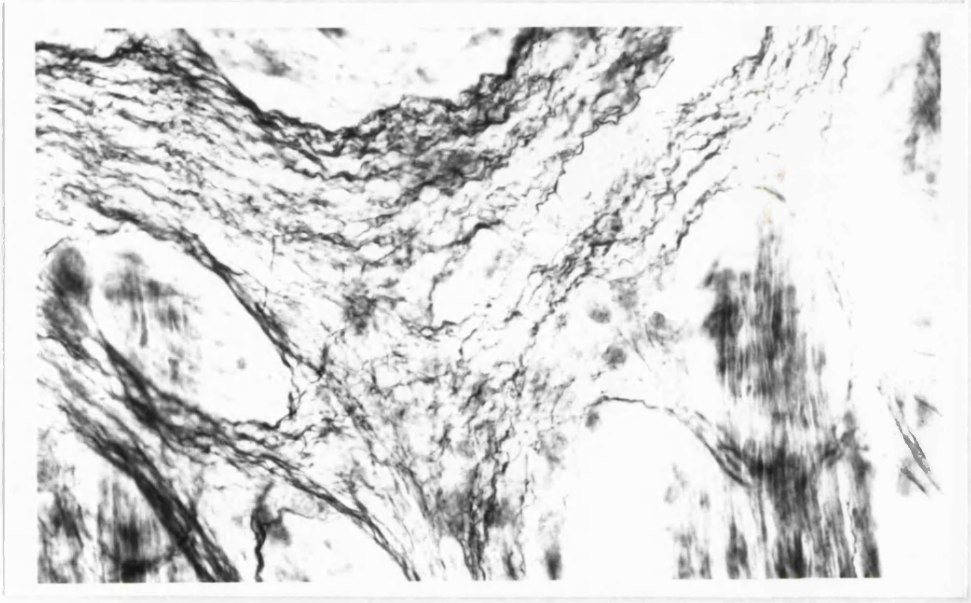


Figure 3. A ganglion from a neonate aged 8 days showing argyrophobes and an absence of argyrophils.

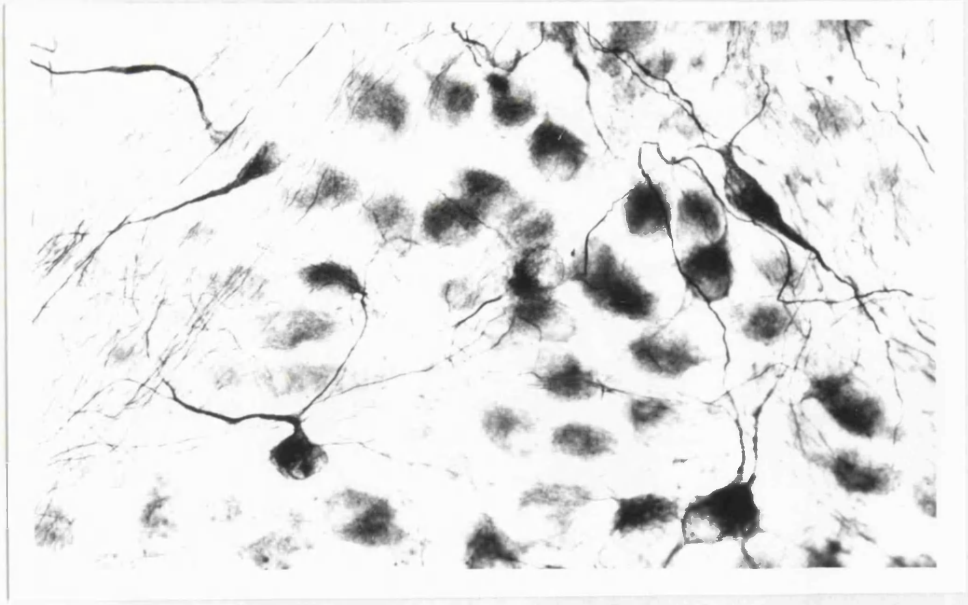


Figure 4. A ganglion containing argyrophobes and several argyrophils with well formed processes in a baby aged 5 months.

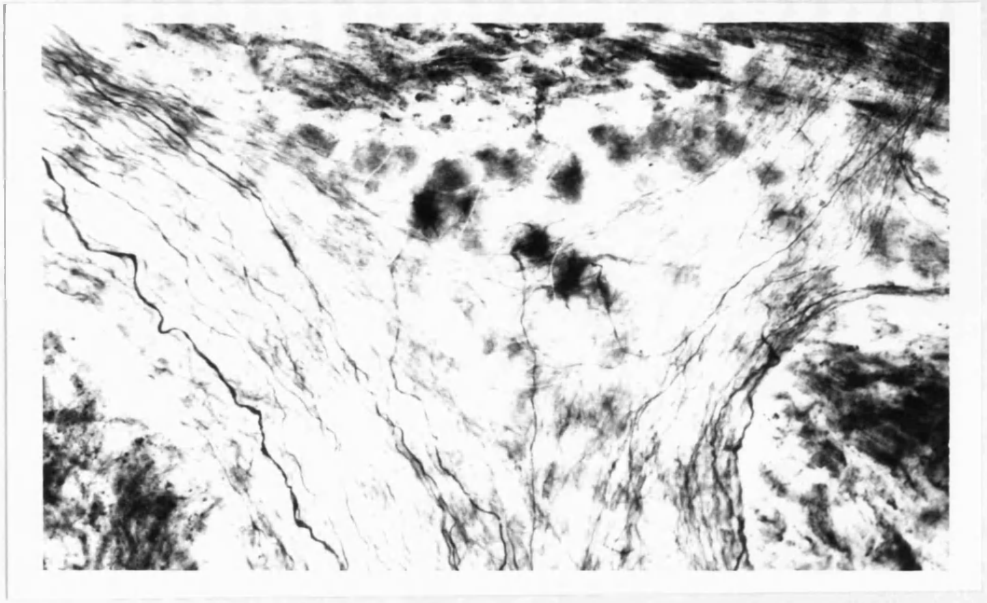


Figure 5. A ganglion in a baby aged 5 months showing poorly impregnated neurons with poor processes.

Figure 6. Condensed barely argyrophilic neurons from a 4-day-old.

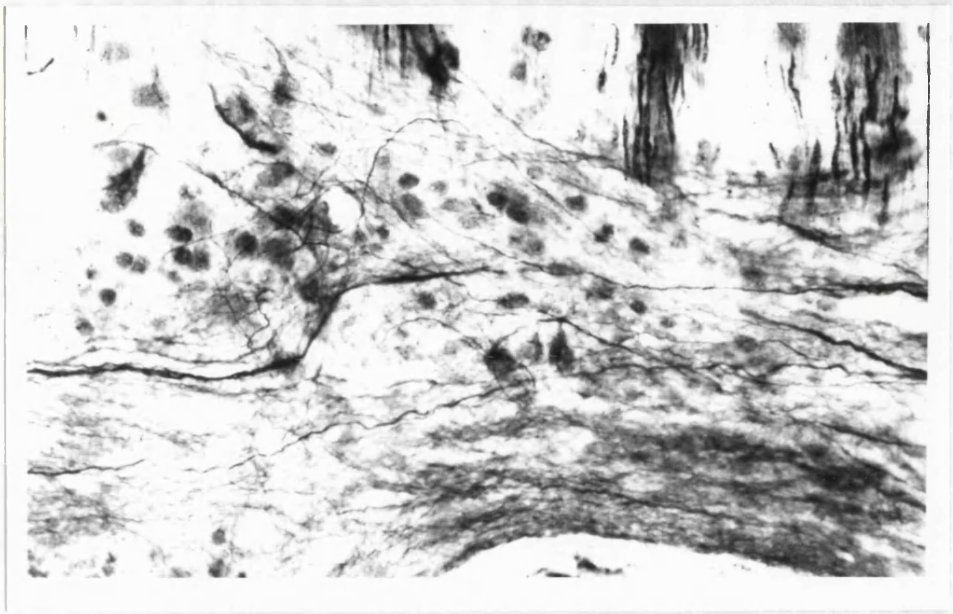


Figure 7. Faint argyrophilia in occasional neurons without appreciable processes. Baby aged 7 months.

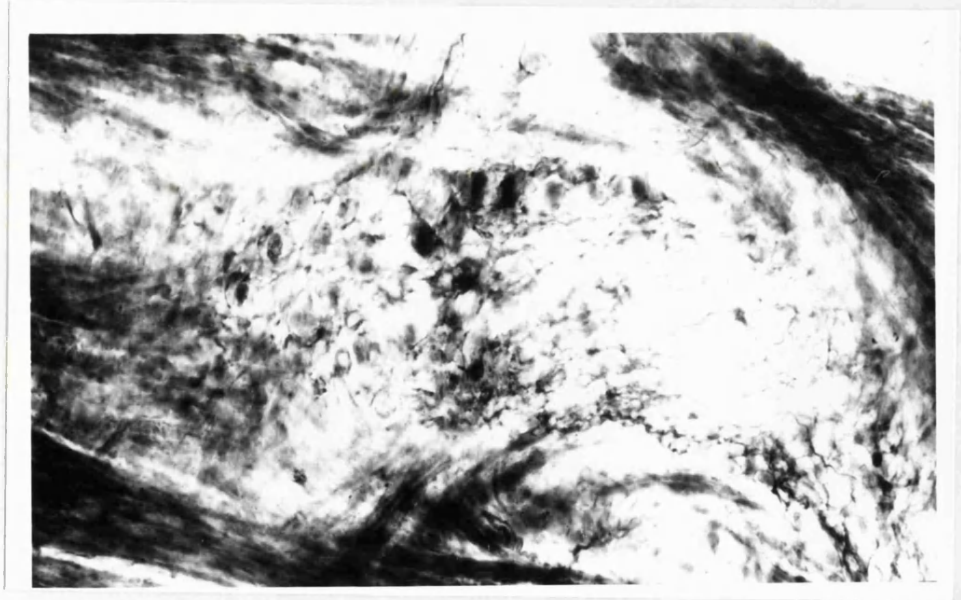


Figure 8. Occasional condensed argyrophil showing no obvious processes. Fragmented twisted axons in the connecting tracts. Baby aged 7 months.

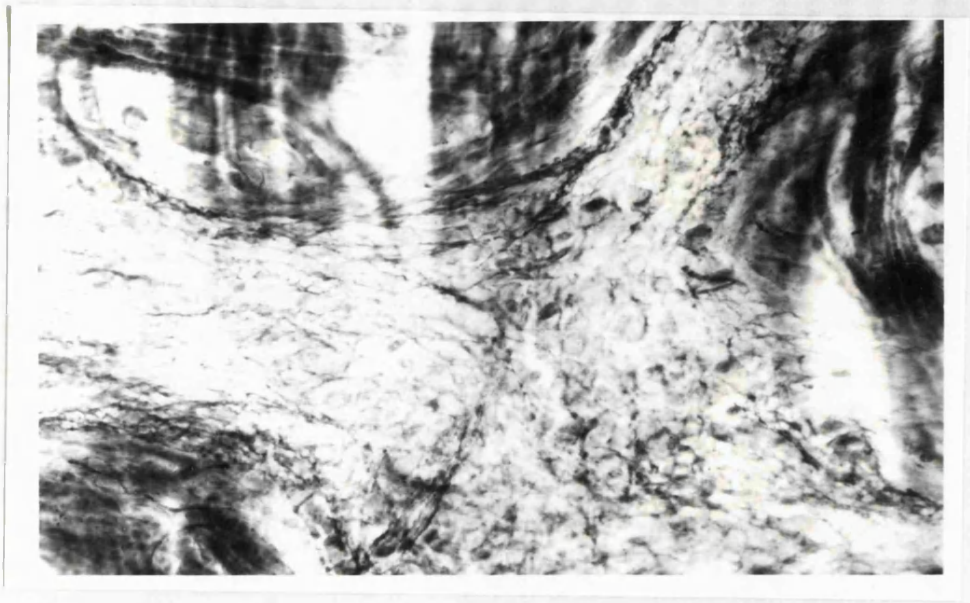


Figure 9. Absence of argyrophilic neurons in a ganglion from a still born term infant. Axons in the connecting tract are also faint and fragmented.

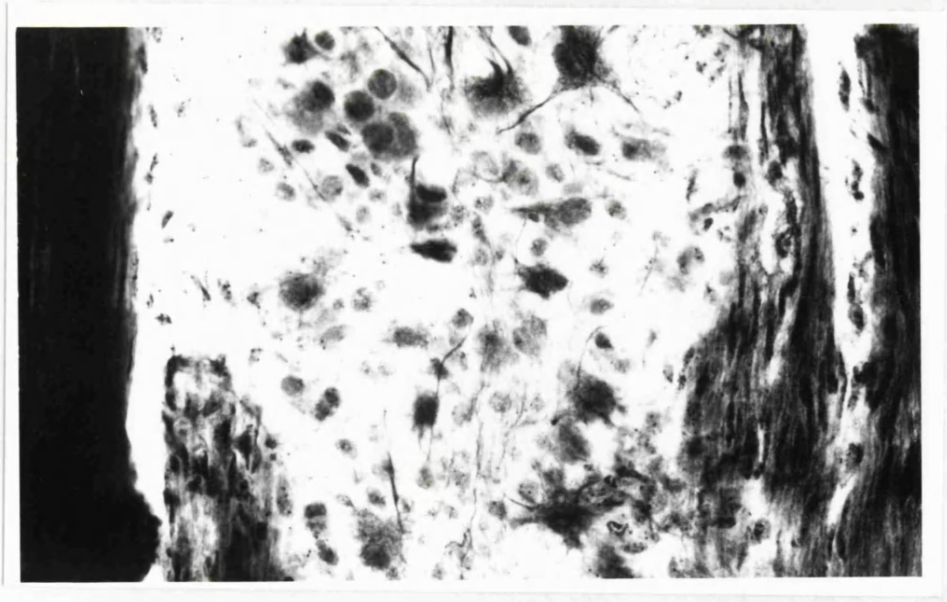


Figure 10. Poorly impregnated spotty barely argyrophilic neurons with occasional processes. The argyrophobic neurons also appear small and ill defined. A baby aged 7 months.

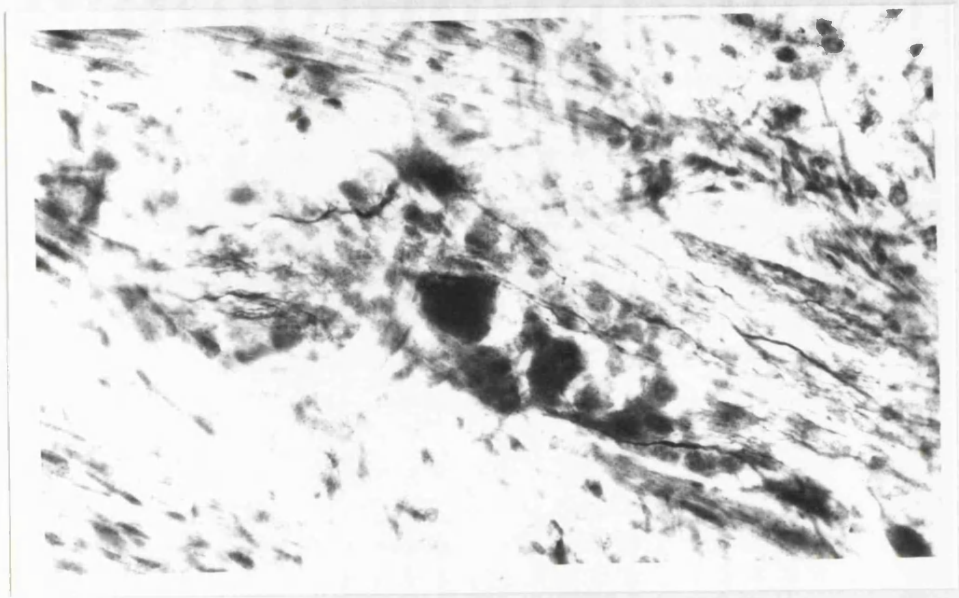


Figure 11. Large, fuzzy and bloated argyrophils showing no evident processes in a 14 year-old child.

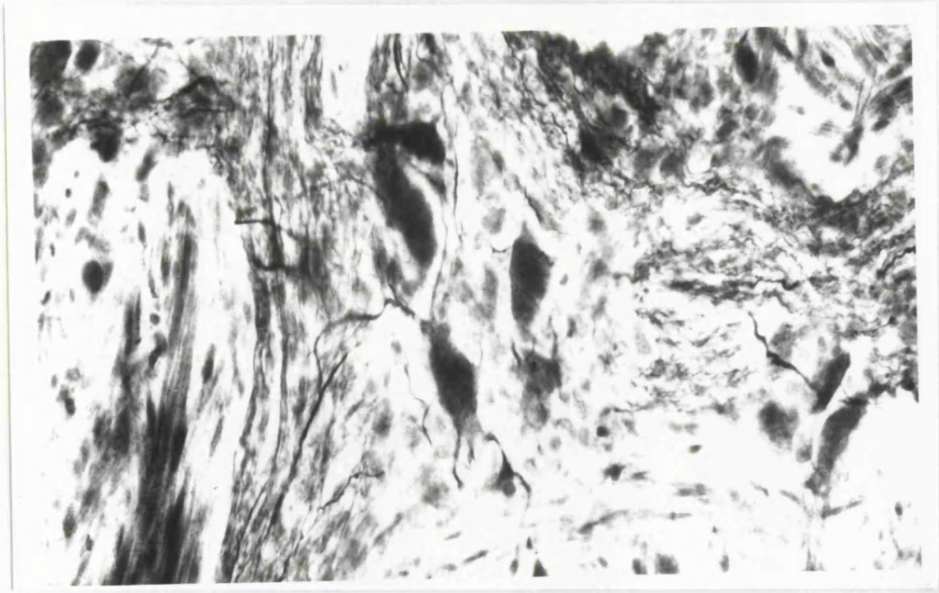


Figure 12. Another ganglion from the 14 year-old boy showing large ill defined argyrophils with occasional processes. Note also poorly formed knobbly and twisted but black axons in the connecting tracts.

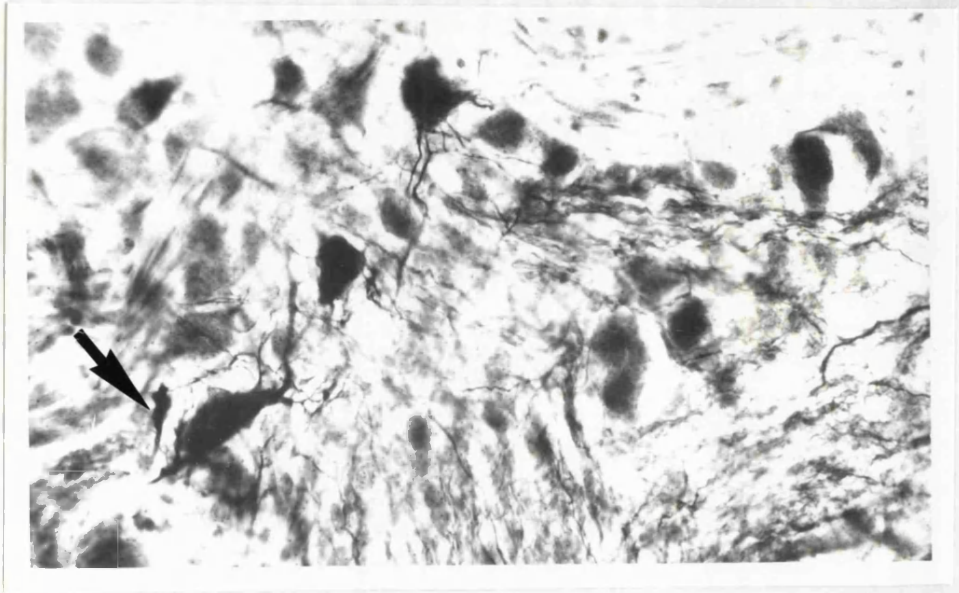


Figure 13. A third ganglion from the 14 year-old boy showing apart from the large and fuzzy neurons also an axonal knot (arrow).

Figure 14. A ganglion from a 20 month-old child with large and ill defined argyrophils showing only occasional stubby and stunted processes.

Of these 48 control samples, 17 had strongly argyrophilic neurons although in two the processes were twisted and in one the neurons appeared condensed. Whilst argyrophobic neurons were plentiful in a further 17, no silver-positive cells could be identified in any of the ganglia included in the sections. In one of the latter group the argyrophobic neurons were small. The three stillborn babies were all in this group with no argyrophilic neurons. In samples from a further two patients only one silver-positive nerve cell was encountered in each after examination of several ganglia comprising only argyrophobes. The remaining 12 specimens contained neurons with faint argyrophilia and of these eight samples showed neurons consisting of swollen, woolly or fuzzy cells (5), large, speckled cells (1) or small, shrunken cells (2).

5.3.2 Relationship of Age and Argyrophilia

The age of the children in the group with good argyrophilic neurons (n=17) ranged from 3 days to 8 years (median 7 months). The age range for infants and children with no or only one argyrophilic neuron(s) (n=16) was 8 hours to 1 year (median 8 weeks). None of the still-born infants (n=3) had silver positive neurons. A variable degree of silver uptake, with only occasional neurons exhibiting strong argyrophilia, was seen in remaining 12 specimens from children aged 1 day to 14 years (median 8 weeks).

In every specimen where argyrophilic cells were observed, in all the ganglia argyrophobic cells outnumbered the argyrophils; the ratios of argyrophobes to argyrophils ranged from 3:1 to 37.2:1 (median 9:1). No clear relationship could be demonstrated between age and the ratio of argyrophobes to argyrophils (Fig.15). Similarly the neuronal numbers appeared not to be age related (Fig.16).

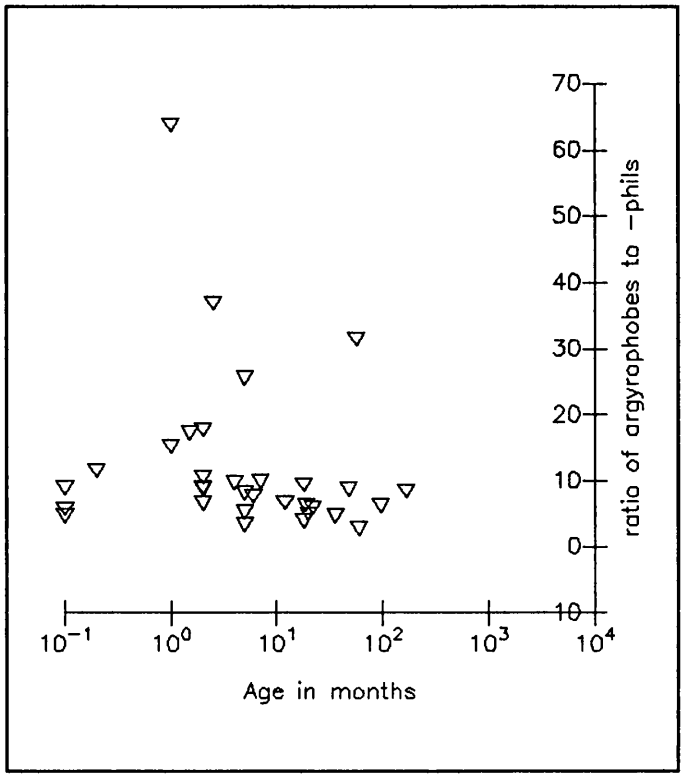


Figure 15. The ratio of argyrophobic to argyrophilic neurons plotted against age (logarithmic scale). A logarithmic scale is used because the majority of patients were young and on an arithmetic scale the values are closely clustered.

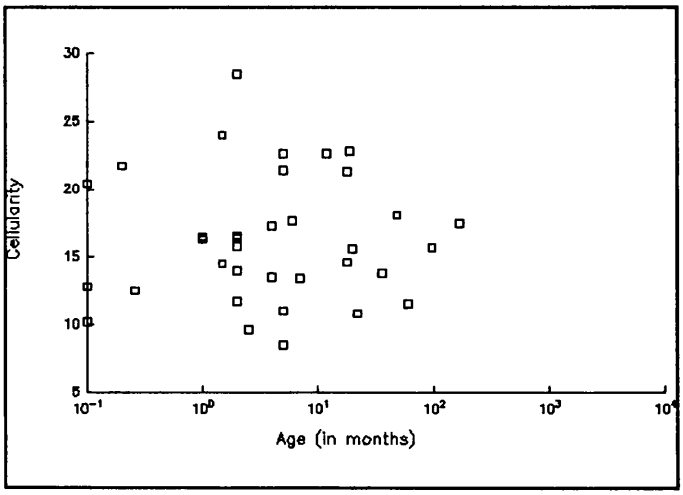


Figure 16. Number of myenteric neurons plotted against age (logarithmic scale).

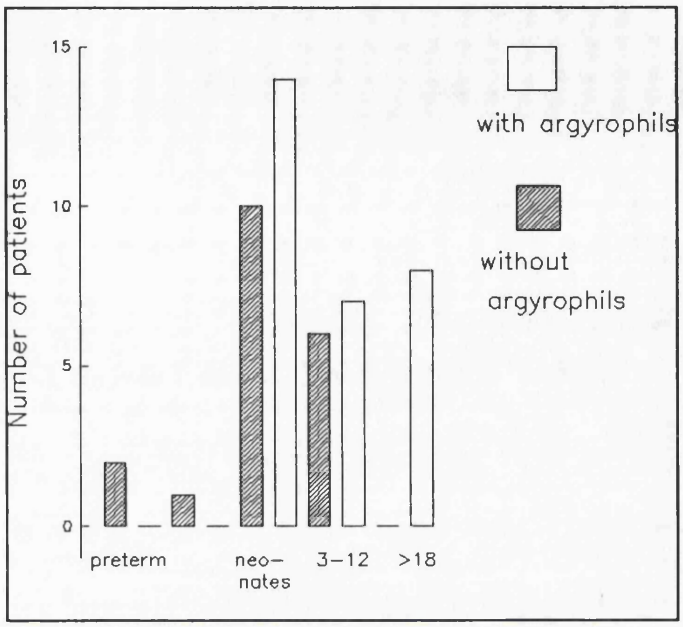


Figure 17. The number of patients with argyrophilic neurons and those without argyrophilic neurons in relation to age. Preterm, neonates, children aged between 3 and 12 months and those over 18 months.

5.4 Results in the Ileal Specimens

The morphological variations in the samples from patients with pseudo-obstruction were similar to, but less marked than in the normal controls. These included indistinct, swollen argyrophilic cell bodies with thin, tangled or fragmented processes as well as strongly argyrophilic 'spidery' neurons with distinct linear processes. In six of the 16 samples no argyrophilic neurons were seen and three had only occasional condensed argyrophils with short fragmented processes. The age of the patients with no silver-positive cells ranged from 5 weeks to 12 years (median 4 years). The three patients with only occasional, condensed silver-positive cells were siblings (an elder brother aged 14 months and his twin sisters aged 10 months at the time of the biopsy).

In the specimens in which argyrophilic cells were seen, the ratios of argyrophobic to argyrophilic cells ranged from 3:1 to 10:1 (median 5:1).

5.5 Discussion

Metal impregnation methods are notoriously finicky and consistent results can be difficult to reproduce. The specificity of these techniques relies heavily on stringent attention to detail in their performance. To control this aspect, samples with known strongly argyrophilic neurons must be included in each staining batch. Appearances in the intestine may differ between necropsy material and surgically resected intestine due to effects of muscle contraction in the latter. This can be overcome by allowing the layers of intestinal muscle of surgically resected specimens to relax (for 6-18 hours) in a moist chamber at 4°C prior to fixation.

It has been suggested that argyrophilic neurons are responsible for ordered peristaltic contraction by emitting a specific neurotransmitter (Tanner et al. 1976). However, it is currently thought that argyrophilia merely reflects the neurofilament content of the neuron (Christensen et al. 1990). Although strongly silver-positive neurons may be seen in the sigmoid colon soon after birth, in the majority of specimens argyrophilic neurons appeared only at some time during the first year of life (Fig.17). In contrast, between 1 year and 18 months of age the absence of these neurons was exceptional, and after 18 months argyrophilic neurons were always seen. Thus the presence of argyrophilic neurons appears to reflect a developmental process involving intrinsic intestinal innervation. Consequently the absence of argyrophilia may be of pathological significance only after the first year life reflecting an arrest of this maturation process.

Three patients with absent argyrophilic neurons in the terminal ileum were older than 18 months (8, 8 and 12 years). In all of these patients abnormalities were seen on routine histological or histochemical techniques. Two were noted to have hypoganglionosis with only scanty, small ganglia. The third child was also hypoganglionic and showed grossly reduced transmural AChE activity. In particular the circular muscle coat appeared denervated and on ultrastructural examination empty and vacuolated nerve tracts were found. Thus silver staining in these specimens did not add to the diagnosis but merely reinforced observations made using routine techniques.

The other three infants with no argyrophilic neurons in the terminal ileum were aged 5 weeks, 3.5 months and 7 months. Routine histology and electron microscopy in these patients was unhelpful. In the light of control data the absence of silver-positive neurons cannot be regarded of pathological significance.

Silver staining in the three siblings with scanty, condensed argyrophilic neurons in the terminal ileum may reflect a pathological abnormality but equally could represent a variant of the normal morphological appearance at a particular stage in the development of the intestinal innervation, since all these babies were less than one year of age and similar condensed small neurons were encountered in the controls.

In the control material wide variations in morphology of the myenteric plexus were seen. In one 14 year-old boy with cerebro-spinal deformities, the myenteric plexus contained fuzzy, swollen poorly impregnated cells with inadequate, often bulbous processes. This appearance could possibly be the result of the same process affecting

both ENS and CNS.

A wide variation in the proportions of argyrophilic and argyrophobic neurons was seen in the terminal ileum from patients with intestinal pseudo-obstruction, but similar variations were also noted in the control specimens of colon as well as in a number of other intestinal resections, from various sites, not detailed here. It is apparent that there is a considerable variation in silver staining in normal enteric neurons. Until this is clearly defined the silver impregnation technique is unlikely to provide any striking new insights into the pathogenesis of intestinal motor disturbances in childhood. Ideally control material should have consisted of tissue from the terminal ileum, but because of the lack of any obvious differences between the appearances of the myenteric plexus in the terminal ileum and the colon, further investigation was not considered likely to be fruitful.

This study has shown an enormous variability in neuronal morphology using silver staining in the normal myenteric plexus and highlights the need for considerable experience and understanding of the range of normality in the interpretation of this technique. Indeed, at least some of the changes regarded as pathological in other series, such as blurring of neuronal cell outline and apparent disorganisation of cell processes, was encountered frequently in our control specimens. It is also important to consider the age of the patient in assessing the significance of an absence of argyrophilic neurons, since these cells may normally be absent, at least in the sigmoid colon up to 1 year of age. In the older child, however, the absence of silver-positive cells should be regarded abnormal.

Silver staining particularly in the adult patients is regarded as an important tool in the identification of morphological mostly degenerative abnormalities (Schuffler 1989) not appreciated on routine sectioning. However, the information gained using these techniques is limited and the investigation requires a large tissue sample. For these reasons there is a clear need to explore other methods for examining intestinal innervation. With increasing understanding of the physiology of intestinal motor activity and the connections between the extrinsic and intrinsic neural pathways (Furness and Costa 1987; Furness et al. 1989), silver staining is likely to become obsolete. However, the concept of tangential sectioning allows the assessment of a larger volume of the intermuscular plexus and could be adapted usefully, for instance, to immunostaining techniques (see Chapter 8).

Chapter 6. Light and Electron Microscopical Appearances in Intestinal Myopathies

6.1 Introduction

Although comparatively little is known about the myopathic varieties of CIIP, it appears that they often involve the whole gastrointestinal tract, and may affect other organs such as the gall bladder and urinary tract (Christensen et al. 1990; Malagelada 1990). The examples with multiorgan involvement are now often termed hollow visceral myopathies (Krishnamurthy and Schuffler 1987).

Intestinal myopathies are more commonly recognised in adult practice (Schuffler et al. 1977a,1977b; Faulk et al. 1978; Lewis et al. 1978; Jacobs et al. 1979; Smith JA et al. 1982; Anuras et al. 1983,1986a; Smout et al. 1985; Venizelos et al. 1988; Alstedt et al. 1988; Colemont and Camilleri 1989; Rodriques et al. 1989; Martin et al. 1990;) but are also increasingly diagnosed in the paediatric age group (Puri et al. 1983; Milla et al. 1983; Bagwell et al. 1984; Anuras et al. 1986b; Vargas et al. 1988; Schuffler et al. 1988a; Nonaka et al. 1989). Both sporadic and familial examples are seen, and the mode of inheritance as well as the patterns of intestinal and other organ involvement are variable. Schuffler and Pope (1977b) described an autosomal dominant type with oesophageal dilatation, megaduodenum, redundant colon and megacystis. An autosomal recessive type (Anuras et al. 1983) is characterised by dilatation of the small intestine with multiple small intestinal diverticula. These patients may have ptosis and external ophthalmoplegia, but do not have megacystis. Other familial visceral myopathies involving single families are also reported (Jacobs et al. 1979; Foucar et al. 1985; Anuras et al. 1983,1986a; Alstead et al. 1988) and

in some kindreds the absence of male-to-male transmission raises the possibility of an x-linked dominant inheritance (Faulk et al. 1978; Rodriques et al. 1989). The most severe, almost universally fatal type of intestinal myopathy is described as the megacystis, microcolon, intestinal hypoperistalsis syndrome (Puri et al. 1983), although some investigators believe it to be the result of neural defects (Berdon et al. 1976; Armoury et al. 1977; Wiswell et al. 1979; Kirtane et al. 1984; Bindl et al. 1989). In this condition mainly affecting females, the bowel is shortened, the proximal small bowel is dilated whilst the distal intestine is narrowed and malrotated. The mode of inheritance for this disorder is not entirely clear. The original report by Berdon et al. (1976) described five female infants two of whom were sisters and Puri et al. (1983) also reported affected siblings indicating a possible autosomal recessive condition. Currently in the 10th edition of McKusack' Mendelian Inheritance in Man (1992) this disorder is considered to be autosomal recessive but clearly needs to be distinguished from milder dominantly inherited intestinal myopathies involving the urinary tract. Sporadic examples of intestinal myopathy have also been reported (Schuffler et al. 1981; Schuffler 1989). Degenerative enteric leiomyopathy seen in native African children has been observed (Kaschula et al. 1987) often in association with inflammation of the muscularis propria and has been postulated to have an immune or toxic aetiology.

As described in Chapter 2, intestinal myopathy is associated with characteristic electrophysiological findings, but the morphological data, particularly that obtained by conventional histology are frequently unrewarding. Even with electron microscopy, pathological abnormalities may be sparse or absent, and when present are often difficult to distinguish from artifact.

Fibrosis of the muscularis propria, and vacuolation of smooth muscle cells in the muscle coats are the most frequently described changes. When gross these changes may be recognised by light microscopy (Schuffler et al. 1977; Rodriques et al. 1989; Anuras et al. 1983,1986a; Smout et al. 1985), but the identification of lesser degrees of fibrosis and smooth muscle vacuolation requires ultrastructural examination (Puri et al. 1983; Milla et al. 1983; Krishnamurthy and Schuffler 1987; Lake 1988). These changes may be more pronounced in the longitudinal (Schuffler et al. 1977,1981; Jacobs et al. 1979; Anuras et al. 1983,1986a; Rodriques et al. 1989) or in the circular muscle coat (Shaw et al. 1979; Lake et al. 1988; Nonaka et al. 1989). In some examples abnormalities are seen equally in both layers of the muscularis propria (Smith JA et al. 1982; Smout et al. 1985; Anuras et al. 1986a; Schuffler et al. 1988; Alstead et al. 1988a) and may occasionally involve the muscularis mucosae (Alstead et al. 1988). Accumulation of sarcoplasmic glycogen has also been described (Dieler et al. 1990) and intramyocytic inclusions (Martin et al. 1990) have been found in one kindred with autosomal dominant inheritance, having a myopathy confined to the internal anal sphincter. Other reported abnormalities include non-uniform electron density of the myocyte cytoplasm (Schuffler et al. 1977a; Shaw et al. 1979; Smith JA et al. 1982; Alstead et al. 1988) and discontinuity of myocytic plasma membranes (Schuffler et al. 1977a).

In this chapter the light and electron microscopical appearances seen in the muscle coats in CIIP are addressed, in an attempt to delineate those changes that might be specific to intestinal myopathy. A major problem in interpreting the ultrastructural changes stems from various constraints in the material examined, which apply particularly to resected specimens of human intestine. The surgical procedures

involved in their removal are lengthy, ligation of vessels prior to excision results in the tissue being exposed to possible ischaemic damage, and the relatively large samples required for proper orientation mean that fixation for electron microscopy can be distinctly suboptimal. All these factors may influence the ultrastructural appearances so that defining changes that are of specific pathological importance from induced artifacts may be very difficult. This is almost certainly the reason why apparent ultrastructural abnormalities are seen in cases without evidence of primary functional abnormality of smooth muscle as identified by electrophysiological studies and described in Chapter 2.

Thus, in this chapter attention is focused on those samples from patients with electrophysiological evidence of intestinal myopathy (see Chapter 2) but also includes other specimens from patients with CIIP of neurogenic or undetermined origin. One sample of functionally normal colon excised during colonic interposition for oesophageal atresia is also included. In an attempt to identify artifacts additional samples were examined from two animal experiments (using rats and piglets) involving short or longer periods of intestinal ischaemia of known duration, performed in separate studies on free radical production and induction of necrotizing enterocolitis.

The intention is to define microscopic changes occurring only in proven cases of myopathy that might truly reflect primary smooth muscle disease. Changes that occur equally in neuropathic or known ischaemic smooth muscle are, diagnostically non-specific and possibly artifactual. Apparently specific myopathic changes encountered in patients with CIIP symptoms, but not categorised as myopathic or

neurogenic by electrophysiological studies (the undetermined group) might then be designated as myopathic by morphological criteria.

6.2 Materials and Methods

6.2.1 Animal Tissue

6.2.1.1 Rat Experiment: Effect of ischaemia (short duration) on enteric smooth muscle

The tissues employed were the byproduct of an experiment to investigate whether intestinal ischaemia, with or without reperfusion, could affect membrane lipid peroxidation produced by an increase in free radical production. This experiment provides information on possible ultrastructural alterations induced by short term ischaemia of known duration. The study (performed by Drs. PJ Milla and K Lindley, Institute of Child Health) required the removal of samples of small intestine from male Wistar rats for biochemical analysis, but parts of these samples were donated for the present investigation. The procedures used produced small intestinal ischaemia for 10 minutes by ligation of the superior mesenteric artery in six rats. In addition, the intestine in three of the six was reperfused for 5 minutes after release of the ligature. Intestine from three control animals in which the artery was not ligated was also examined.

The animals (aged 55 weeks) were sacrificed by cervical dislocation and proximal jejunum was removed of which a ring of bowel measuring 3mm from each animal was fixed at room temperature for 24 hours in 2.5% glutaraldehyde in 0.1M cacodylate buffer at pH 7.4 containing 2.5mM calcium chloride. Blocks of tissue from each ends of the specimen including the full thickness of the bowel wall were

then processed into Araldite resin for electron microscopy (see Appendix).

Ultrathin sections were cut, contrasted with uranyl acetate and lead citrate (see Appendix) and viewed by transmission electron microscopy. The appearances of the circular and longitudinal muscle layers and the myenteric plexus were scrutinised for any alterations and photographed. The mucosal morphology was also examined.

6.2.1.2 Pig Experiment: Effect of ischaemia (prolonged) on enteric smooth muscle

The tissues examined were from an experiment investigating the induction of necrotizing enterocolitis in newborn piglets by prolonged (48-72 hours) segmental ligation of mesenteric vessels (performed by Dr. P Sibbons, Institute of Child Health). Samples of ileum were kindly donated for this investigation, which provides information on the ultrastructure of the intestine following prolonged circulatory arrest of known duration.

Under general anaesthesia using halothane, oxygen and nitrous oxide delivered through a close-fitting mask, six normal and one low birth weight newborn piglets from an outbred commercial herd (Large White/Landrace strain) were subjected to ligations of various vessels supplying the terminal ileum. The samples of ileum were recovered when the animals were killed 3 days after vascular ligation.

The mesenteric vascular arcades supplying the bowel are formed from groups of vessels, each comprising an artery, a vein and one or two efferent lymphatics. The lymphatics alone were ligated in five piglets, the vein in another animal and in the low birth weight piglet a combined arterial and lymphatic tie was used. Rings of

ileum (3mm width) supplied by the ligated vessels and samples of unaffected ileum were fixed and processed in the manner described above (6.2.1.1). The muscularis propria and the myenteric plexus were examined in detail.

6.2.2 Human Tissues From Patients with CIIP

6.2.2.1 Patients

Samples of intestine from a total of 50 patients with CIIP were investigated. These included the 26 patients in whom electrophysiological studies were performed (see Chapter 2) and 24 others. In addition, a sample of functionally normal colon taken at surgery for colonic interposition in the treatment of a child with oesophageal atresia was examined.

In Table 1 data are set out grouping these patients into those with myopathic disease (the 11 patients with electrophysiological evidence of myopathy as well as the patient with combined myopathy/neuropathy detailed in chapter 2); those with neuropathic disease (the 11 patients with electrophysiological evidence of neuropathy described in Chapter 2, as well as three patients with HD, three with hypoganglionosis, three with IND - see Chapter 3); and those with undetermined motility disturbance. The patients with undetermined motility disturbance comprised the three patients with normal traces on electrophysiological study (see Chapter 2) and 15 others in which electrophysiological studies had not been performed.

The colonic interposition sample is identified separately in the Table 1. In addition, the numbers of small and large intestinal samples, and the methods of investigation (light microscopy alone, or both light and electron microscopy) are

included in Table 1.

Table 1. Samples examined by light or electron microscopy

		Small gut		Large gut	
Disease Category	Number of Patients	LM only	LM&EM	LM only	LM&EM
Myopathy	12	0	11	0	8
Neuropathy	20	0	13	0	10
Undetermined	18	2	9	2	12
Colonic Interposition	1	0	0	0	1
Total	51	2	33	2	31

6.2.2.2 Methods

Full thickness specimens of intestine were obtained during exploratory laparotomy, surgical decompression or intestinal resection. The samples included pieces of full thickness intestine measuring 2cm length and 2cm width, sleeves of the complete intestinal circumference averaging 2cm in length, and lengths of up to 20 cm of resected intestine. The intestine was opened and a strip parallel to the long axis of the bowel extending from one resection margin to the other was taken, pinned flat on cork in 10% formalin in phosphate buffer and processed routinely into paraffin wax.

An adjacent longitudinal strip of bowel in blocks of 1cm length was snap-frozen in hexane cooled to the temperature of solid carbon dioxide (-90°C) and stored at -30°C to -40°C for histochemical studies. A full thickness slice of 3mm width and 1 cm length was fixed in 2.5% glutaraldehyde (see above 6.2.1.1) placing flat and with the mucosa down on card.

(a) Light Microscopy

Longitudinal blocks comprising the entire specimen (and on occasions additional transverse blocks) were processed into paraffin wax. Sections (3μm) from these blocks were stained with H&E to assess gross morphology.

Cryostat sections were cut from the snap-frozen intestine and stained with H&E, periodic acid Schiff (PAS) after celloidin protection, Gomori one-step trichrome and picrosirius. The activities of acid phosphatase using the Gomori lead precipitation method and acetyl cholinesterase were also assessed on cryostat sections (details of the methods are found in Appendix).

(b) Electron Microscopy

A full thickness slice of 3mm width and 1 cm length was fixed and processed as described above in 6.2.1.1.

6.3 Results

6.3.1. Rat Experiment

Ultrastructurally, the smooth muscle cells within the muscularis propria and muscularis mucosae appeared healthy with well formed caveoli, and clearly defined

dense bodies and dense bands. Slight looseness of the contractile filaments within the myocyte cytoplasm was seen and mitochondria appeared mildly dilated (Fig.1). No abnormality was discernable in the nerves within the enteric plexuses nor in those in the muscularis or in the lamina propria. The mucosa appeared healthy and no morphological alterations could be detected in the enterocytes.

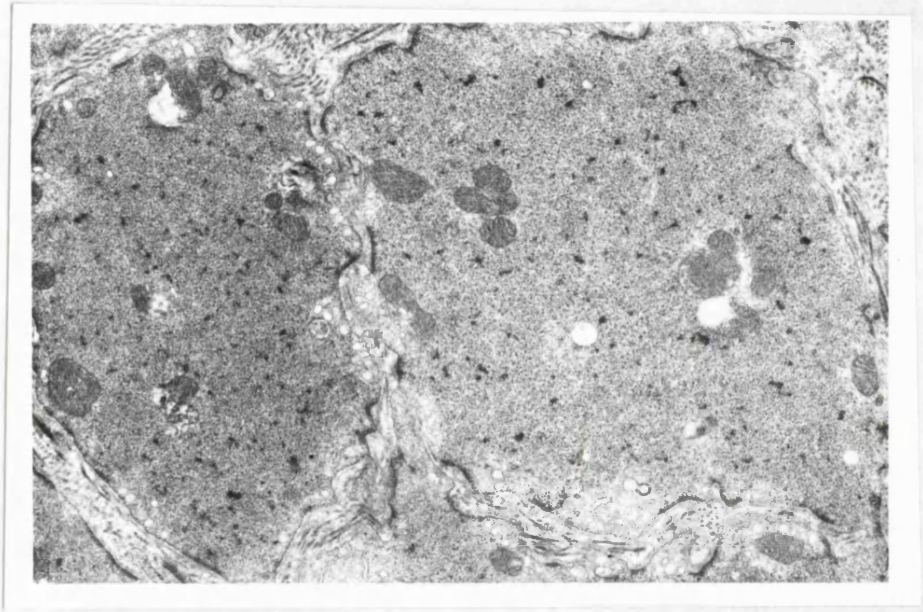


Figure 1. Electron micrograph of ischaemic reperfused rat intestine showing good preservation of smooth muscle cells. Occasional mitochondria show evidence of vacuolation but most are well preserved with well formed cristae. Normal caveolae, dense bodies and bands and well organised myofilaments. Magnification x10900

These appearances were seen in intestine from all the animals examined including those with intestinal ischaemia with or without reperfusion. Thus it was concluded that acute ischemia of up to 10 minutes duration did not produce changes that are

identifiable on routine electron microscopy.

6.3.2 Pig Experiment

Venous ligation alone was insufficient to produce changes identifiable on routine ultrastructural examination. A general looseness and disorganisation of the myofilaments with a loss or reduction in dense bodies were found in the circular muscle of intestine removed from piglets where lymphatics had been occluded (Fig.2). In addition, sub-sarcolemmal blebbing was apparent in these specimens. Increased amounts of rough endoplasmic reticulum and a prominent Golgi apparatus, together with accumulation of a lipid-like substance within the myocytes was also a feature. This appearance was not seen in the same animal in a block of intestine taken from intestine with a normal vascular supply away from the occluded segment. No abnormalities were seen in the nerves.

In the low birth weight piglet where NEC was produced by a combined arterial/lymphatic tie, the intestine adjacent to the NEC lesion showed gross disorganisation of the muscle (Fig.3) and proliferation of endoplasmic reticulum (Fig.4). Severe disruption of plasma membranes and disorganisation of myofilaments with an obvious loss of dense bodies was noted in the myocytes. However, dense bands remained in the intact portions of the plasma membranes, although caveoli were sparse throughout. Mitochondria were ill-defined with damaged cristae and the smooth muscle cells were separated by dilated empty spaces. Gross subsarcolemmal blebbing was seen throughout. Due to the degree of destruction of the muscle, the nerves were also harder to trace, but contained normal filaments and neurosecretory granules. It was sometimes hard to discern whether a structure was an abnormal

empty nerve process or a large sub-sarcolemmal bleb, but generally these structures could be traced back to the muscle cytoplasm strongly suggesting that the latter interpretation was correct.

Intestine not submitted to vascular occlusion showed no abnormality (Fig.5).

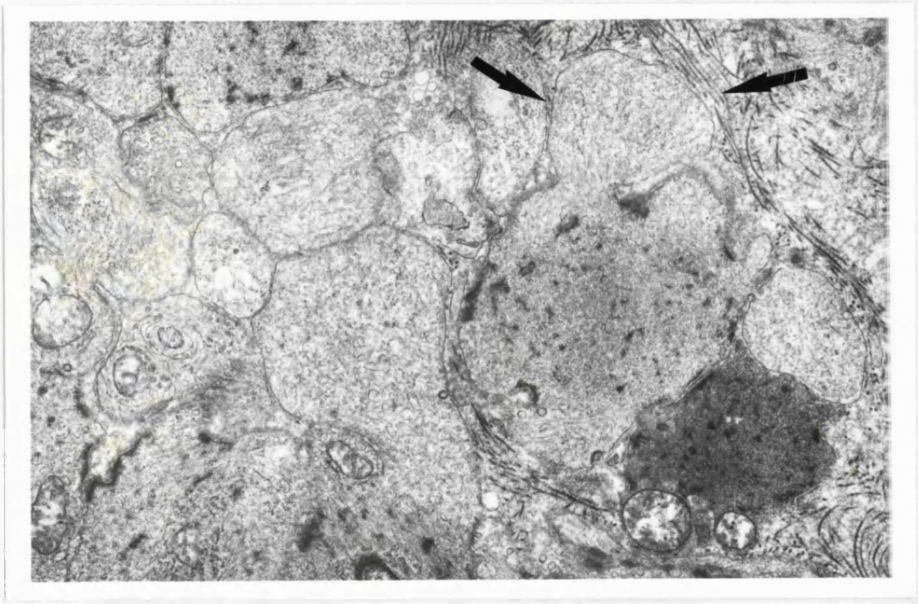


Figure 2. Electron micrograph of circular muscle in a piglet subjected to lymphatic occlusion. Note the prominant subsarcolemmal blebbing (arrows) with loosely distributed myofilaments. Magnification x10900

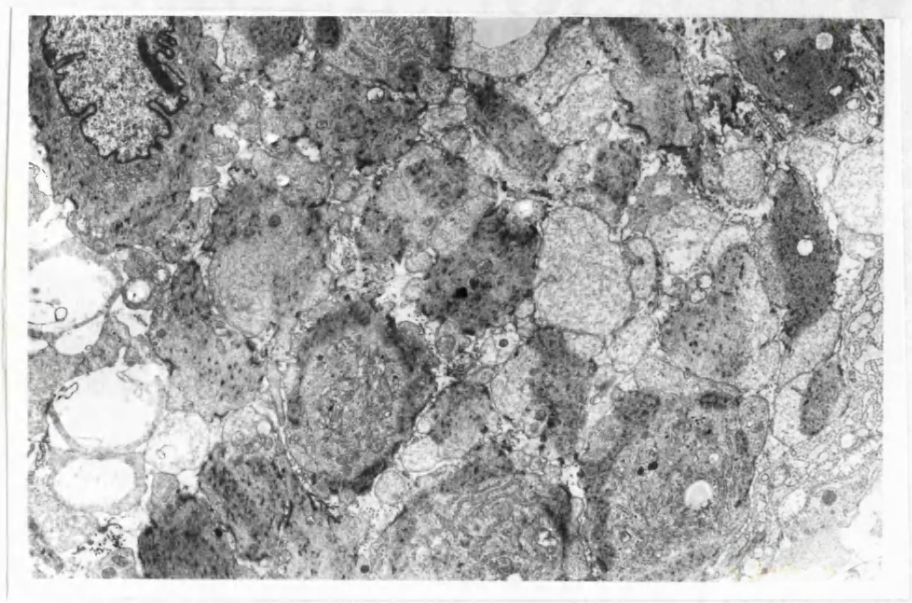


Figure 3. Ultrastructure of the circular muscle in a low birth weight piglet subjected to combined arterial/lymphatic ligation showing gross destruction of the smooth muscle. Vacuolation and prominant blebbing is seen together with proliferation of endoplasmic reticulum. Magnification x3600

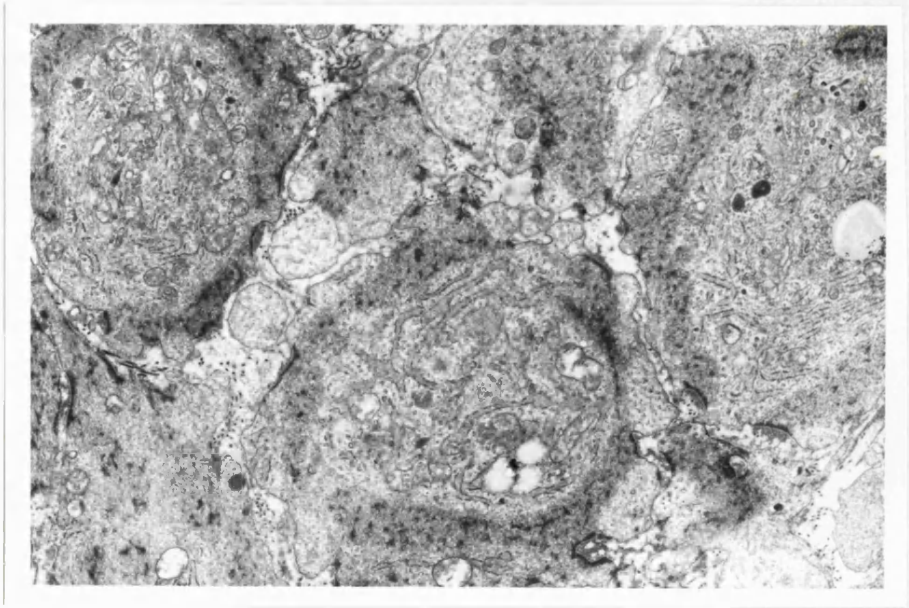


Figure 4. A higher magnification of the smooth muscle from the piglet shown in Fig.3 illustrating the proliferation of the endoplasmic reticulum. Magnification x7300.

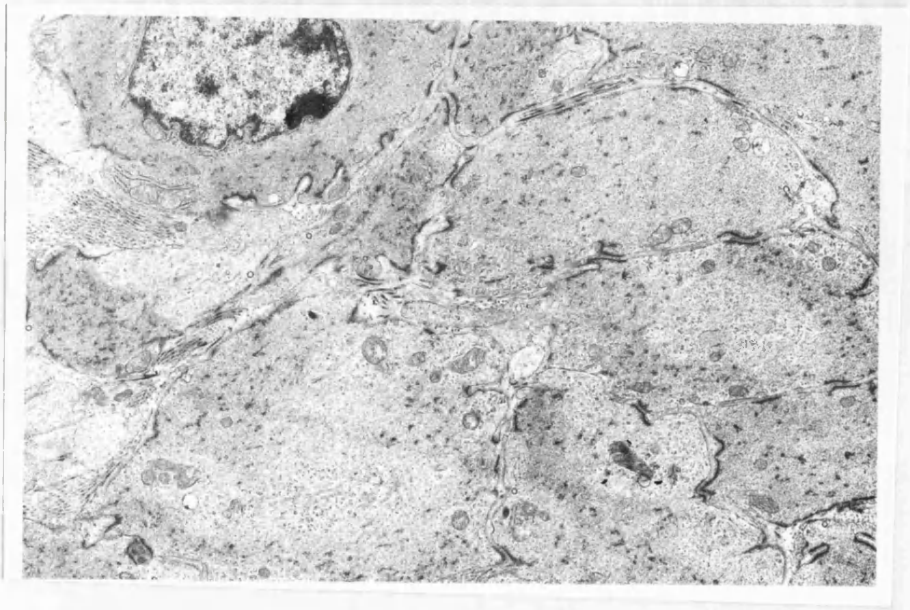


Figure 5. Intestine not subjected to vascular occlusion from the same piglet as seen in Figs. 3 and 4. Magnification x5500

Table 2a. Correlation of light microscopical appearances with putative primary diagnosis

Disease Category	Number of Patients	Light Microscopy: Fibrosis	Myocyte Atrophy	Myocyte Vacuolation
Myopathy	12	3	3	2
Neuropathy	20	0	0	0
Inter-position	1	0	0	0
Unde-termined	18	5	5	1

6.3.3 Human Resected Intestine

Light microscopical abnormalities were seen in eight patients (see Table 2a).

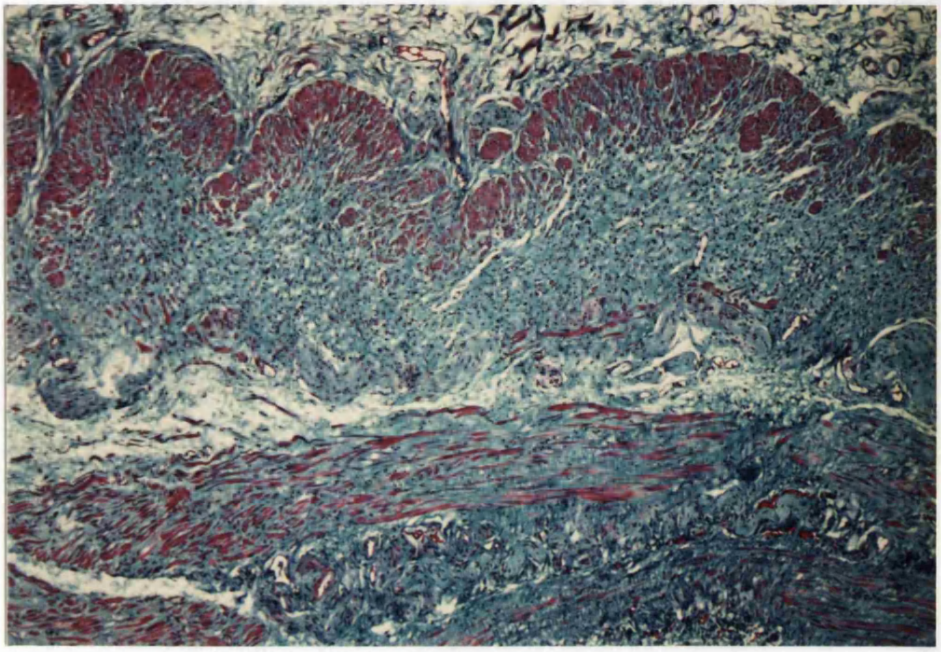


Figure 6. Trichrome stained muscularis propria showing prominent fibrosis (green) and a gross atrophy of smooth muscle cells (red) particularly in the circular muscle. Magnification x570

6.3.3.1 Fibrosis and Atrophy of the Smooth Muscle Cells in the Muscularis Propria on Light Microscopy

Gross fibrosis and atrophy of smooth muscle cells in the muscularis propria, especially in the circular muscle, was seen in eight patients using routine histological techniques (Fig.6). In six patients the fibrosis was seen in all the specimens examined including small and large intestine. In two others the fibrosis affected only a distal segment of the rectum.

6.3.3.2 Vacuolation of Individual Myocytes of the Muscularis Propria on Light Microscopy

In three patients the smooth muscle cells appeared vacuolated. However, only post-mortem tissue was examined from one of these patients. In routine sections no vacuolation was seen in the muscularis propria from the remaining 47 patients.

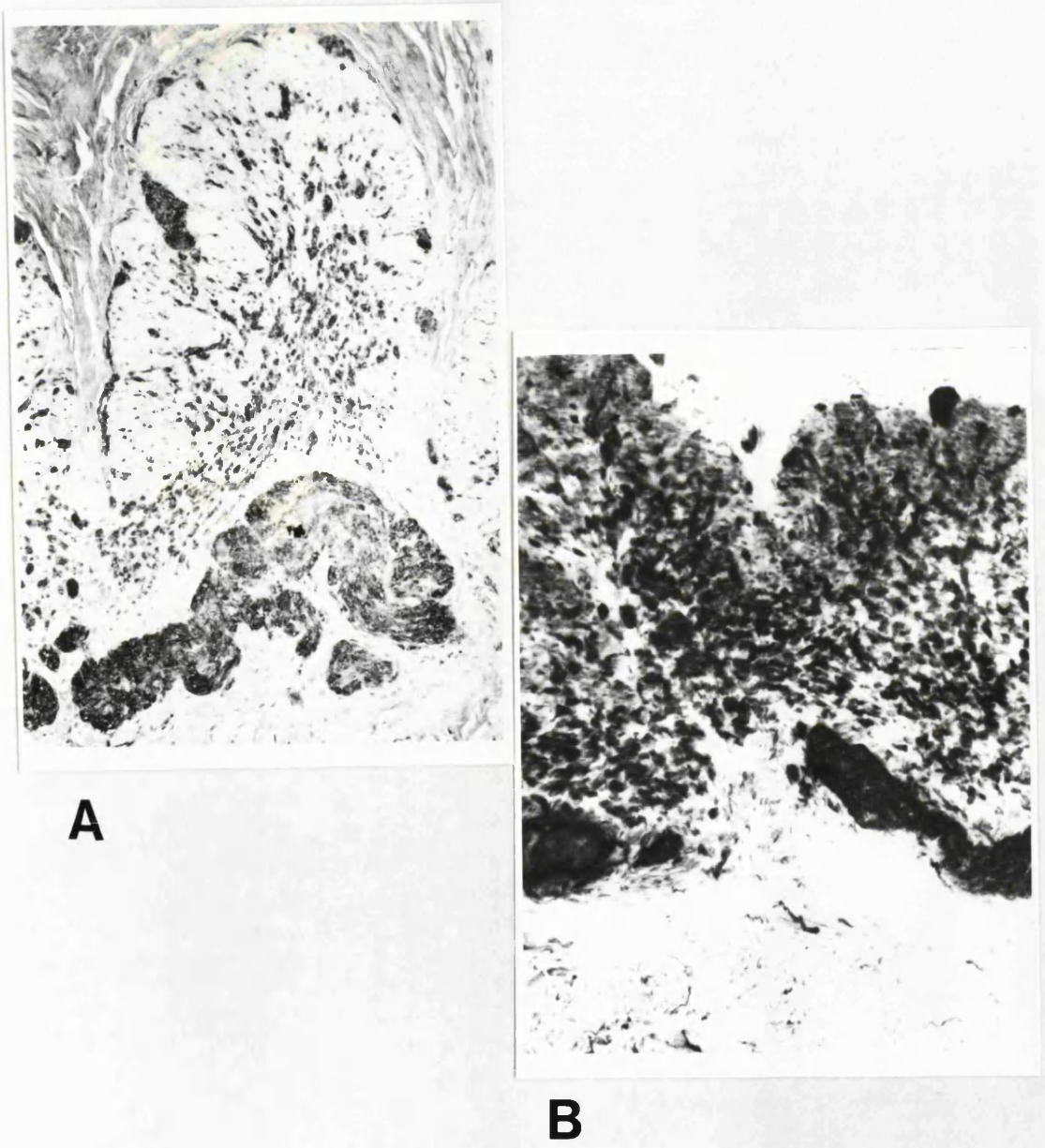


Figure 7. Photomicrographs showing pronounced proliferation of nerves in the circular muscle in the same patient as illustrated in Fig.6. (A) immunostaining for PGP 9.5 Magnification x120 (B) AChE-activity. Magnification x120

6.3.3.3 Light Microscopical Abnormalities of the Innervation of the Muscularis Propria Associated with Fibrosis

In all the samples from two patients with gross fibrosis of the muscularis propria and smooth muscle cell atrophy (Fig.6) there was a profound proliferation of nerve fibres within the muscle layers (Fig. 7 a and b). In one of these patients the nerve proliferation was more pronounced in the areas adjacent to the myenteric plexus whilst in the other it was distributed throughout the smooth muscle layers. In both of these patients there was also increased acid phosphatase activity within the myocytes indicating the presence of active lysosomes.

6.3.3.4 Electron Microscopical Changes

A variety of ultrastructural alterations of the smooth muscle cells in the muscularis propria were encountered (see Table 2b). In particular the mitochondria showed a range of apparent abnormalities. They were occasionally amorphous and often appeared dilated with poorly preserved cristae. In most patients some degree of mitochondrial dilatation was evident (Fig.8) and in 20 the mitochondria were grossly bloated with poor preservation of the cristae. This appearance was more noticeable in the circular than in the longitudinal muscle coat. From nine of these 20 patients the sample examined was a large resection specimen and from two patients multiple biopsies were obtained during an exploratory laparotomy.

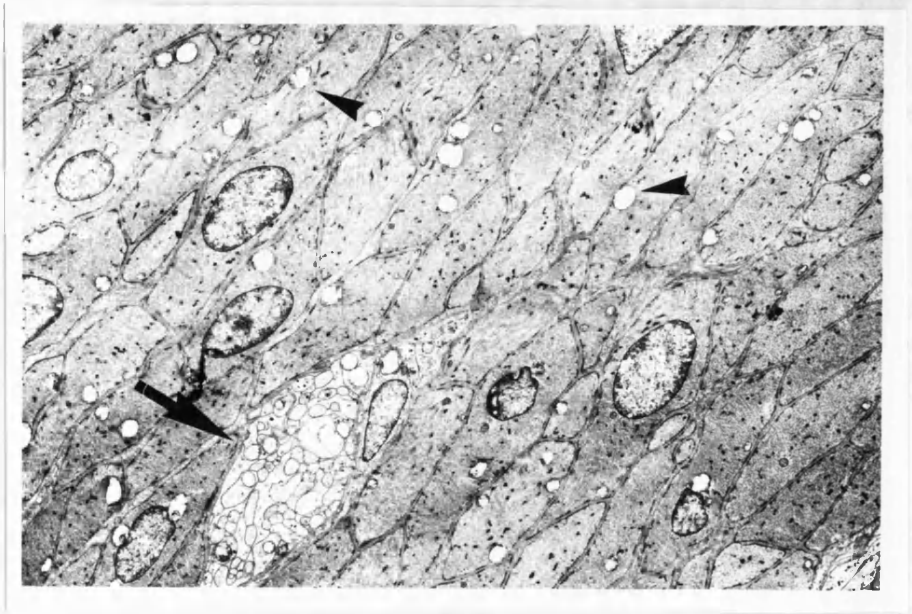


Figure 8. Relatively normal circular muscle. The smooth muscle cells are closely applied with little connective tissue between them. A nerve varicosity is seen (arrow). Note the vacuolar appearance of the mitochondria (arrow heads). Magnification x2800.

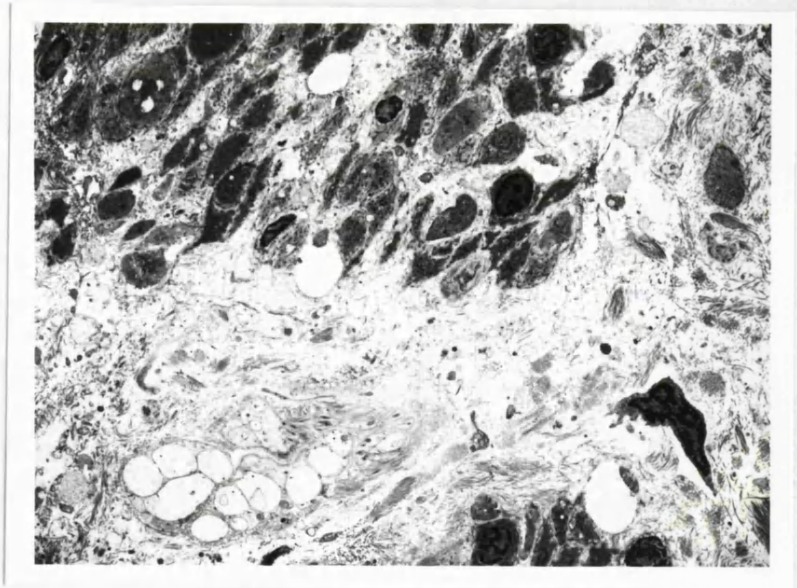


Figure 9. Gross fibrosis of the circular muscle and severe atrophy of the myocytes. Magnification x1600

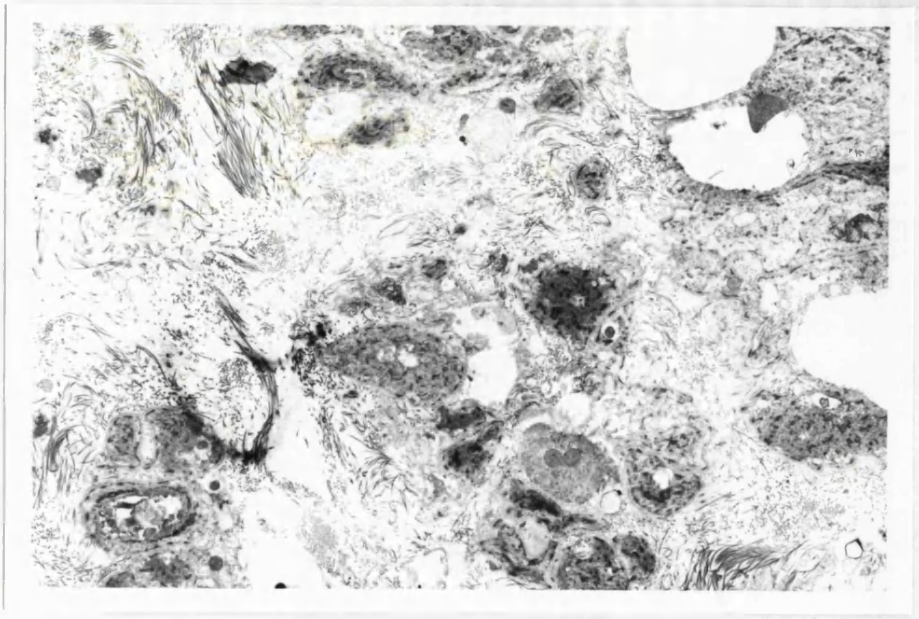


Figure 10. Profound increase in connective tissue with only occasional atrophic smooth muscle cells remaining. Magnification x3600

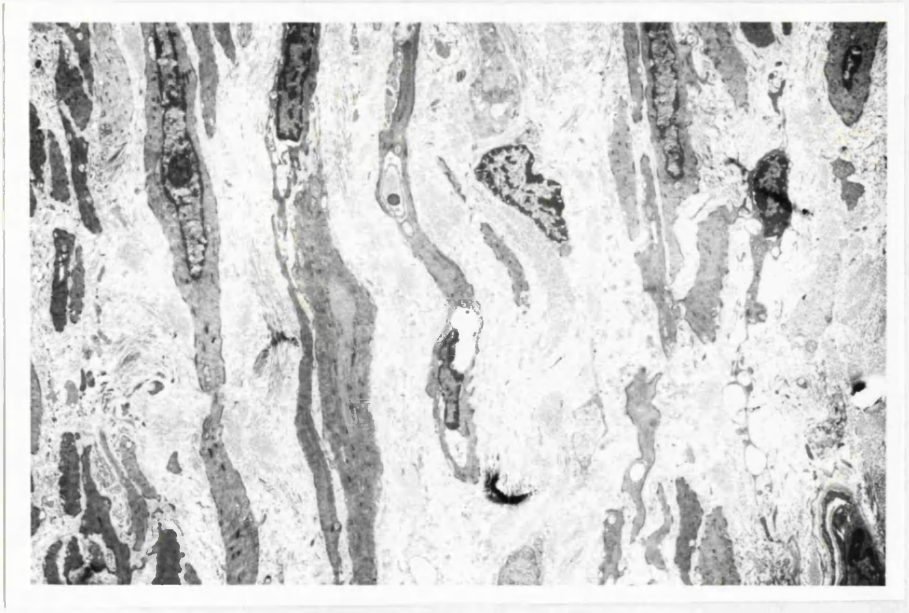


Figure 11. Prominent increase in connective tissue between atrophic and spindly smooth muscle cells. Magnification x5500

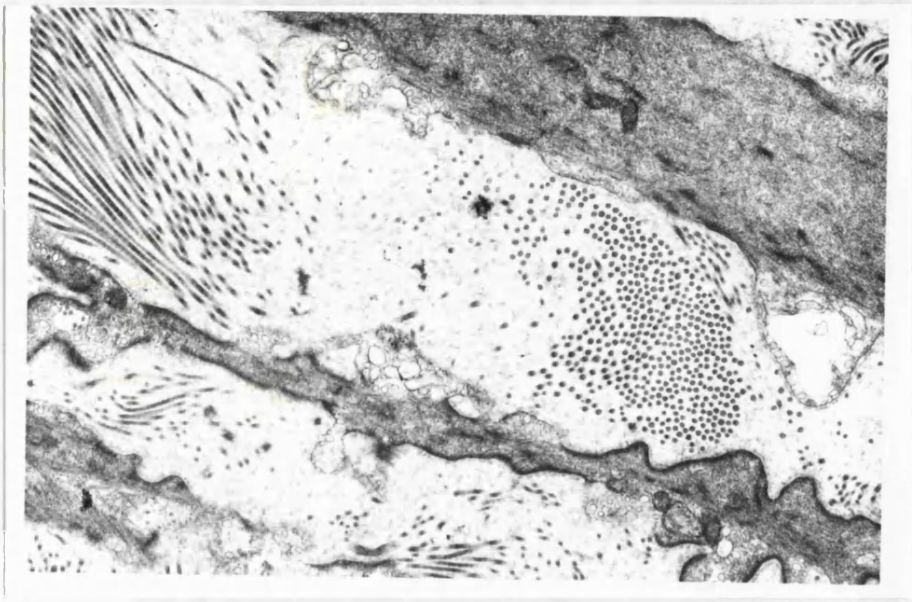


Figure 12. Higher magnification of the spindly and atrophic myocytes seperated by a gross increase in connective tissue. Magnification x10900

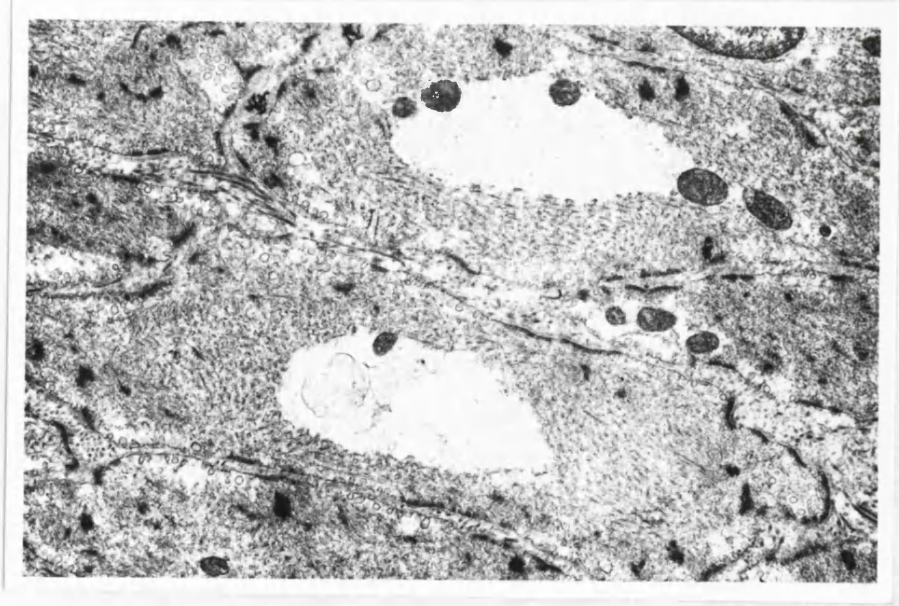


Figure 13. Central vacuolation of the myocytes and general looseness of the myofilaments. Mitochondria relatively well preserved. Magnification x10900

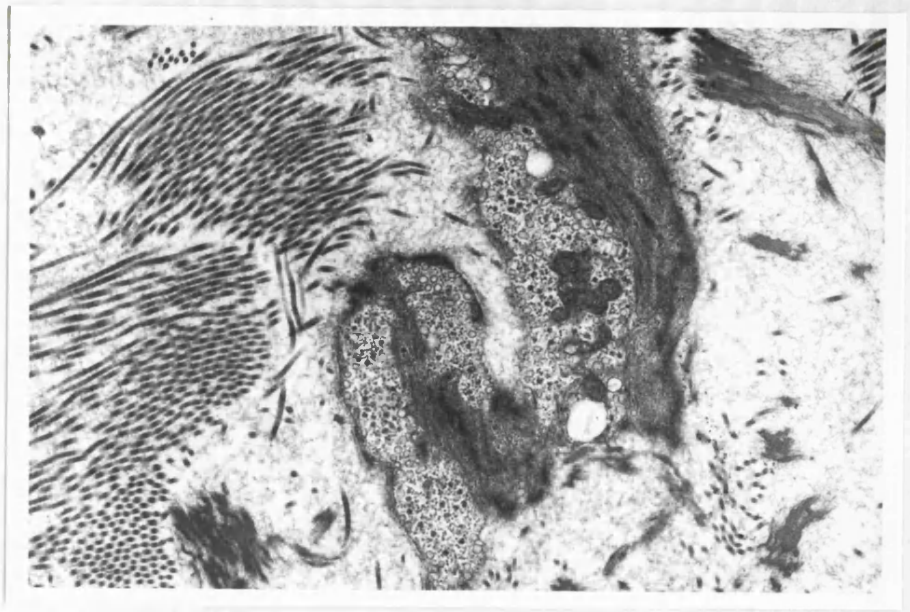


Figure 14. Marked increase in glycogen within the smooth muscle cell cytoplasm. Magnification x10900.

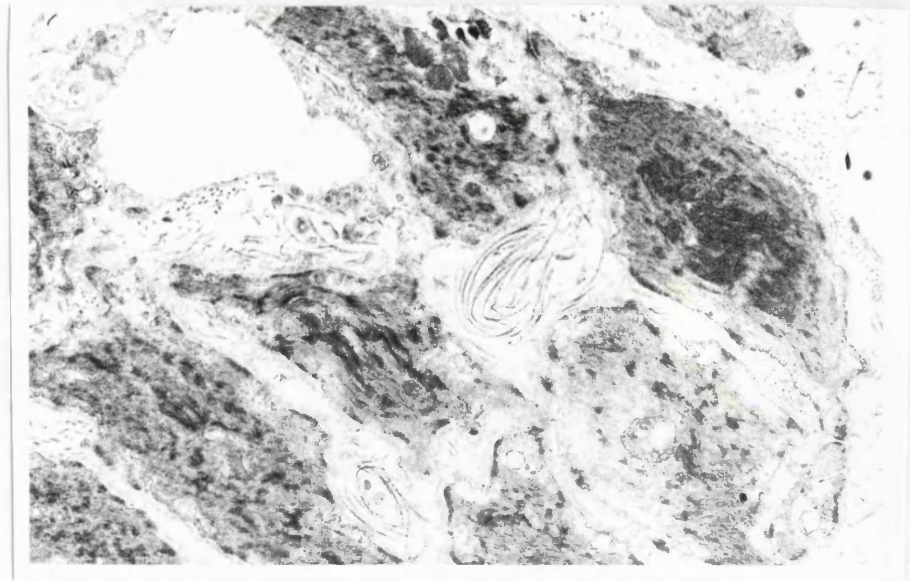


Figure 15. Dense bodies forming sheets in some smooth muscle cells and appear to have undergone proliferation. Magnification x5500.

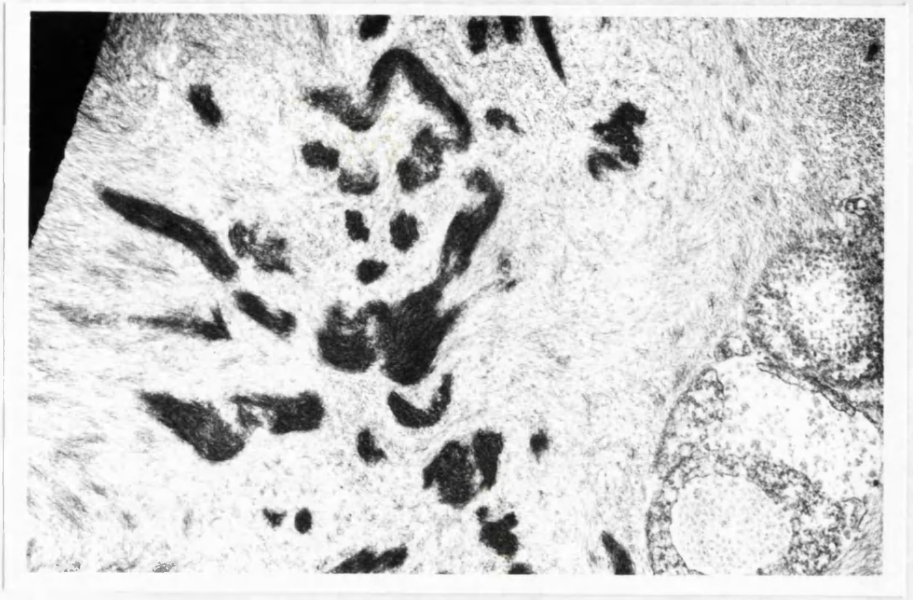


Figure 16. Higher magnification of the expanded and apparently proliferated dense bodies. Magnification x14500

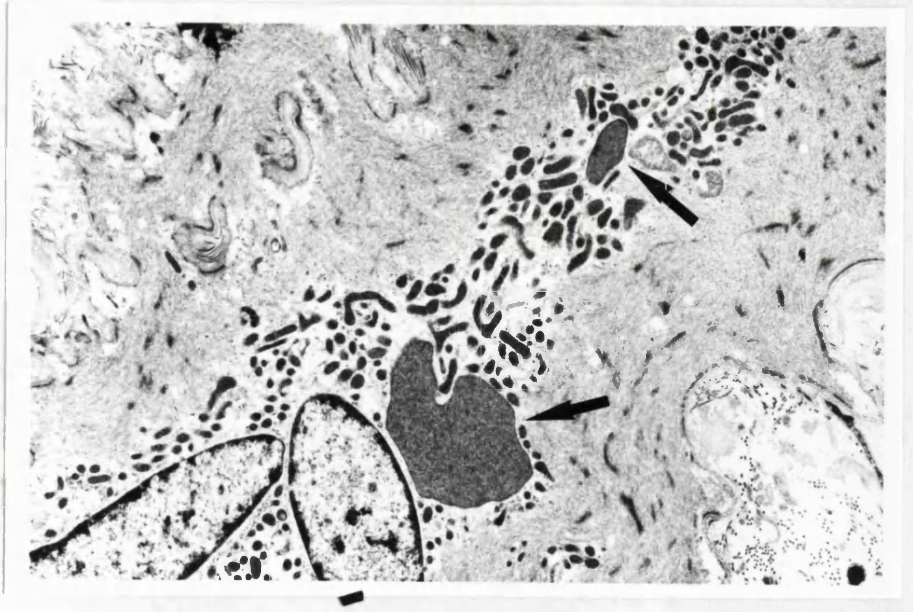


Figure 17. An obvious increase in centrally placed mitochondria. Note also amorphous mitochondria with no apparent cristae (arrows). Magnification x5500

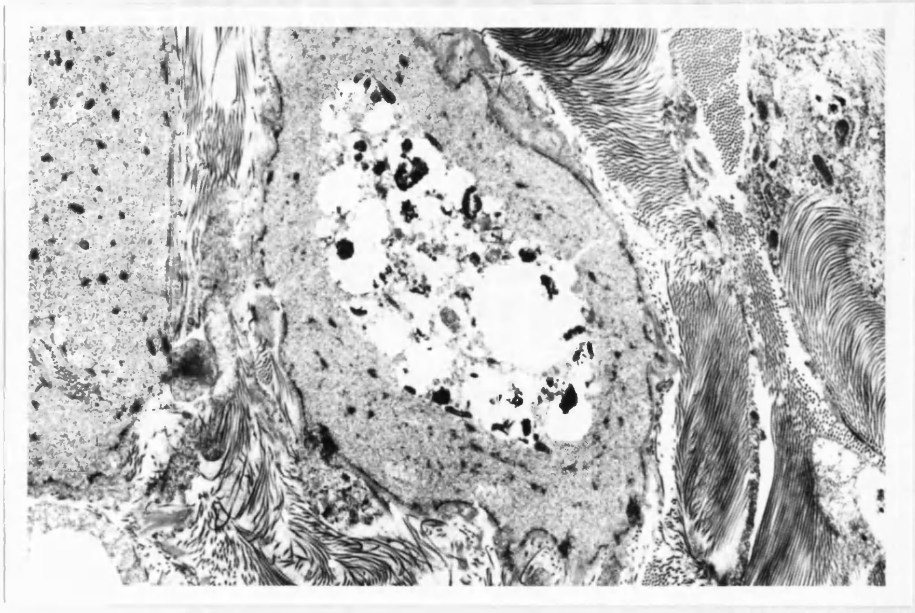


Figure 18. A smooth muscle cell showing autophagic activity is illustrated. Note hugely dilated lysosomes which contain electron dense degradation products. Magnification x5500

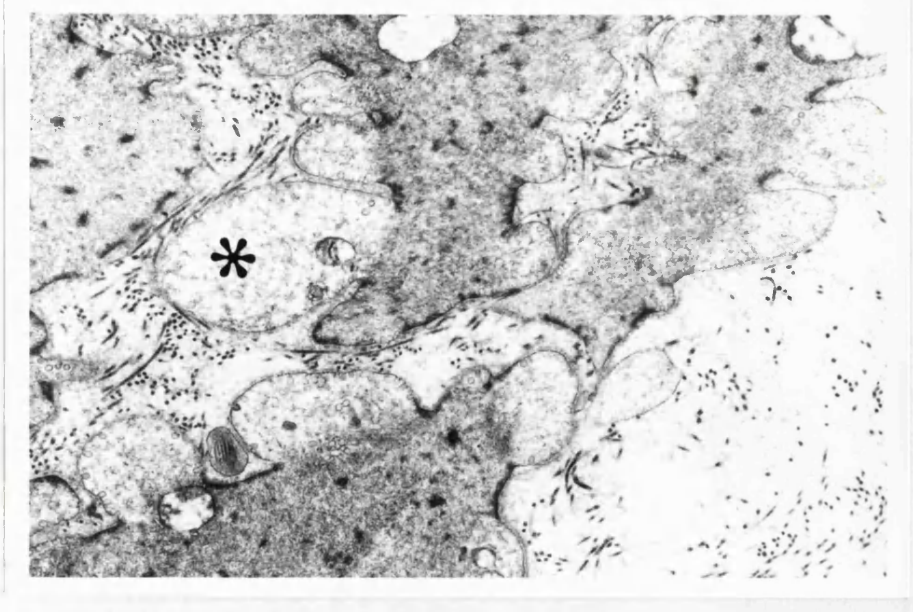


Figure 19. Myocytes showing pronounced subsarcolemmal blebbing (asterisk). Magnification x10900

Table 2b. Correlation of electron microscopical appearances with putative primary diagnosis

Disease Type	No. Pts	Electron Microscopy: Fibr-osis	Myocyte Atrophy	Myocyte vacuol-ation	Auto-phagic activ-ity	Blebs	Mito-chon-drial abn.
Myo-pathy	12	7	3	5	1	3	2
Neuro-pathy	20	0	0	0	0	11	10
Inter-position	1	0	0	0	0	0	1
Unde-termined	18	7	5	6	1	2	7

Differences in the distribution and organisation of the myofilaments were also evident and the amount of intercellular collagen varied. The myocyte ultrastructural appearances were of three types:

(a) 'Disorganisation of the myofilaments' (myocyte vacuolation; Table 2b) was seen in 11 patients in three of whom profound fibrosis and atrophy was evident on light microscopy. The ultrastructural appearances of fibrosis and atrophy are depicted in Figures 9-12. Light microscopy in three had shown smooth muscle vacuolation, but these included the post-mortem samples from one patient. This ultrastructural type was characterised by an increase in connective tissue between the myocytes and profound disorganisation of the contractile filaments resulting in a moth-eaten appearance due to frank cytoplasmic holes in the centres of the myocytes (Fig.13).

In some the holes were shown to contain glycogen and in samples from three patients this accumulation of glycogen was prominent and confined not only to the vacuoles (Fig.14). Proliferation of intermediate filaments and peripheral displacement of dense bodies was obvious. In two patients dense bodies appeared in fused sheets (Figs.15 and 16) rather than individually in the central regions in a number of myocytes. The disorganisation of myofilaments in one patient was accompanied by a marked proliferation of mitochondria (Fig.17) and in another an apparent loss of dense bodies. Numerous atrophic, electron- dense and spindle-shaped smooth muscle cells were also identified (Figs.11 and 12) in samples showing disorganisation of myofilaments from six patients.

b) 'Autophagic activity' was seen in all the samples from two patients with gross fibrosis, smooth muscle cell atrophy, acid phosphatase activity within the myocytes and proliferation of nerves on light microscopy. This ultrastructural type was characterised by the presence of active lysosomes containing electron-dense products of degradation in the smooth muscle cells (Fig .18). The myofilaments were grossly disarranged and the myocytes had almost completely lost their smooth muscle cell characteristics. The collagen between the myocytes, many of which appeared atrophic, was also grossly increased. Moreover, frequent but ultrastructurally unremarkable nerves were detected between the smooth muscle cells in the circular muscle. In one of these patients the dense bodies formed continuous sheets and appeared to have undergone proliferation (see Figs.15 and 16).

(c) 'Subsarcolemmal blebbing' was seen in the intestine from 16 patients. The muscle had a honeycomb appearance, with the plasma membrane which was thrown

into folds around peripheral vacuoles containing no contractile filaments or organelles (Fig.19).

Other Associated Ultrastructural Abnormalities

Apart from mitochondrial changes seen in most specimens, as noted above, in one patient, the mitochondria were rounded and contained 'tubular' arrays without obvious normal cristae (Fig.20). This mitochondrial appearance was not seen in the intestine from her twin sister biopsied on the same day, nor was it found in any other intestine examined.

In one patient with a normal electrophysiological trace, the smooth muscle appeared extremely fragmented with ill- defined plasma membranes, grossly disorganised myofilaments and hugely bloated mitochondria.

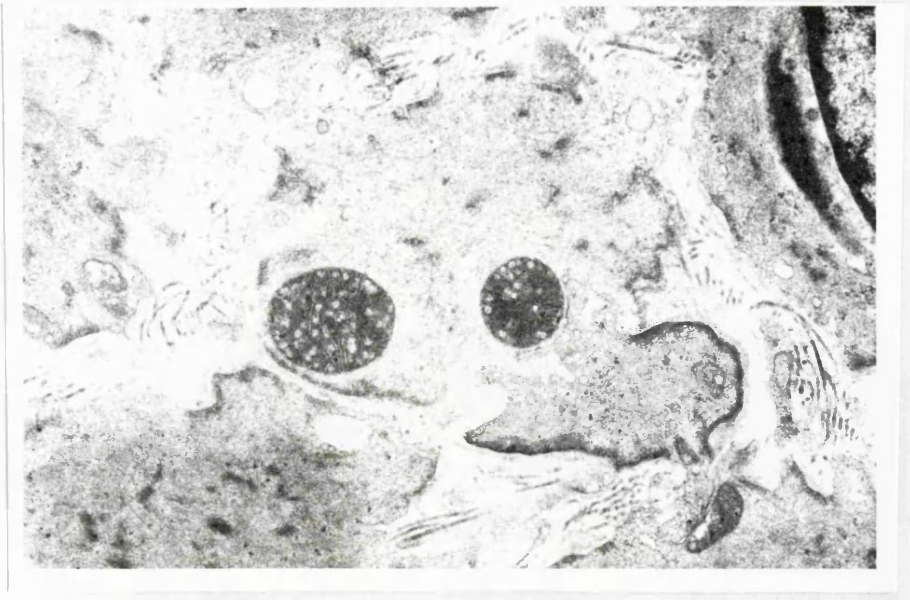


Figure 20. Tubular arrays inside globular and rounded mitochondria. Magnification x14500.

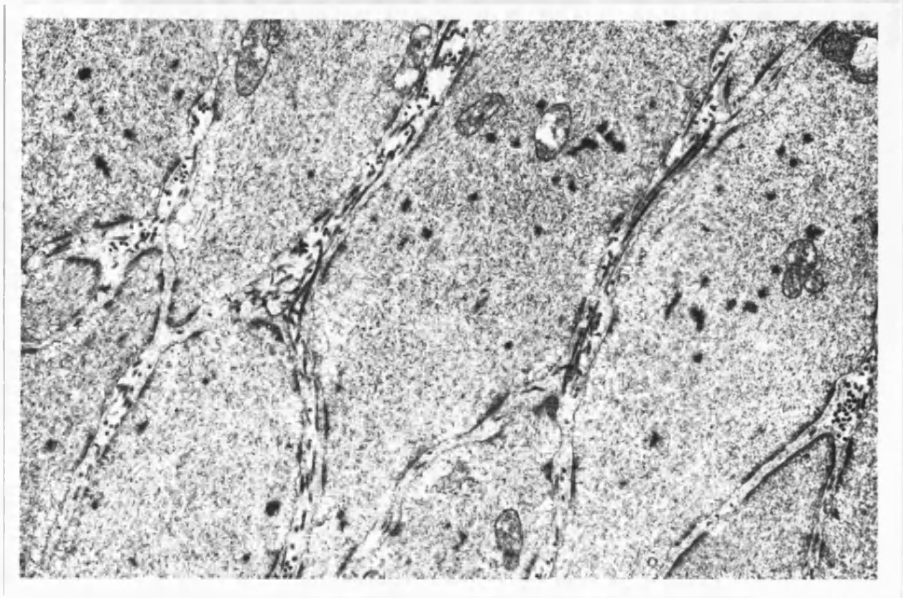


Figure 21. Smooth muscle cells closely applied in the circular muscle coat from the colonic transposition specimen showing relatively good preservation. Mild looseness of the myofilaments can be seen and the mitochondria appear slightly vacuolated. Magnification x10900

Apart from mild mitochondrial dilatation no ultrastructural abnormality was detected in the colonic transposition specimen (Fig.21). The myofilaments were well aligned and the plasma membranes and caveoli were well preserved. The muscle cells were in close contact with each other and there was a minimal amount of collagen between them. Nerves were readily identified and contained dense-cored and empty neurosecretory granules in normal amounts.

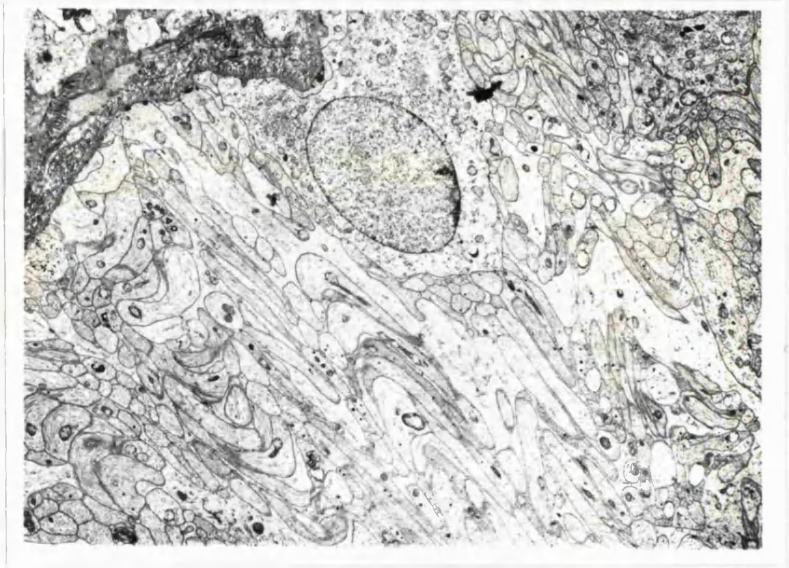


Figure 22. Electron micrograph of myenteric plexus showing a relatively unremarkable neuron and well preserved axons and supporting cell processes. Magnification x2800

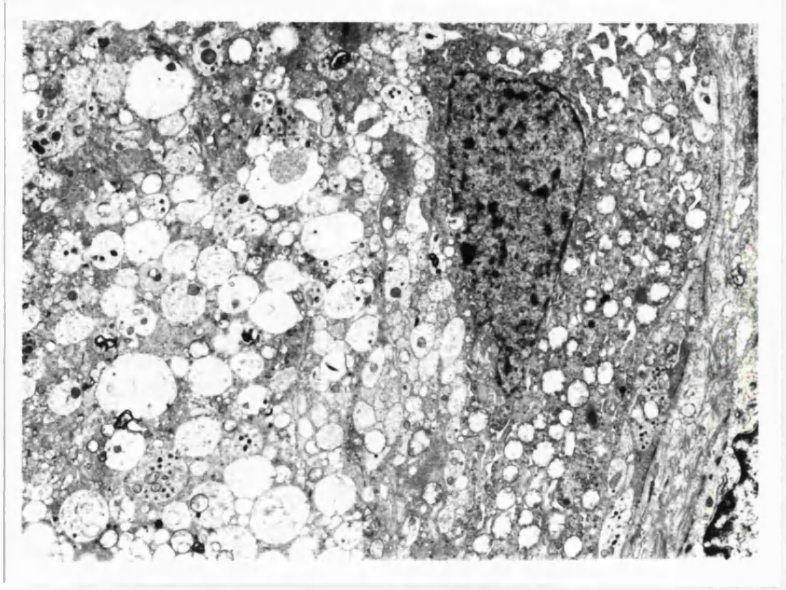


Figure 23. Poorly preserved myenteric plexus. A vacuolated and 'degenerate' neuron and dilated bloated axons and supportin cell processes. Magnification x5500

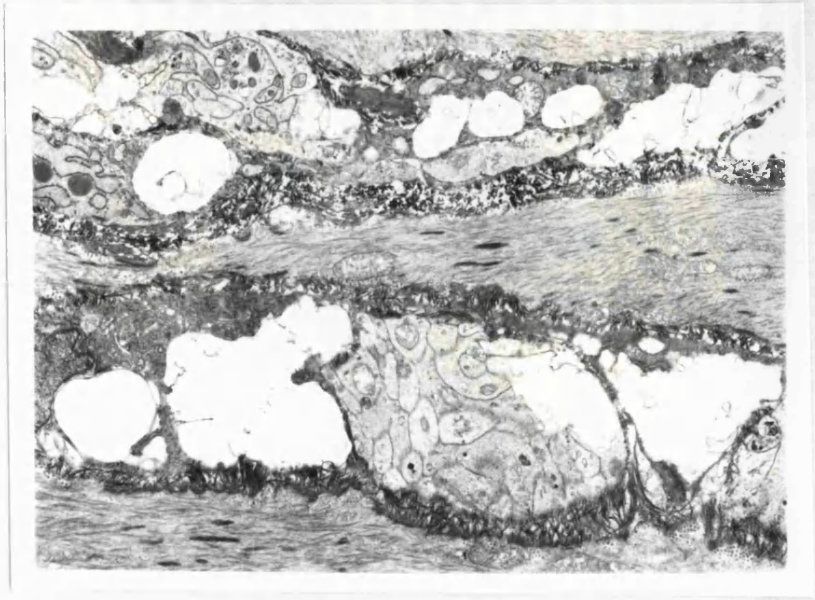


Figure 24. Well preserved smooth muscle cells in the circular muscle. The nerve tracts, however, show gross vacuolation with a loss of neural elements. Magnification x5500

In general neural structures seemed better preserved than myocytes (Fig.22) but dilated axons and processes were seen in four samples (Fig.23), in all but one of which gross subsarcolemmal blebbing of smooth muscle cells was also noted. Two of these patients had neuropathic manometry. Condensed neurons and tightly packed small nerve cell or supporting cell processes were seen in four further patients, three of whom had neuropathic electrophysiology. Vacuolated nerve tracts (Fig.24) were seen in intestine from another three patients and in two patients both nerves and smooth muscle were grossly ill defined. One of the patients with poorly defined intestinal tissue components had showed evidence of transmural inflammation on a previous sample.

6.3.3.5 Correlation between the Light and Electron Microscopical Appearances, and the Putative Primary Diagnosis

(a) Specific Changes

Fibrosis, atrophy and central vacuolation, either on light microscopy and EM, EM alone or light microscopy alone, was seen in seven patients with electrophysiological evidence of myopathic disease (see Tables 2a and 2b). These changes were not seen in any of the 12 patients with electrophysiological neuropathic disease and thus appear to be specific morphological markers of myopathy.

Excess glycogen was seen only in proven myopathic patients and this change was accompanied by ultrastructural central vacuolation in all the three patients in which it was observed.

(b) Non-specific Changes

Other changes (subsarcolemmal blebbing, irregularities in mitochondrial appearance) were seen more frequently in the neuropathic group than in the myopathic thus appear not to be markers of myopathic disease.

(c) New Sub-group

In one patient from the group with myopathic electrophysiology there was striking evidence of lysosomal autophagocytosis. Many myocytic lysosomes in specimens from both large and small intestine contained electron-dense degradation products. These changes were accompanied by gross fibrosis, smooth muscle cell atrophy as morphological evidence of myopathic disease, and there was also proliferation of

nerves in the muscle layers (identified by their AChE-activity and on EM) and acid phosphatase activity in the myocytes.

This same association of lysosomal autophagic activity, nerve fibre proliferation with fibrosis and myocyte atrophy was also seen in one patient in the undetermined group who had not been investigated electrophysiologically. Whilst these patients are clearly myopathic, the associated changes in lysosomes and enteric nerves seems to distinguish them as a distinct morphological sub-group.

(d) Morphological Evidence of Myopathy in the Undetermined Group

In those 18 cases in which electrophysiological studies were not performed (the undetermined group), on light microscopy fibrosis and atrophy was seen in five patients (see Table 2a). On electron microscopy fibrosis was seen in seven and it was accompanied by atrophy in five, central vacuolation (disorganisation of myofilaments) in six and autophagic activity in one (see Table 2b). Thus on purely morphological criteria these seven patients can be considered as examples of intestinal myopathy.

6.4 Discussion

From the animal experiments performed, it is evident that short term ischaemia (10 min), as shown in the rat model, is insufficient to produce significant ultrastructural abnormalities, despite clear biochemical evidence of increased peroxidation in these samples (Lindley, personal communication).

However, more prolonged vascular occlusion (48-72 hours) is associated with profound ultrastructural changes in smooth muscle, as exemplified in the porcine

model. This was particularly evident when lymphatics as well as the arterial and venous supply were cut. Many of the changes encountered, such as subsarcolemmal blebbing, disruption of plasma membranes and mitochondrial disruption, were similar to those seen in some of the human specimens, and they can therefore be considered as non-specific artifacts of probable ischaemic origin. In surgical specimens from human subjects the introduction of ultrastructural artifacts is almost inevitable. Many surgical procedures, such as the pull-through operation for the treatment of HD or IND, involve vascular ligation during mobilisation of the bowel to be resected, and this can result in prolonged periods of ischaemia. In all the specimens of this type examined here 'ischaemic' times in excess of 45 minutes were usual and it is not surprising that this should affect smooth muscle ultrastructure.

Even with intestinal biopsy specimens where operative manipulation is minimised, or in the sample of colon obtained during the colonic interposition procedure for oesophageal atresia in which the intrinsic blood supply was preserved, the method of fixation may introduce artifacts. Relatively large blocks of tissue need to be taken for orientation purposes, which is less than ideal for ultrastructural preservation. In addition, dissection of larger specimens takes at least several minutes. The practice of attaching the specimens of bowel to a card, mucosa downwards, to facilitate orientation may also be a factor explaining the relatively worse ultrastructural preservation of the circular muscle compared with the longitudinal muscle coat.

However, despite the obvious caution required in the interpretation of apparent ultrastructural abnormalities in the diagnosis of intestinal pseudo-obstruction, this study did confirm the importance of some electron microscopical changes in the

diagnosis of intestinal myopathy.

Fibrosis of the muscularis propria and atrophy of smooth muscle cells, which appeared small and spindle shaped with electron-dense cytoplasm, was seen in eight of the 12 patients with electrophysiological evidence of myopathic disease, either by both electron and light microscopy, or by light or electron microscopy alone. These changes were never seen in the 20 neuropathic patients and can thus be regarded as specific markers of myopathy.

In addition, central vacuolation of myocytes sometimes associated with glycogen accumulation within the vacuoles as well as peripheral increase in glycogen (Figs.13 and 14), was seen in five patients with myopathic electrophysiology but in none of the neuropathic patients. These changes too, appear to be indicative of myopathic disease. Central vacuolation needs to be distinguished from subsarcolemmal blebbing. Although, when present subsarcolemmal blebbing is a dramatic change, it appears to be quite non-specific. It was seen in three of the myopathic patients, but also in 11 of the neuropathic patients. Since blebbing was also encountered in the piglets with long-term ischaemia it can be regarded as almost certainly due to the surgical procedure or to poor fixation.

Mitochondrial changes were almost universal in the human material examined, as well as in the porcine specimens. It seems likely, therefore that those are fixation artifacts or are induced by ischaemia and have no relevance to the diagnosis of myopathy.

Of the 18 patients in the undetermined group in whom electrophysiological studies

were either normal or not done, fibrosis, atrophy or central vacuolation of the smooth muscle cells were seen in seven. It seems reasonable to regard these seven patients as having intestinal myopathy on morphological grounds alone.

It is of interest that in two of the patients with marked fibrosis and myocyte atrophy indicative of myopathic disease, there was a marked proliferation of nerves within the muscle layers and evidence of lysosomal autophagic activity. These two patients appear to form a morphologically identifiable subset of myopathic disease having some features in common with patients described by Smout et al. (1985), Kaschula et al. (1987) and Rode et al. (1992). The possible significance of nerve fibre proliferation in the muscularis is discussed in more detail in Chapter 8.

Despite the identification of apparently specific morphological markers of myopathic disease (fibrosis of muscularis propria, atrophy and central vacuolation of the myocytes and autophagic activity) it is evident that a significant proportion of subjects with electrophysiological evidence of smooth muscle dysfunction show no diagnostic structural abnormalities of the smooth muscle coats even by electron microscopy. It is clear that some more subtle defects must account for their disease, an one possible further line of investigation is identified in Chapter 7.

Chapter 7. Immunostaining of Contractile Proteins: A New Approach to the Investigation and Diagnosis of Intestinal Myopathies

7.1 Introduction

In Chapter 6 it was stated that in patients with intestinal pseudo-obstruction associated with myopathic disease, specific morphological abnormalities in the enteric musculature often elude detection even by electron microscopy. A different approach to this problem has therefore been attempted by investigating contractile proteins present in the myocytes of the intestinal wall to see whether alterations in these elements might be related to abnormal intestinal function.

In this investigation immunohistochemical techniques using commercially available antisera to contractile proteins and their isoforms have been applied. Immunohistochemical methods rather than biochemical assays were employed so that any abnormalities in the distribution of contractile proteins could be identified topographically. One polyclonal and seven monoclonal antibodies to various contractile proteins and their isoforms have been applied to full-thickness sections of snap-frozen intestinal wall from patients with myopathic, neuropathic and undetermined types of intestinal pseudo-obstruction. In addition, sections of intestine obtained at post-mortem from patients dying from non-gastrointestinal causes were included. Before detailing these investigations, a survey of present knowledge regarding the function and distribution of contractile proteins is presented and information of the antibodies to these proteins available commercially is given.

7.1.1 Function and Distribution of Contractile Proteins

The mechanisms involved in smooth muscle contraction are incompletely understood but it is accepted that contraction involves the formation of cross-bridges between thick (myosin) filaments and thin (actin) filaments (Hartshorne 1987). This is associated with hydrolysis of adenosine triphosphate (ATP) promoted by magnesium ion-dependant ATP-ase in the presence of calcium ions (Hartshorne 1987). The interaction between actin and myosin is facilitated by a number of other proteins, some of which are associated with the thin filaments. The proteins associated with both thick and thin filaments are referred to as contractile proteins, all of which exist in different isoforms.

The thick myosin filaments are phosphorylated in the relaxed state and dephosphorylated in the contracted state. Myosin consists of myosin heavy chains (four isoforms), myosin regulatory light chains (two isoforms) and myosin alkali light chains (four isoforms) (Murphy 1992). All these isoforms of myosin are expressed in mature smooth muscle cells.

Actin, which is the major component of thin filaments, exists in at least six different isoforms, each of which is encoded by a separate gene and which are expressed in a tissue-specific pattern. The isoforms are structurally very homologous but differ in their amino acid sequences particularly at the amino terminus (Hartshorn 1987; Sawtell and Lessard 1989). They show about 90% overall homology but this is reduced to 50-60% homology if only the first 18 amino acids are considered (Gown et al. 1985). For instance, the four muscle specific actins differ in only eight of the 375 amino acid residues (Sawtell and Lessard 1989). The isoforms show no structural

differences between species but there are interspecies variations in the mRNAs that encode them. The amino terminal region is thought to be the major antigenic region of the proteins and it is also this part of the protein which interacts with myosin. The isoforms of actin can be separated on isoelectric focusing into three bands (alpha, beta and gamma) and further subdivided by their tissue distribution into three alpha isoforms (localised in smooth muscle, skeletal muscle and cardiac muscle respectively), and two gamma isoforms (localised in smooth muscle and the cell cytoplasm of most cell types). The beta isoform is also cytoplasmic and is present in all cells, although it is not thought to be involved in muscle contraction (Hartshorne 1987). The major isoactins in mature intestinal smooth muscle are alpha and gamma smooth muscle actin; the gamma isoform is the largest constituent and alpha smooth muscle isoactin, although less abundant, is nevertheless an important component (Fatigati and Murphy 1984).

As well as actin, thin filaments also contain tropomyosin and caldesmon or filamin (Lehman et al 1987). Tropomyosin exists in isoforms (Hartshorne 1987) which in smooth muscle, are of two similar subunits (beta and gamma) that form homodimers (beta:beta or gamma:gamma). The role of tropomyosin in smooth muscle is unknown, but in skeletal muscle it regulates contraction in association with troponin. Although no troponin is found in smooth muscle, tropomyosin appears to be essential for smooth muscle contraction.

Caldesmon is a calmodulin-binding protein and exists as two proteins of different molecular weights (70kD and 150kD; Hartshorne 1987). In the absence of calcium ions, caldesmon binds to actin and inhibits its interaction with myosin to produce

muscle relaxation. In the presence of calcium ions and calmodulin, caldesmon dissociates from actin, allowing interaction between actin and myosin to occur and cause muscle contraction.

The role of filamin is unclear, but it is known to bind with polymerised actin thereby inhibiting its activation (Perry and Grand 1979, Hartshorne 1987). The formation of actin-filamin complexes may thus help to regulate contraction by reducing the availability of actin for interaction with myosin (Perry and Grand 1979, Hartshorne 1987).

The intermediate filament proteins desmin, which is present in large amounts in intestinal smooth muscle, and vimentin are unlikely to be involved in smooth muscle contraction. Their role is probably related to maintaining the size, shape and the volume of myocytes. Thus these proteins are not regarded as contractile proteins, despite their intimate association with filaments observable on ultrastructural examination.

Total actin-myosin ratios, as well as the proportions of contractile protein isoforms, vary in smooth muscle from different tissues (Fatigati and Murphy 1984). Variations in the proportions of these isoforms also occur during muscle development. The ontogeny of contractile protein isoforms has been studied largely in developing smooth muscle of pulmonary arteries and the aorta (Owens and Thompson 1986; Glukhova et al. 1990) as well as in chicken gizzard (Kuroda 1985), but not in the intestine. Non-muscle cytoplasmic actin isoforms predominate in undifferentiated smooth muscle cells in the early embryo, but smooth muscle-specific isoforms

(gamma and alpha smooth muscle actins) are most abundant in mature fully differentiated cells (Kuroda 1985; Owens and Thompson 1986; Otey et al. 1987; Glukhova et al. 1990), the shift from cytoplasmic isoactins to muscle specific isoforms occurring gradually during maturation. An increase in smooth muscle myosin heavy chain and a change from the low molecular weight (70kD) caldesmon to the high molecular weight caldesmon (150kD) is also seen with increasing maturity (Glukhova et al.1990).

The reasons for the variations in the proportions of isoproteins during development and in different smooth muscles is incompletely understood. However, the different cytoplasmic actins appear to correlate with proliferative, synthetic and secretory cell functions (Murphy 1992). These proteins, like desmin and vimentin, are probably also involved in the maintenance of cell shape and volume. In contrast, the smooth muscle-specific actin isoforms modulate the type of contraction appropriate for a particular type of smooth muscle (Fatigati and Murphy 1984). For instance, alpha smooth muscle actin predominates in arterial muscle with a high degree of tonic activity, whilst gamma smooth muscle actin predominates in intestinal muscle showing phasic activity (Fatigati and Murphy 1984). The localisation of the isoactins also differ within the cytoplasm of an individual cell, muscle specific isoforms occurring centrally and cytoplasmic isoforms near the cell membrane (Shires and Rubenstein 1989).

Not only are variations in the proportions of isoproteins seen during development but they also occur in response to various acquired stimuli. For example, cholesterol feeding of prairie dogs results in shifts of isoactins in the gall bladder (Li et al.

1990). Exposure to an oxygen-rich atmosphere causes proliferation of pulmonary arterial smooth muscle, which is associated with changes in the proportions of isoactins (Coflenski et al. 1988). In addition, certain drugs affect actins (Gottlieb et al. 1991); for example cytochalasin D attaches to actin filaments causing their depolarisation and adriamycin (Doxorubicin) depresses the synthesis of sarcomeric alpha actins (Lewis and Gonzalez 1990). In atheromatous arterial lesions there is a reduction in the alpha smooth muscle actin content of the affected vessel wall (Owens and Thompson 1986).

7.1.2 Specificity of the Commercially Available Antibodies to Contractile Proteins Employed

Monoclonal Antibodies

(i) 1A4 (Sigma, Dorset, UK). This antibody reacts exclusively with alpha smooth muscle actin. It was raised against the unique synthetic aminoterminal decapeptide of alpha smooth muscle actin.

(ii) CGA7 (Universal Biologicals Ltd, London, UK). This antibody reacts selectively with both alpha and gamma smooth muscle isoactins. However, the specific epitope with which it reacts is not known.

(iii) HHF35 (Universal Biologicals Ltd, London, UK) reacts in a selective manner with pan-muscle (smooth, skeletal and cardiac) alpha and gamma actins. However, the epitope with which it reacts is not known.

(iv) FIL2 (Sigma, Dorset, UK). An antibody raised against chicken gizzard cytoskeletal proteins recognises the 250 Kd actin cross-linking protein, filamin

(v) CALD5 (Sigma, Dorset,UK). An antibody raised against turkey gizzard caldesmon. It localises both high (120-150kD) and low (71-80 Kd) molecular weight caldesmon protein, recognising an epitope in the calmodulin non-binding part of the aminoterminal end of the protein.

(vi) TM311 (Sigma, Dorset, UK). An antibody raised against chicken gizzard tropomyosin and recognising both the 36kD and 39 Kd proteins.

(vii) DE-R-11 (Dako, High Wycombe, UK). This antibody reacts with the 53 Kd intermediate filament protein desmin in muscle cells. It recognises an 18 Kd rod piece of the molecule.

Polyclonal Antibody

An antibody to myosin (Sigma, Dorset, UK). This antibody was raised against myosin (heavy and light chains) from bovine uterine smooth muscle.

7.2 Materials and Methods

7.2.1 Materials

(a) Surgical samples

A total of 17 small intestinal and 36 large intestinal specimens from 35 patients were studied. Large bowel only was available from 18 patients, small from 13 and both large and small from four patients. The surgical material was from patients diagnosed

on clinical, electrophysiological or histopathological criteria as having intestinal myopathy (11 patients, 21 samples), intestinal neuropathy (18 patients, 26 samples), or undetermined pseudo-obstructions (six patients, one sample from each). The ages of these patients ranged from 15 days to 48 years, 11 were between 10-16 years and three were adults (27,34 and 48 years).

(b) Post-mortem samples

A total of nine post-mortem samples, six from colon and three from small bowel, were also examined. These tissues were obtained from patients who died from non-gastrointestinal causes. The age of these patients ranged between 8 weeks and 33 months. One specimen was from a baby born at 28 weeks gestation who died at the age of 11 weeks.

7.2.2 Methods

Immunohistochemical studies were performed on cold (-20°C) acetone-fixed cryostat sections (10μm thick) of snap-frozen tissue. The avidin-biotin peroxidase complex (ABC) method (Hsu et al. 1981) was employed using the primary antibodies listed above (7.1.2). 1A4 was used at a titre of 1:2000, CGA7 at 1:20, HHF35 at 1:20, FIL2 at 1:100, CALD5 at 1:40, TM311 at 1:400, DE-R-11 at 1:100 and antimyosin at 1:100.

In addition a number of controls were used including neural markers, which are detailed in Chapter 8, 'irrelevant' antibodies and the omission of the primary antibody. The irrelevant antibodies were a polyclonal antibody (at titre of 1:75) to alpha-fetoprotein (Dako, High Wycombe, UK) and a monoclonal antibody, clone

GB3, (at titre of 1:160) to epithelial basement membrane glycoprotein (600kD; Sera-lab, Sussex, UK).

The biotinylated antimouse and antirabbit antibodies and the ABC complex were obtained from Dako, High Wycombe, UK.

All immunostaining was performed without blocking the endogenous peroxidase. This was based on preliminary studies using several blocking techniques, which showed that blocking seriously compromised immunostaining. Blocking was attempted prior to the application of the primary, secondary or tertiary antibodies in the ABC method. The blocking reagents (also see Appendix) used were:

* 0.3% hydrogen peroxide in methanol.
* 2.5% periodic acid followed by 0.2% sodium borohydride
* 10 mM Glucose with 100 units of glucose oxidase.

The analysis of immunostaining was performed microscopically by noting immunoreactivity in the smooth muscle cells of the intestinal muscle layers. Comparisons in intersample intensity in immunostaining were made in samples from patients with CIIP and controls, and was recorded as absent, decreased, normal or increased.

7.3 Results

In this section the immunostaining of normal intestine is reported first, followed by the results in the intestine from patients with CIIP.

7.3.1 Muscle Markers in Normal Intestine

Immunostaining in the normal intestine for alpha smooth muscle actin (1A4) showed strong reaction in the intestinal muscle layers (muscularis mucosae, circular- and longitudinal muscle) with a clean background. Similarly smooth muscle in the walls of blood vessels was strongly immunoreactive. Neural elements were negative but an occasional fibroblast was stained.

The appearances were similar with HHF35 (pan muscle alpha and gamma actin antibody) and CGA7 (smooth muscle alpha and gamma actin antibody). With these antibodies more background staining was seen and a few more fibroblasts appeared immunoreactive than with the antibody 1A4. Also, using CGA7, very slight positivity could be detected in the myenteric neurons. The overall immunostaining with CGA7 was weaker than with HHF35 or 1A4.

Tropomyosin (TM311) immunoreactivity was strong and confined to the intestinal smooth muscle layers and the vessel walls. The level of background staining was comparable to that of CGA7.

The antibodies to filamin (FIL2) and caldesmon (CALD5) showed weak immunostaining in all muscle layers and blood vessel walls. They also showed some background staining and numerous positive fibroblasts. Neurons in the enteric plexuses were slightly immunoreactive.

All muscle layers and blood vessels were strongly positive with the antibody to desmin (DE-R-11). No staining was seen in the neural elements and the background

was clear.

Immunostaining with the polyclonal antibody to myosin was moderately positive in all smooth muscle elements, and also produced high levels of background staining. Fibroblasts and neuronal perikarya were immunoreactive. The heavy background staining probably reflects the relative non-specificity of this polyclonal antibody which may well cross-react with myosin in cells other than myocytes.

The negative controls using irrelevant antibodies:

GB3 (to control the monoclonal systems) showed a clean background with no neural staining and with only very faint positivity in the muscle layers.

Alpha-fetoprotein (to control the polyclonal system) again produced a clear background. Fibroblasts stained positively but smooth muscle and neural elements were negative.

7.3.2 Muscle Markers in CIIP

Using the battery of antibodies described to contractile proteins on 53 samples of intestine from 35 patients (11 myopathic, 18 neuropathic, six undetermined), seven patients were identified with one or more irregularities in the immunostaining pattern compared with controls (see Table 1). In all seven patients myopathic disease was confirmed by electrophysiological and/or specific light and/or electron microscopical morphological criteria.

With one exception (Table 1; patient 1), the precise nature of the contractile protein

abnormality seen by immunostaining could not be identified exactly due to the incomplete specificity of the antibodies involved. In all seven patients CGA7 (selective for alpha and gamma smooth muscle actins) immunostaining was reduced (n=2) or completely absent (n=5) in the circular muscle coat or in both muscle layers of the muscularis propria. In three of these patients it was also absent in the muscularis mucosae. However, staining with HHF35 (selective for pan muscle alpha and gamma actins) was normal in all these cases. In addition, in three of these patients staining for 1A4 (specific for alpha smooth muscle actin) was absent.

In three patients with abnormal CGA7 immunostaining, including one of those with deficient alpha smooth muscle actin immunostaining, abnormal immunoreactivity was also identified for other contractile proteins. In one tropomyosin (TM311) was deficient in the circular muscle, in the second filamin (FIL2) was deficient in the circular muscle, and in the third FIL2-immunostaining was actually enhanced in both layers of the muscularis propria.

Table 1. Abnormality in contractile protein immunostaining

	1A4			HHF35			CGA7			CALD5			FIL2			TM311		
Pt	CM	LM	MM	CM	LM	MM	CM	LM	MM	CM	LM	MM	CM	LM	MM	CM	LM	MM
1	-ve						R											
2	-ve						-ve	-ve					I	I		-ve		
3							-ve	-ve										
4							-ve	-ve	-ve				-ve					
5	-ve						-ve	-ve	-ve									
6							-ve	-ve	-ve									
7	R							R										

CM circular muscle
LM longitudinal muscle
MM muscularis mucosae

R reduced
I increased
-ve negative

Observations recorded indicate differences from controls

Figure 1. H&E stained paraffin section of jejunum from patient 1 showing normal small bowel wall and in particular unremarkable muscularis propria. Magnification x64

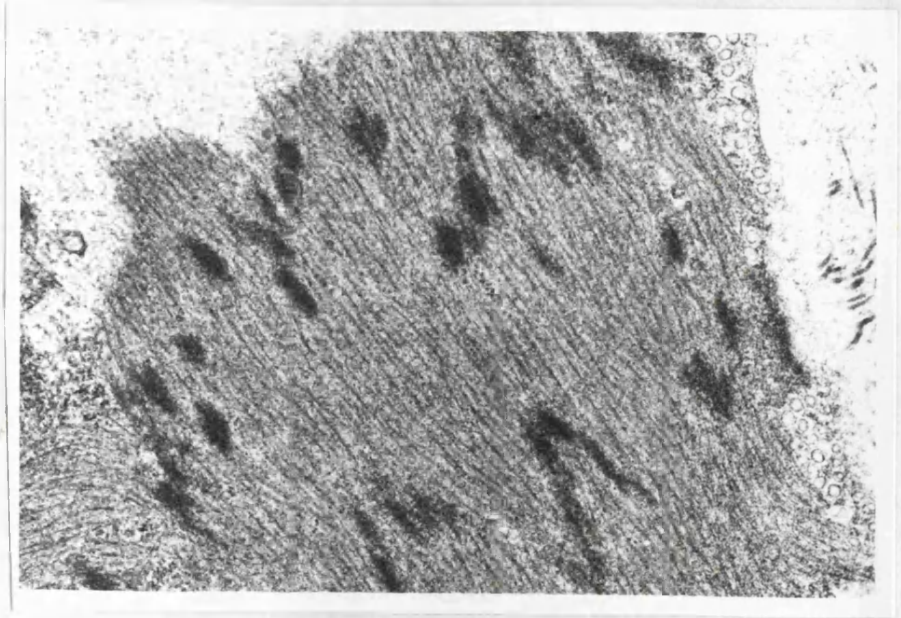


Figure 2. Electron micrograph of a typical myocyte in the bulk of the circular muscle from patient 1 showing normal myofilaments especially the thin filaments appear plentiful. Magnification x27300

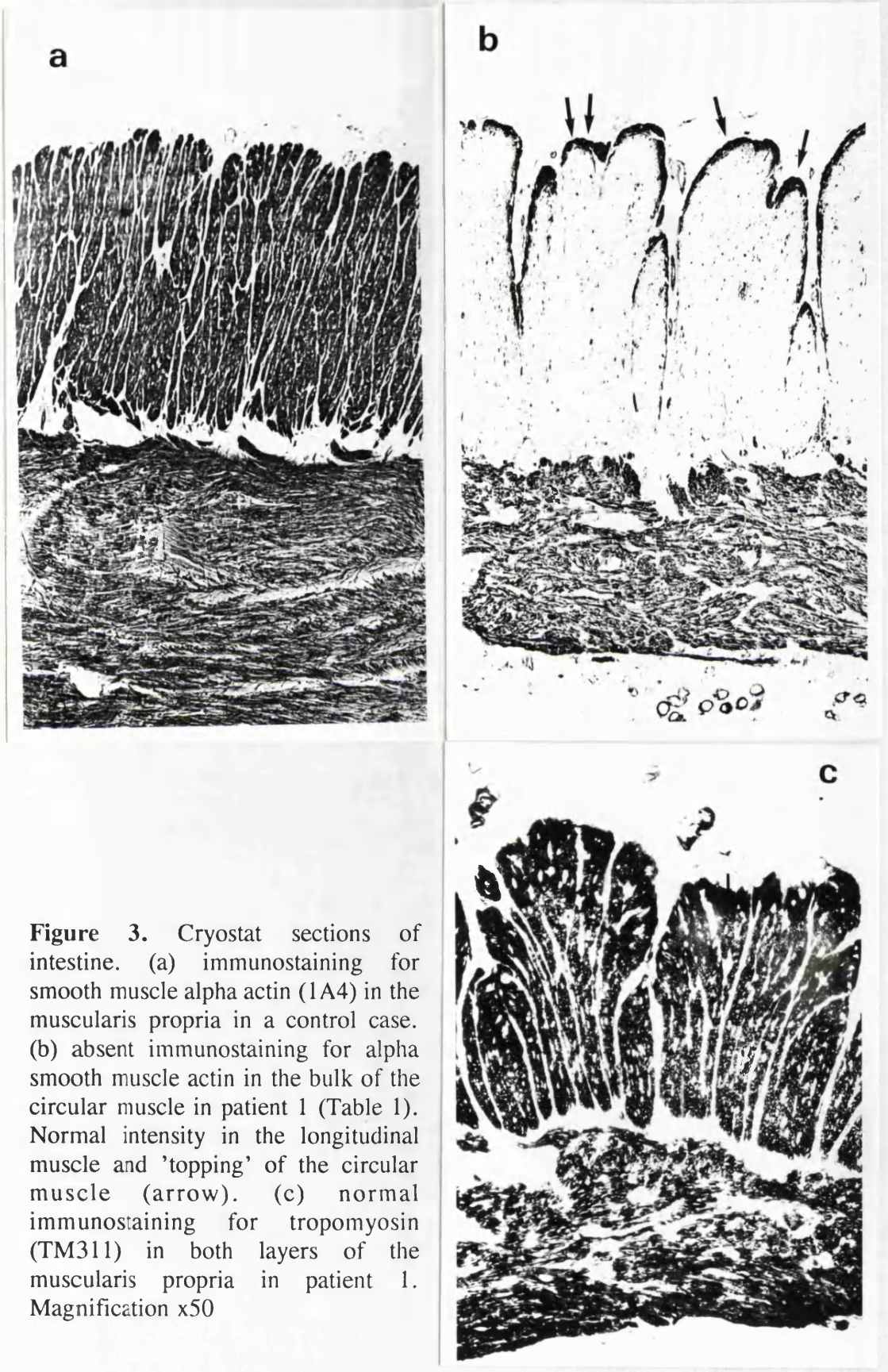


Figure 3. Cryostat sections of intestine. (a) immunostaining for smooth muscle alpha actin (1A4) in the muscularis propria in a control case. (b) absent immunostaining for alpha smooth muscle actin in the bulk of the circular muscle in patient 1 (Table 1). Normal intensity in the longitudinal muscle and 'topping' of the circular muscle (arrow). (c) normal immunostaining for tropomyosin (TM311) in both layers of the muscularis propria in patient 1. Magnification x50

The most easily interpreted contractile protein abnormality was seen in patient 1. This was an adult woman with a history of pseudo-obstruction since childhood. Electrophysiological studies were indicative of myopathic disease 1and the patient required a jejunostomy for the management of her symptoms. During this procedure a full-thickness sample of jejunum was removed for pathological examination. On light microscopy no morphological abnormality was detectable (Fig.1) and the ultrastructural appearance was also unremarkable (Fig.2). Smooth muscle alpha actin (1A4) immunostaining revealed a pattern quite different from the controls (Fig.3). 1A4 staining was absent within the smooth muscle cells in the bulk of the circular muscle, with the exception of its innermost layer adjacent to the submucosa, where the smooth muscle cells stained strongly positive. In contrast, immunostaining for smooth muscle alpha actin (1A4) was normal in the smooth muscle cells throughout the longitudinal muscle, muscularis mucosae and in the smooth muscle of the blood vessel walls (Fig.3). Immunostaining, in the circular muscle, for tropomyosin (TM311; Fig.3), filamin (FIL2), caldesmon (CALD5), desmin (DE-R11) and myosin was of the same intensity as in the other intestinal muscle layers. Similarly, all muscle coats showed normal immunostaining with the antibody to pan-muscle alpha and gamma actins (HHF35). However, the pattern of immunoreactivity with the antibody to smooth muscle alpha and gamma actin (CGA7) was markedly reduced in the circular muscle although not absent like 1A4. CGA7 reactivity was present in normal intensity in the longitudinal muscle, muscularis mucosae and blood vessel walls.

7.3.3 Neural Markers

The results of immunostaining for neurofilaments is considered in the next chapter, but negative immunoreactivity of smooth muscle elements was seen with the neurofilament antibodies. The antibody for neural cell adhesion molecule in general reacted with neural elements only and spared the intestinal musculature. A variable degree of stippled immunostaining for muscarinic receptors was seen in the intestinal smooth muscle as well as in the enteric neuronal perikarya and along the axons. These results are discussed in greater detail in Chapter 8.

7.4 Discussion

In this study, a number of monoclonal and a polyclonal antibody to contractile filaments have been applied to full-thickness specimens of snap-frozen intestine from controls and from patients with symptoms of CIIP. Abnormalities were identified by immunostaining in surgical samples of intestine from seven patients, all of whom had definite evidence of myopathic disease by electrophysiological (Chapter 2) or morphological (Chapter 6) criteria. On the other hand, no immunohistochemical abnormalities with contractile protein antibodies were seen in any of the other specimens examined including those from patients with neuropathic pseudo-obstruction and those samples obtained at necropsy from patients dying of non-intestinal disease (controls).

In these seven patients abnormalities in contractile proteins, particularly in isoactins, were recognised, and in two of them there were additional abnormalities of filamin and in one of tropomyosin. In three patients the smooth muscle actin

abnormality could be narrowed more precisely to an apparent deficiency in the alpha smooth muscle actin isoform, although in two of them this could not be proved to be the only abnormality. In one patient, however, a remarkable and unique feature was a deficiency of alpha smooth muscle actin immunostaining alone confined to the circular muscle. Despite the clear demonstration of this deficiency by immunohistochemistry, no significant ultrastructural differences were shown in the myofilament composition of the circular muscle and other smooth muscles of the gut wall. This is therefore the first description of a biochemical, as opposed to a structural, abnormality in CIIP. Confirmation of the absence of this protein or the presence of an altered protein by assay was not possible due to insufficient tissue for analysis. It should be noted, that immunohistochemistry has clear advantages over biochemical assay in that protein abnormalities confined to specific components of heterogeneous tissues such as the intestine can be accurately localised. Moreover, the biochemical analysis could reflect an impure smooth muscle preparation, in which the vascular and neural elements would not have been excluded.

Isoform specific antibodies to contractile proteins are generally not commercially available. Unless the antibody is made against the specific aminoacid sequence in the amino terminus, cross reactivity with other isoforms is likely (Otey et al. 1987). Since the antibody 1A4 is directed against the aminoterminus of a synthetic decapeptide of alpha smooth muscle actin it is, therefore, specific for this isoform (Skalli et al. 1986). Unfortunately no other isoform-specific antibody is currently available.

The exact epitopes identified by antibodies such as HHF35 and CGA7, both of which are said to identify alpha and gamma muscle isoactins, are unknown but HHF35 appears less specific than CGA7 in that it recognises actins of skeletal and cardiac muscle, as well as smooth muscle (Gown et al. 1985; Tsukada et al. 1987). This relative non-specificity may account for the normal staining with HHF35 in the five patients with absent CGA7 staining in this study. In patient 1, the fact that some reactivity with CGA7 was noted in the circular muscle, probably reflects that gamma smooth muscle isoactin is present and is immunostained but the weakness of the reaction is accounted for by the deficiency of alpha smooth muscle actin, proven by the negative 1A4 staining. Immunoreactivity was entirely normal with HHF35, again presumably reflecting the reactivity of this antibody with a whole variety of other actins. A difference in immunoreactivity between the various muscle layers in the same specimen or between specimens from different patients, probably does indicate significant abnormalities in the isoform content, as shown in these seven patients described here (see Table 1). In particular the abnormality detected with 1A4 against alpha smooth muscle actin is particularly informative due to the high degree of specificity of this antibody.

The absent immunostaining for an actin isoform could reflect either an acquired phenomenon (Coflenski et al. 1988; Lewis and Gonzalez 1990; Li et al. 1990; Gottlieb et al. 1991), or a specific developmental defect. In view of the life-long duration of symptoms in these patients, the latter appears more likely.

This concept is supported by consideration of the normal differentiation of intestinal smooth muscle. Cytodifferentiation of smooth muscle cells in the rat gut (Kedinger et al. 1990) proceeds in a specific order: cells expressing actin (alpha and gamma) appear in the mesenchyme of the 15 day fetal rat in the circular-muscle-forming area and in the presumptive longitudinal muscle 48 hours later. Such cells are noted in the muscularis mucosae shortly after birth and become fully established 2 weeks later. A distinct cell layer of actin-expressing cells arises in the perinatal period in the innermost part of the circular muscle adjacent to the submucosa. In humans the intestinal smooth muscle layers develop in the same order although the timing is different. The circular muscle develops at 9 weeks gestation, the longitudinal by 12 weeks and the muscularis mucosae is evident after the 21st gestational week (Desa 1991). It is probable that the development of the innermost circular muscle layer follows the same pattern as in the rat and appears later.

More intense immunoreactivity ('topping') can be seen in the innermost layer of the circular muscle compared to the bulk of the circular muscle using a variety of antibodies and this layer may even be recognised in routinely stained sections. The difference in staining may signify the separate origin of this layer from the rest of the circular muscle. In patient 1, strong 'topping' was seen with the antibody to alpha smooth muscle actin, while the bulk of the circular muscle was unstained; the other muscle layers were stained normally. One possible mechanism for this would be a transient developmental block, at the crucial time of circular muscle development. The temporary nature of this block would allow normal later

development of the longitudinal muscle, muscularis mucosae and the innermost layer of the circular muscle.

Since the ratios and composition of contractile isoproteins reflects specific functions of smooth muscle cells, it is reasonable to conclude that the absence of a particular protein, or the presence of altered forms, might adversely affect normal smooth muscle contraction. Whilst a clearly identified deficiency in a specific contractile protein isoform is shown in patient 1, in the other six patients with abnormal immunostaining the defects are less well defined and the possibility that more than one protein is involved remains open. Nevertheless, changes in contractile protein content are evidently a fruitful area for further research into the underlying defects in intestinal myopathies, and point to possible abnormalities at the molecular and functional levels rather than mere structural aberrations useful for description only. There is clearly a need for the production of specific antisera against all the known isoforms of the contractile proteins in order to pursue this line of enquiry (see Chapter 9).

Chapter 8. The Role of Neural Markers in CIIP

8.1 Introduction

Histochemical and morphometric studies (Chapters 3 and 4) have identified diagnostic criteria for well established forms of neuropathic CIIP, such as HD, IND and hypoganglionosis. However, it is true that many patients with electrophysiological evidence of neuropathic enteric disease (Chapter 2) show no recognisable morphological changes in enteric innervation by conventional light and electron microscopical techniques.

The successful demonstration of subtle immunohistochemical abnormalities in myopathic disease was described in Chapter 7. A similar study has been attempted for intestinal neuropathies, focussing particularly on antibodies to probe the integrity of nerve cells and their processes (antibodies to neurofilament; NF) and the interface between nerves and smooth muscle cells (antibodies against neural cell adhesion molecule; N-CAM, and muscarinic receptors). The properties and roles of NF, N-CAM and muscarinic receptors are first reviewed and the antibodies used in this study are then described.

The antibodies (against NF, N-CAM and muscarinic receptors) have been applied to cold (-20°C) acetone- fixed cryostat sections of snap-frozen samples of full-thickness intestine obtained from patients with neuropathic, myopathic and undetermined forms of CIIP, as well as from apparently normal control samples obtained at post-mortem (as previously described in Chapter 7), and from fetal examinations.

8.1.1 Function and Distribution of NF, N-CAM and Muscarinic Receptors

8.1.1.1 Neurofilaments (NF)

NF are cytoskeletal intermediate filament proteins of three different molecular weights (light 70kD, medium 160kD and heavy 200kD), each of which exist in dephosphorylated or phosphorylated forms (Schlaepfer 1987). NF transcription, translation, assembly and alignment occur in the nerve cell body. The filaments are transported down the axon to the axon terminus, where they are degraded; the degradation products are then transported back to the perikarya. The rate of NF movement along the axon is regulated by NF phosphorylation (Schlaepfer 1987). The role of NF is to is to maintain nerve cell integrity by providing a framework to support the long axons. They also serve to anchor the microtubular system important in axonal transport (Hollenbeck 1989).

In the mature brain dephosphorylated NF are seen in the perikarya and large primary dendrites, but not in axonal processes. On the other hand moderately and strongly phosphorylated NF are found in axons but not in the cell bodies, whilst weakly phosphorylated NF are distributed uniformly along the neuraxis (Nixon and Sihag 1991). NF in the axons of mature enteric nerves differ from those in the brain in that they are mostly weakly phosphorylated (Eaker et al. 1990). In the newborn myenteric plexus, however, neurons do show positive immunostaining with antibodies to phosphorylated medium and heavy neurofilaments (Eaker and Sallustio 1992). Thus the degree of NF phosphorylation in enteric nerve cells may be a marker of a disturbed ontogenic process.

In neurodegenerative disorders such as Alzheimer's disease and following nerve

injury, the degree of neurofilament phosphorylation is increased in neuronal cell bodies (Leigh et al. 1989; Nixon and Sihag 1991; Schlaefper 1987). Axonal transport is slowed by general phosphorylation, or by phosphorylation of specific sites in the protein (Nixon and Sihag 1991) and thus abnormal phosphorylation could lead to pathological accumulation of perikaryal NF. It has been postulated that hyperphosphorylation at carboxy-terminal sites may promote abnormal accumulation in the axons (Nixon and Sihag 1991) and that a decrease in carboxy-terminal phosphorylation may cause NF to move faster in some neuropathies. In the intestine, where mature nerve cells normally contain weakly phosphorylated NF, alteration in the state of phosphorylation to heavily phosphorylated forms, apart from being a marker of immaturity, might also be an indicator of nerve injury or a neurodegenerative process. It could also reflect pathological defects in NF metabolism.

8.1.1.2 Neural Cell Adhesion Molecule (N-CAM)

N-CAM is a transmembrane glycoprotein found in nerve cells and glia, which regulates cell to cell interactions by homophilic polyvalent binding mechanisms (Nybroe et al.1988). It comprises at least thirty isoforms of 180kD, 140kD and 120kD molecular weights which arise from alternate splicing of mRNA transcribed from a single gene (Patel et al.1989) mapped to chromosome 11q23 in man (Nybroe et al. 1988). The length of the cytoplasmic portion of the molecule dictates the molecular weight of the protein (Walsh 1988).

Adhesion molecules are important in cell to cell recognition during embryogenesis and development (Nybroe et al. 1988). N-CAM is expressed in skeletal myoblasts and

myotubules in developing muscle but not in innervated adult myofibres. However, in denervated muscle cells in spinal muscular atrophy N-CAM is expressed. Immunohistochemically N-CAM has also been shown to be expressed transiently in fetal rat intestinal smooth muscle (Akeson et al. 1988) but not in the adult gut.

8.1.1.3 Muscarinic Receptors

Muscarinic receptors on smooth muscle cell membranes are the main receptors for acetyl choline released from cholinergic nerves to initiate smooth muscle contraction (Goyal 1989). The receptor proteins ranging in molecular weight between 51kD and 66kD comprise at least three pharmacologically different (M1, M2, M3) and five different molecular forms (m1, m2, m3, m4, m5) each encoded by a different gene. Muscarinic receptors have a single chain of 460-590 amino acids which span the plasma membrane seven times thus creating four extra cellular domains, seven helical hydrophobic transmembrane domains and four intracellular domains.

Muscarinic receptors are found on the soma and dendrites of many cholinergic neurons and on some axonal endings. In smooth muscle M1, M2 and M3 are present but contraction is mainly mediated through M3 receptors. Muscarinic receptor agonists and antagonists have been utilised in the pharmacological treatment of gastrointestinal disease (Goyal 1989). The M3 receptor agonist bethanecthol improves oesophageal motor activity and prokinetic agents such as metoclopramide, domperodone and cisapride enhance gastrointestinal transit by releasing endogenous acetyl choline (Goyal 1989). M1 on the myenteric neurons has been postulated to be involved in the prokinetic effects of these drugs by improving co-ordination of the contractions (Goyal 1989).

Abnormal muscarinic receptor content or distribution may be responsible for some forms of chronic idiopathic intestinal pseudo-obstruction (Bannister and Hoyes 1981). Diminished muscarinic binding sites were found, using radiolabelled receptor agonists or antagonists, in the mucosa of the dilated part of intestine from a patient with pseudo-obstruction with no histological or ultrastructural abnormalities (Rossowski et al. 1988). In contrast the muscularis propria contained increased concentrations of M2 binding sites but the M1 receptor concentration was normal.

A monoclonal antibody (M35) to muscarinic receptors has been raised (Andre et al. 1984, 1987), which reacts with antigenic determinants present on the active receptor. This antibody is an agonist to muscarinic receptors in gastric smooth muscle (Moummi et al. 1988) and is probably a pan-muscarinic receptor antibody recognising at least receptors M1 and M2 (Moummi et al.1988; Matsuyama et al. 1988). It is not clear, however, if M3, the major receptor mediating smooth muscle contraction, is identified by M35. This antibody is the only commercially available muscarinic receptor antibody.

8.1.2 Specificity of the Commercially Available Antibodies Used

(i) NR4 (Sigma, Dorset, UK) was raised against pig spinal cord NF and recognises both phosphorylated and dephosphorylated light molecular weight (68kD) NF.

(ii) NN18 (Sigma, Dorset, UK) was raised against pig spinal cord NF and recognises both phosphorylated and dephosphorylated medium molecular weight (160kD) NF.

(iii) NE14 (Sigma, Dorset, UK), an antibody raised against pig spinal cord NF. This

antibody reacts with phosphorylated heavy (200kD) molecular weight NF.

(iv) N52 (Sigma, Dorset, UK) was raised against the carboxyterminal tail segment of enzymatically dephosphorylated porcine NF heavy-subunit, recognising phosphorylated and dephosphorylated heavy molecular weight (200kD) NF.

(v) BF10 (Boehringer Mannheim UK, Lewes, UK), was derived from a solubilised raw extract of diseased Alzheimer brain. Medium (160kD) molecular weight phosphorylated NF are recognised by this antibody.

(vi) SM132 (Sternberger Baltimore, Maryland, USA) identifies dephosphorylated heavy (200kD) molecular weight NF.

(vii) RT97 (A gift from Prof. Anderton, Institute of Psychiatry, London,UK) demonstrates phosphorylated heavy (200kD) molecular weight NF.

(viii) Eric1 (A gift from Prof. Walsh, Guy's Hospital, London, UK). An antibody to N-CAM which was cloned from c-DNA library. This antibody recognises an epitope on the polypeptide backbone of N-CAM thus detecting most (all) isoforms of N-CAM.

(ix) M35 (Chemunex, Paris, France). This antibody was raised against affinity purified calf forebrain muscarinic receptors.

8.2 Materials and Methods

8.2.1 Materials

The tissue samples examined were the same as those used in the investigation of contractile proteins (see Chapter 7), namely nine control intestines and 53 specimens of intestine from 35 patients with CIIP (11 with myopathic, 18 with neuropathic and 6 undetermined CIIP). Because insufficient tissue remained in the tissue blocks for full immunohistochemical examination in a further 12 patients (seven with neuropathic and five with undetermined forms of CIIP), NF immunostaining was performed only with clones NR4 and NN18 . The immunoreactivity of N-CAM was also assessed in samples of intestine from six abortuses (12-23 weeks gestation). Ethical committee approval for this study was obtained.

8.2.2 Methods

ABC immunostaining was performed on cold acetone (-20°C) fixed cryostat sections of snap-frozen samples of full- thickness intestine (see Chapter 7 and Appendix). Endogenous peroxidase was not blocked since this inhibited the specific immunostaining. The monoclonal antibodies listed above were used at a titre of 1:320 (NR4), 1:2000 (NN18, NE14, N52), 1:80 (BF10), 1:8000 (SM132), 1:40 (RT97), 1:15000 (Eric1) and 1:1800 (M35). For controls see Chapter 7.

Microscopy

Microscopical assessment of the myenteric plexus was made by noting the differences in the immunostaining of axons, neuronal cell bodies and glia with these antibodies. The presence or absence and the type (linear or tangled, see below and Figs.1 and 2) of NF-positive nerve fibres in the circular muscle coat was also recorded. In addition,

the presence or absence of immunostained smooth muscle cells was also noted.

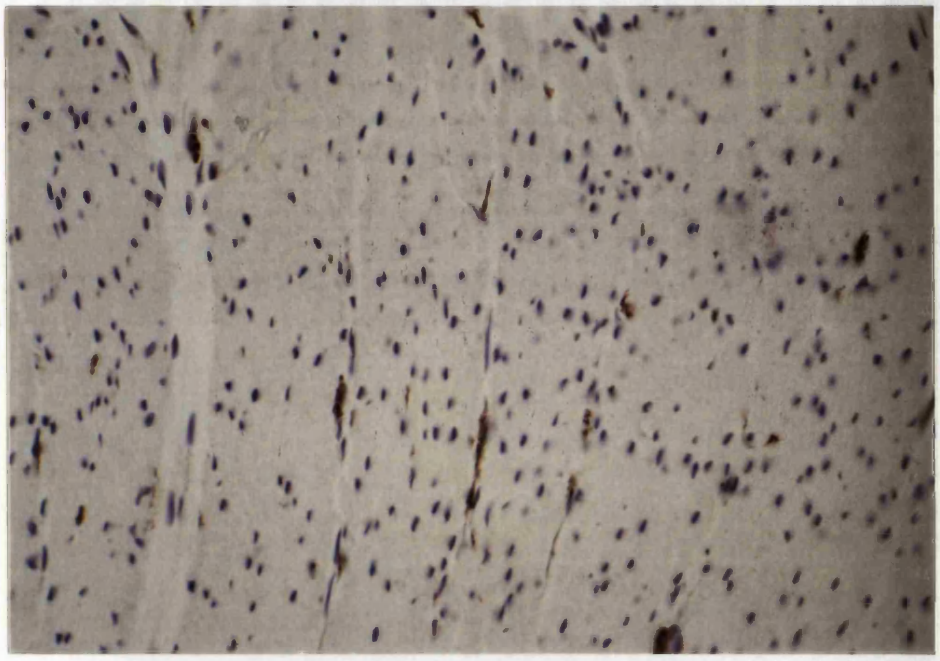


Figure 1. Neurofilament immunostaining (NR4) showing an increase in 'linear' nerves in the circular muscle coat in a patient with HD. Magnification x640. In normal circular muscle no or only a very occasional fibre is seen.

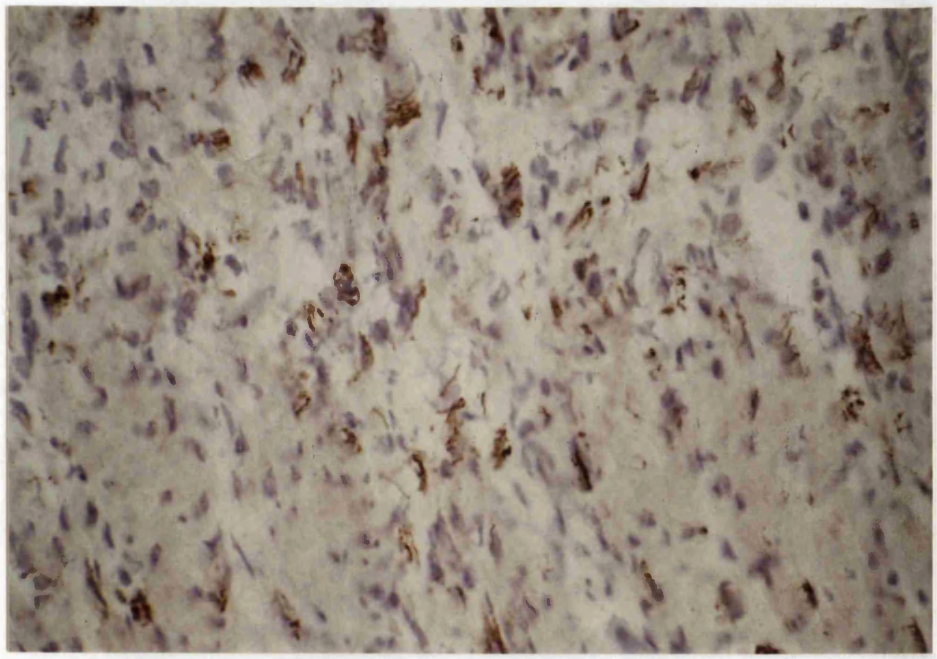


Figure 2. Circular muscle showing a marked increase in short brush-like 'tangled' NF-positive (NR4) nerves. This patient has both histopathological and electrophysiological evidence of myopathy. Magnification x640

Morphometric analysis

In selected samples (detailed below) morphometric analysis was made, employing the Magiscan (Joyce Loebel). Using a x40 objective, nerves in ten manually chosen fields of the circular muscle in each sample were automatically quantified. The morphometric program used identified nerves with grey levels within predetermined range chosen during a calibration procedure. It was possible to check microscopically that the structures recognised by the program reflected the nerve content and any discrepancies could be edited out. The results were expressed as the percentage of the area occupied by nerves in the total area analysed.

Samples analysed morphometrically

(a) The degree of NF phosphorylation was analysed morphometrically in intestine from one patient with myopathic disease associated with morphological evidence of autophagic activity and a gross increase in nerve fibres innervating the circular muscle.

(b) Morphometric analysis of N-CAM immunostained nerves and glia in the circular muscle was made in four control samples and 18 surgical specimens. Of the latter samples, eight were from patients with intestinal myopathy, eight from those with neuropathic disease, and two from patients with undetermined pseudo-obstruction.

8.3 Results

8.3.1 NF Immunostaining in Normal Post-mortem Bowel

Using the antibody SM132 (dephosphorylated heavy molecular weight NF), most neuronal cell bodies showed strong immunoreactivity which extended a short but

variable distance into the proximal processes (Fig.3). Fibres in the axon bundles were mostly negative and no fibres were seen in the muscularis propria.

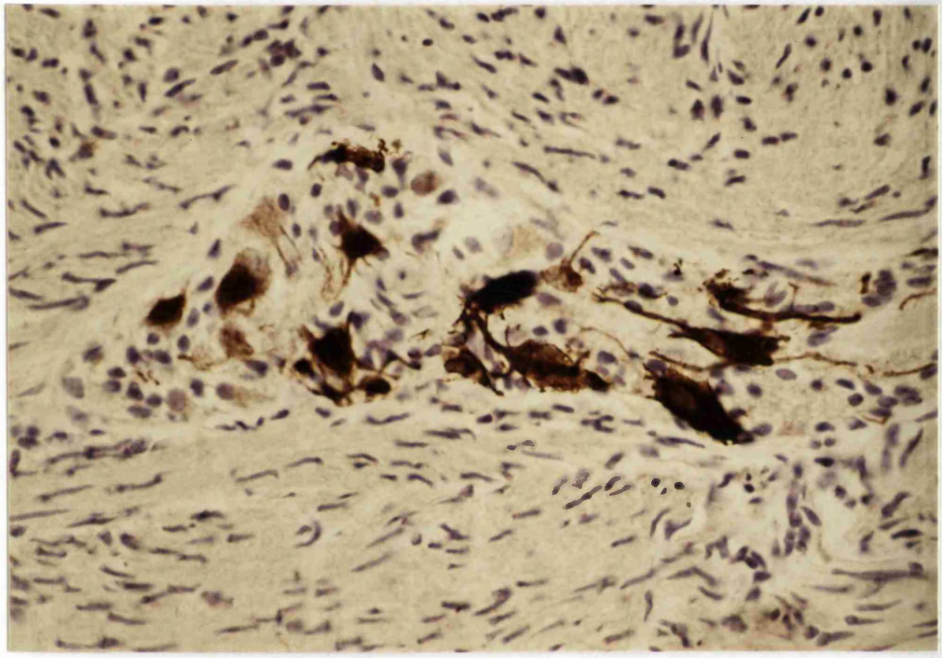


Figure 3. The myenteric plexus showing immunoreactivity in neuronal cell bodies with the antibody SM132. Note the poor immunostaining of processes and the negative nerve bundles. Magnification x400

The antibody NE14 (phosphorylated heavy molecular weight NF) proved rather non-specific. It was difficult to eliminate all background staining but further titering of the antibody allowed recognition of weak specific staining. Fibres in the axon bundles were strongly immunoreactive but the cell body staining was variable, ranging from strongly positive in the minority to negative in the majority. Occasional nerve fibres were detected in the muscularis propria.

With RT97 (phosphorylated heavy molecular weight NF) only very occasional moderately positive neuronal cell bodies were found but the fibres stained strongly (Fig.4). Immunostaining with BF10 (phosphorylated medium molecular weight NF) was very similar to RT97, the only difference being that perikaryal immunoreactivity appeared stronger in the few neuronal cell bodies that exhibited it. Both of these antibodies detected very sparse fibres in the muscularis propria.

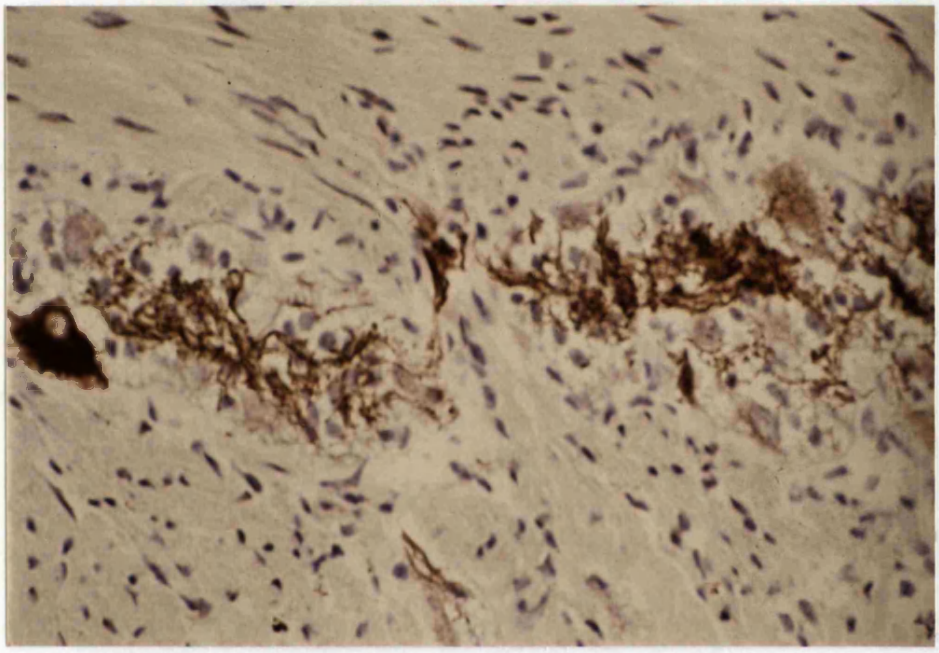


Figure 4. Immunostaining with RT97 showing immunoreactive axons in the axon bundles. The neuronal cell bodies are mostly negative but very occasional cell body is positive. Magnification x400

Nerve fibres and several cell bodies showed strong immunoreactivity with N52 (dephosphorylated and phosphorylated heavy molecular weight NF), NN18 (dephosphorylated and phosphorylated medium molecular weight NF) and NR4

(dephosphorylated and phosphorylated light molecular weight NF; Figs.5 and 6), although negative and weakly positive perikaryal immunostaining was also noted. The normal muscularis propria showed only an occasional immunopositive nerve fibre.

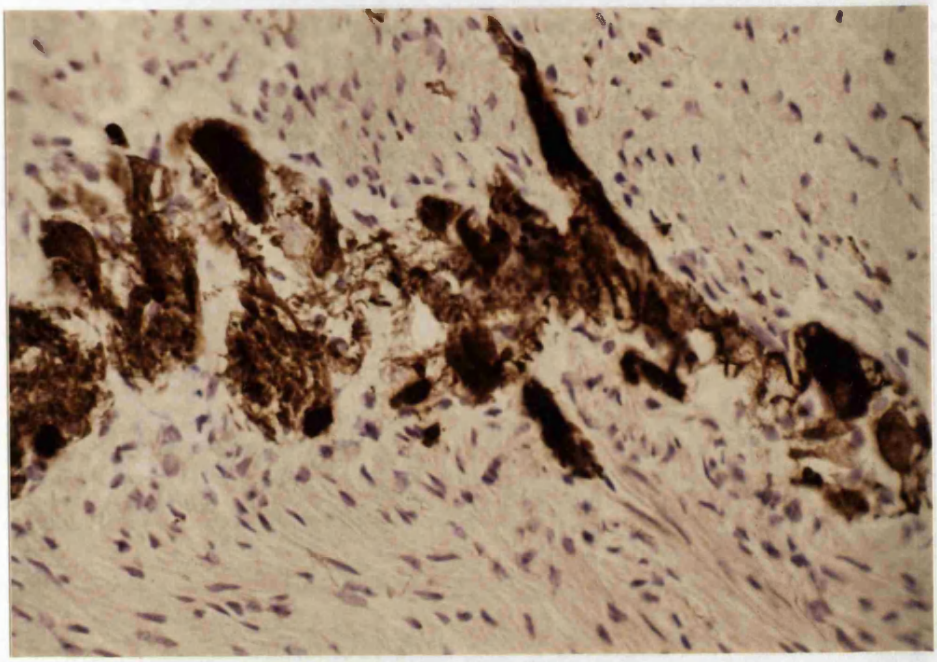


Figure 5. NR4 immunostaining in the myenteric plexus showing several strongly immunoreactive cell bodies and axons. Magnification x400.

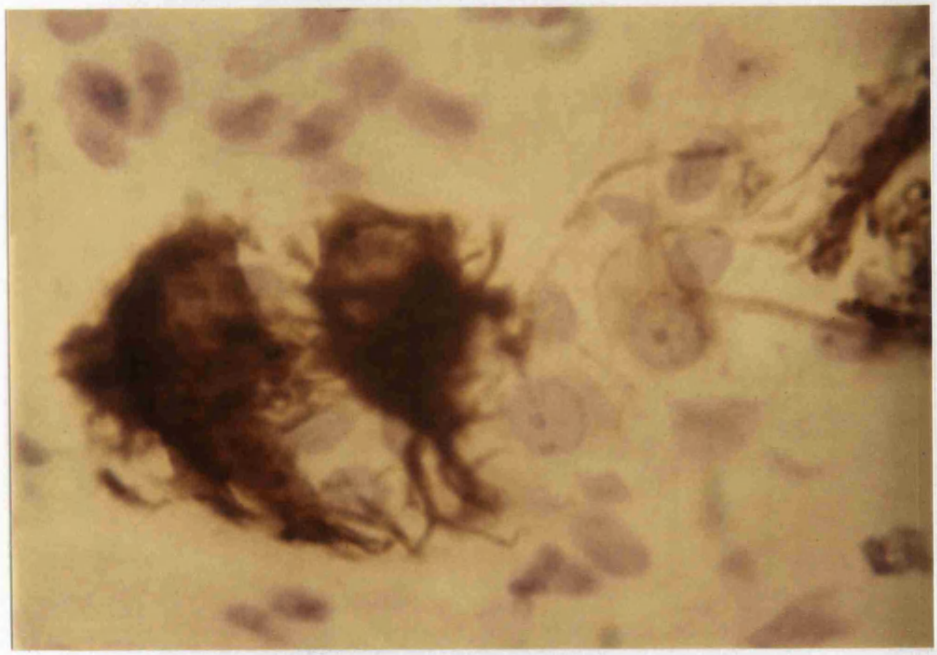


Figure 6. NR4 immunostaining showing 'spidery' immunoreactive neurons at higher magnification (x640)

Due to the presence of numerous white cells with abundant endogenous peroxidase the assessment of immunoreactivity in the lamina propria was impossible. These results are summarised in Table 1.

Table 1. Antibody reactivity with enteric nerve cells

Antibody	Recognition	Cell bodies	Axons
SM132	(D-H)	+++	-ve
NE14	(P-H)	++	+++
RT97	(P-H)	+	+++
BF10	(P-M)	+	+++
N52	(P&D-H)	+++	+++
NN18	(P&D-M)	+++	+++
NR4	(P&D-L)	+++	+++

P Phosphorylated
D Dephosphorylated

L Light NF
M Medium NF
H Heavy NF

-ve Negative

\+ rare NF-positive cells
++ occasional NF-positive cells
+++ many NF-positive cells

8.3.2 NF in Specimens from Patients with CIIP

The most striking observation was an increase in NF immunoreactive nerves (with a number of NF antibodies) in the circular muscle in 13 patients, 11 with myopathic

electrophysiology and two in whom electrophysiology was not performed but the samples showed morphological evidence of smooth muscle disease. This was characterised by numerous tangles of brush-like nerve fibres (Fig.2) scattered throughout the circular muscle layer ('tangled' nerves; see Table 2). In contrast in the circular muscle of controls only very occasional NF-immunoreactive nerves were identified.

In HD (four patients) and IND (two patients) there was also an increase in immunoreactive nerve fibres in the circular muscle with all but one of the NF antibodies. However, the character of this immunostaining was quite different and identified short straight lengths of positively immunostained nerve fibres between the muscle bundles (Fig.1; 'linear' nerves). Similar immunostaining was noted with one or more NF antibodies in one other patient with IND, seven patients with electrophysiological evidence of neuropathic disease, one patient with hypoganglionosis and one with undetermined CIIP (Table 3).

In the myenteric plexus no obvious inter-sample differences, other than those reflecting morphological abnormalities such as the presence of hypertrophied nerve trunks in HD, were seen after immunostaining with any of the antibodies employed. All axons in the axon bundles appeared to be reactive and the immunostaining of neuronal perikarya was the same in all specimens as in controls.

Morphometric analysis (Table 4) of the immunostaining with all but one of the NF antibodies (NN18) was performed in the intestine from one myopathic patient described with autophagic activity in Chapter 6. NN18 staining was excluded because

the background was high (see Fig. 7), the brush-like tangled nerves were palely stained, and the sensitivity of the automated morphometric program was insufficiently reliable to identify them. Although an increase in nerve fibres in the circular muscle was confirmed morphometrically with all the antibodies used, this increase was most pronounced with antibodies to phosphorylated heavy and medium NF. Since practically no immunoreactive nerves were detected in the circular muscle in the controls and most surgical samples morphometric analysis added no significant advantages over subjective analysis.

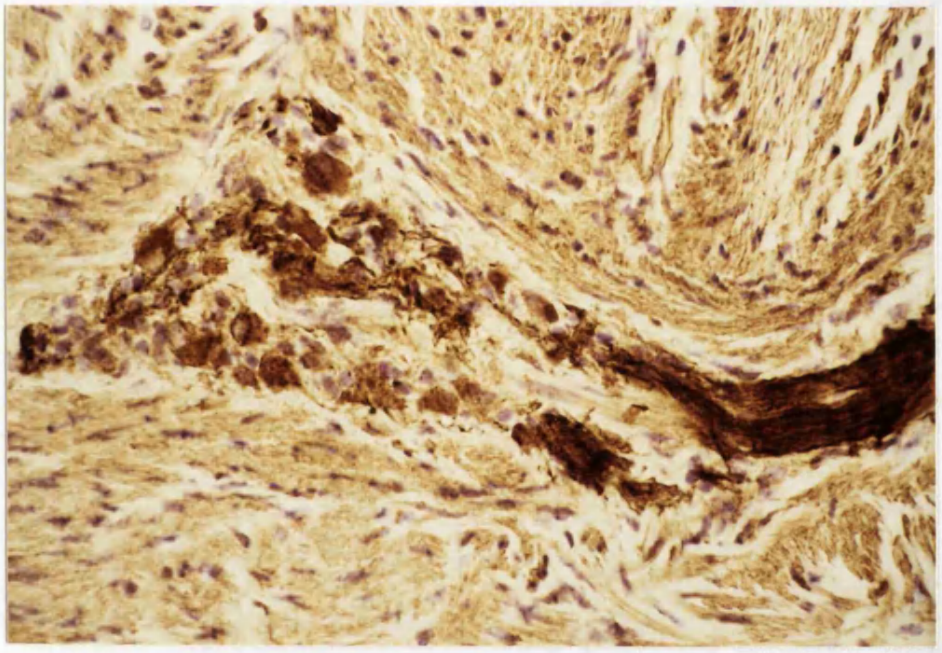


Figure 7. NN18 in the myenteric plexus showing variable immunostaining of neuronal cell bodies. Axons appear strongly positive. Note the high non-specific background reaction in the myocytes of the muscularis propria. Magnification x400

Table 2. Tangled small nerves in circular muscle

Patient	NR4	N52	NN18	SM132	RT97	BF10
PA (M)	-	+	-	-	-	-
PM (M)	+	+	+	+	+	+
CJ (M)	+	+	+	-	+	+
Alo (M)	+	-	-	-	-	+
Is (M)	+	+	+	+	+	+
ZB (M)	+	+	+	+	+	+
JB (M)	+	+	+	+	+	+
AM (M)	+	+	+	+	+	+
LC (M)	+	-	-	-	-	-
ALI (M)	-	-	-	-	-	-
Ala (M)	+	0	+	0	0	0
CW (MH)	+	+	+	-	-	+
Be (MH)	+	0	+	0	0	0
MS (NM)	+	0	+	0	0	0
Rob (UD)	-	0	-	0	0	0
Der (UD)	-	0	-	0	0	0
DC (UD)	-	0	-	0	0	0
LR (UD)	-	0	-	0	0	0

+ increase in tangled nerves
- no tangled or linear nerves
0 not performed
NE14 not analysed

M myopathy
MH morphologically myopathic
UD undetermined
NM myo/neuropathy

Only patients in whom an abnormality was detected and those with incomplete immunostaining are listed here.

Table 3. Increase in linear nerves in circular muscle

Patient	NR4	N52	NN18	SM132	RT97	BF10
Sa (N)	-	+	-	-	-	-
FC (N)	-	+	-	-	-	-
GB (N)	-	+	-	-	tangled	-
AR (N)	-	+	-	-	-	+
Bry (N)	-	+	-	-	+	-
NS (N)	+	0	+	0	0	0
RW (N)	+	0	+	0	0	0
MM (N)	-	0	-	0	0	0
JM (N)	-	0	-	0	0	0
LM (N)	-	0	-	0	0	0
LP (IND)	+	+	+	-	+	+
CT (IND)	+	+	+	-	+	+
MK (IND)	-	+	-	-	-	+
Mar (ho)	+	+	-	-	-	+
Cwe (UD)	-	+	-	-	-	-
HD (x4)	+	+	+	-	+	+

+ increase in linear nerves
- no linear or tangled nerves
0 not performed

ho hypoganglionosis
HD Hirschsprung's disease

N neuropathy
UD undetermined
IND intestinal neuronal dysplasia

Only patients in whom an abnormality was detected and those with incomplete immunostaining are listed here.

Table 4. Morphometry of NF-immunostaining in circular muscle of a patient with intestinal myopathy *

Antibody	Density of nerves (%-age of area analysed)
SM132	0.19
NE14	1.27
RT97	0.48
BF10	1.80
N52	0.22
NN18	Not analysed
NR4	0.34

* Note in normal intestine virtually no NF-positive nerves are identified in the circular muscle.

8.3.3 Neural Cell Adhesion Molecule (N-CAM) Immunostaining

8.3.3.1 N-CAM Immunostaining in Control Bowel

In the normal bowel the antibody Eric1 reacted with nerve fibres in the enteric plexuses immunostaining the periphery of the perikarya, neuronal processes and glial cells (Fig.8). Due to the large quantity of endogenous peroxidase, assessment of nerve fibres in the lamina propria was unreliable, but in the muscularis Eric1 proved extremely avid and gave clean immunostaining with virtually no background (Fig.8). Nerve fibres and glial elements in the circular and longitudinal muscle layers as well as in the muscularis mucosae were well demonstrated. The pattern and number of N-CAM immunostained neural and glial fibres mimicked those of AChE-positive nerves.

However immunostaining for N-CAM did not correspond with that for NF protein, with which nerves in the circular muscle of controls were almost completely unreactive. No N-CAM immunostaining was detected in the smooth muscle cells of any of the intestinal muscle coats.

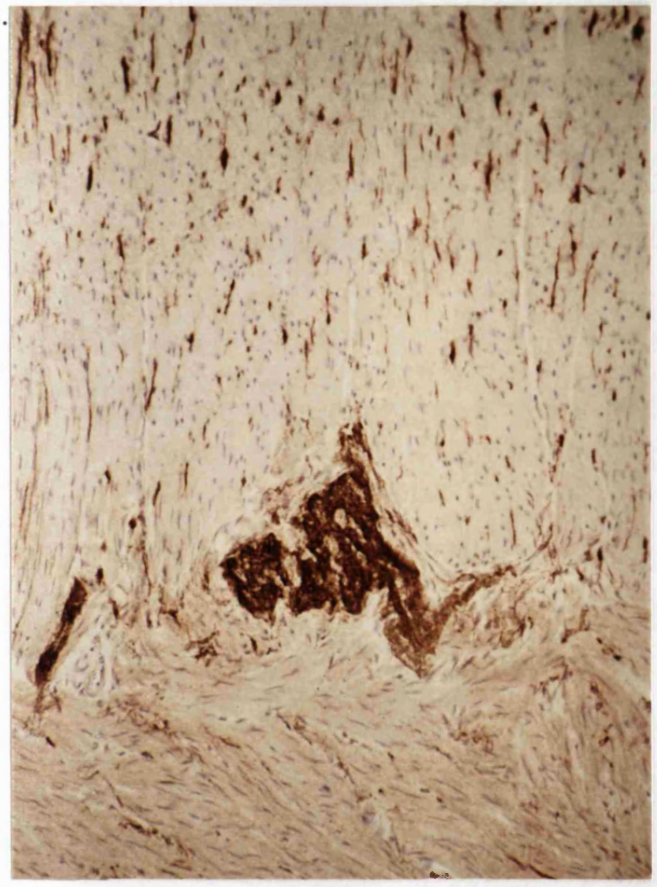


Figure 8. Section of intestine showing normal N-CAM immunostaining in the myenteric plexus and the muscularis propria. Many positive fibres are seen in the circular muscle coat. Magnification x160

Morphometry in Control Intestine (N-CAM)

Values obtained by the automated morphometric method represent the percentage fraction of the total area analysed occupied by the immunostained nerves and glia. The technique required circular muscle at least 0.3mm thick and most post-mortem control samples were too thin. However, four control samples of colon were suitable for morphometry and in these the values ranged from 0.33% to 0.57% (mean 0.48%) (Table 5).

Table 5. Morphometry of N-CAM immunostained fibres in circular muscle

Myopathy (n=8)	Neuropathy (n=8)	Undetermined (n=2)	Controls (n=4)
(Fraction expressed as percentage of area analysed)			
0.64	0.6	0.42	0.55
2.48*	0.44	0.59	0.57
0.21	0.008*		0.49
0.26	0.62		0.33
0.71	0.38		
0.29	0.34		
0.54	0.22		
0.31	0.29		

* Very similar values were seen in the majority of samples. In one, however, a prominent increase in fibres was noted, while in another a virtual absence of fibres was evident (values identified by *). These appearances were readily identifiable even on simple microscopy.

8.3.3.2 N-CAM Immunostaining in CIIP

Microscopy of the Muscularis Propria

In the majority of the 35 patients with CIIP examined, N-CAM immunostaining of nerve and glial fibres in the muscularis propria was very similar to that in normal controls. However, distinct differences were seen in four patients in two of whom there were grossly elevated numbers of N-CAM-positive nerve and glial fibres in the circular muscle. Both patients were those recognised in Chapter 6 as a distinct subgroup of myopathic disease already characterised by autophagic activity, and prominent increases in tangled NF-positive fibres and AChE activity. An increase in N-CAM-positive fibres in the circular muscle is thus a further feature of this

myopathic subgroup.

In the other two patients the abnormality lay in the virtual absence of N-CAM-positive nerve and glial fibres in the circular muscle. One (Sa; Table 6) had aganglionosis; a further abnormality noted in this case was that the usual increase in AChE-positive nerves in the muscularis seen in HD was lacking. The other patient with absent N-CAM immunostaining as well as a corresponding lack of AChE-positive fibres in the circular muscle, was a patient with acquired aganglionosis due to an inflammatory plexopathy (FH; Table 6). This patient had a number of bowel resections showing a gradual loss of enteric neurons accompanied in the initial stages by a dense chronic inflammatory cell infiltration in the enteric plexuses (Fig. 9). The loss of enteric neurons was paralleled by a loss of AChE-positive elements (Fig. 10) and negative immunostaining for protein gene product 9.5 (PGP 9.5; a neural marker) in the muscularis propria. An IgG class antibody in this patient's serum reacted with enteric nerve cells in sections of neonatal porcine intestine, while no such immunostaining was observed with control serum (Figs.11 and 12). These findings suggest that the intestinal denervation in this patient was an autoimmune process.

As in controls, in patients with CIIP the pattern of N-CAM immunostained nerves and glia in the circular muscle was generally similar to that by AChE histochemistry (Table 6). However, in two patients with hypoganglionosis (GB and Han; Table 6) absent or reduced AChE activity was accompanied by normal N-CAM immunostaining. Patient GB also had an increase in NF-positive nerves in the circular muscle (seen as 'tangled' with RT97 and 'linear' with N52; Table 3). In patient Han NF immunostaining was entirely normal.

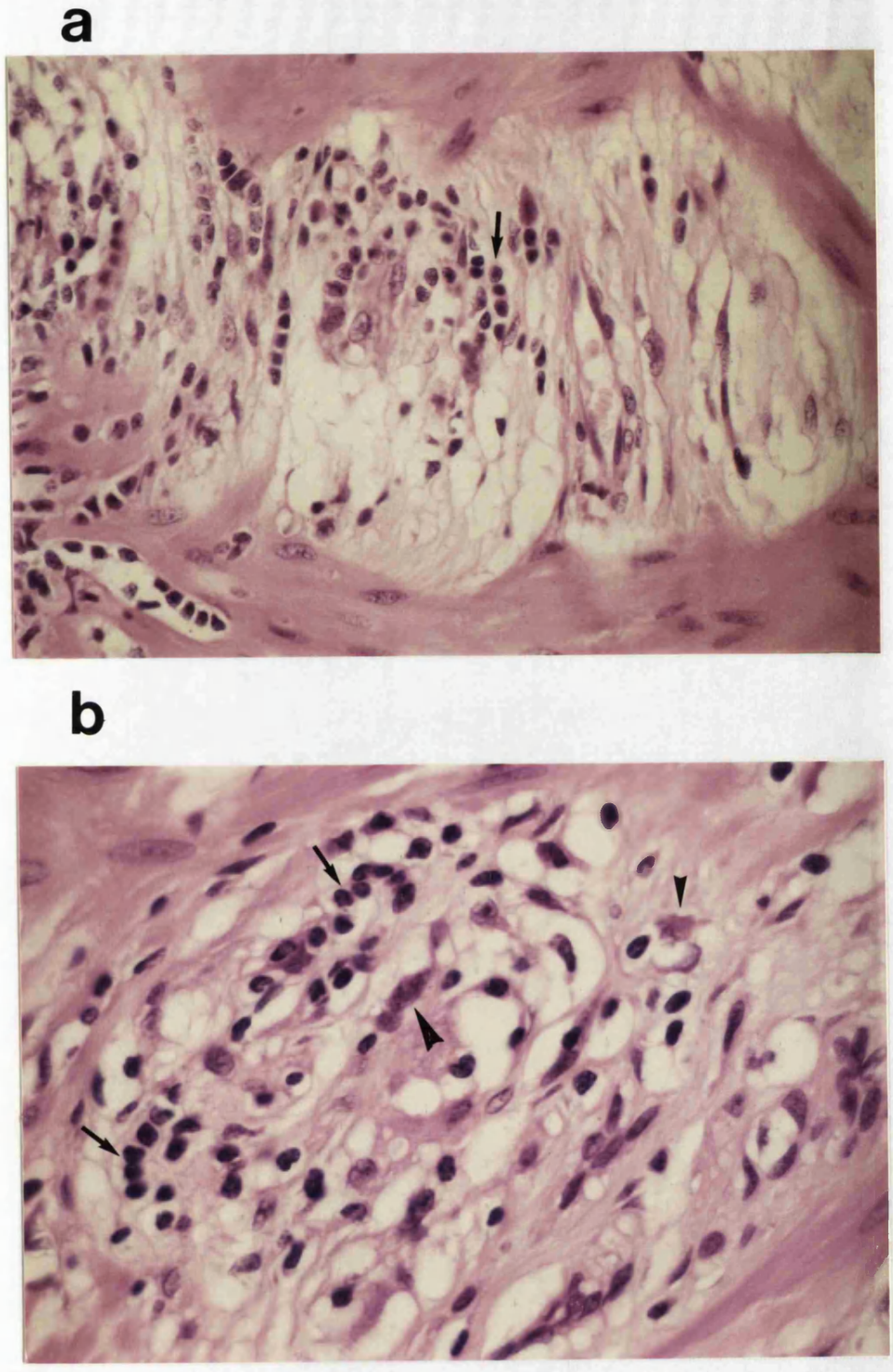


Figure 9 a & b. Abnormal vacuolated myenteric ganglia in the ileum from the patient with acquired hypoganglionosis. Neurons are seen but they are condensed and degenerate (arrow heads). Observe the presence of a marked mononuclear cell infiltrate (arrows). Magnification (a) x400 (b) x640

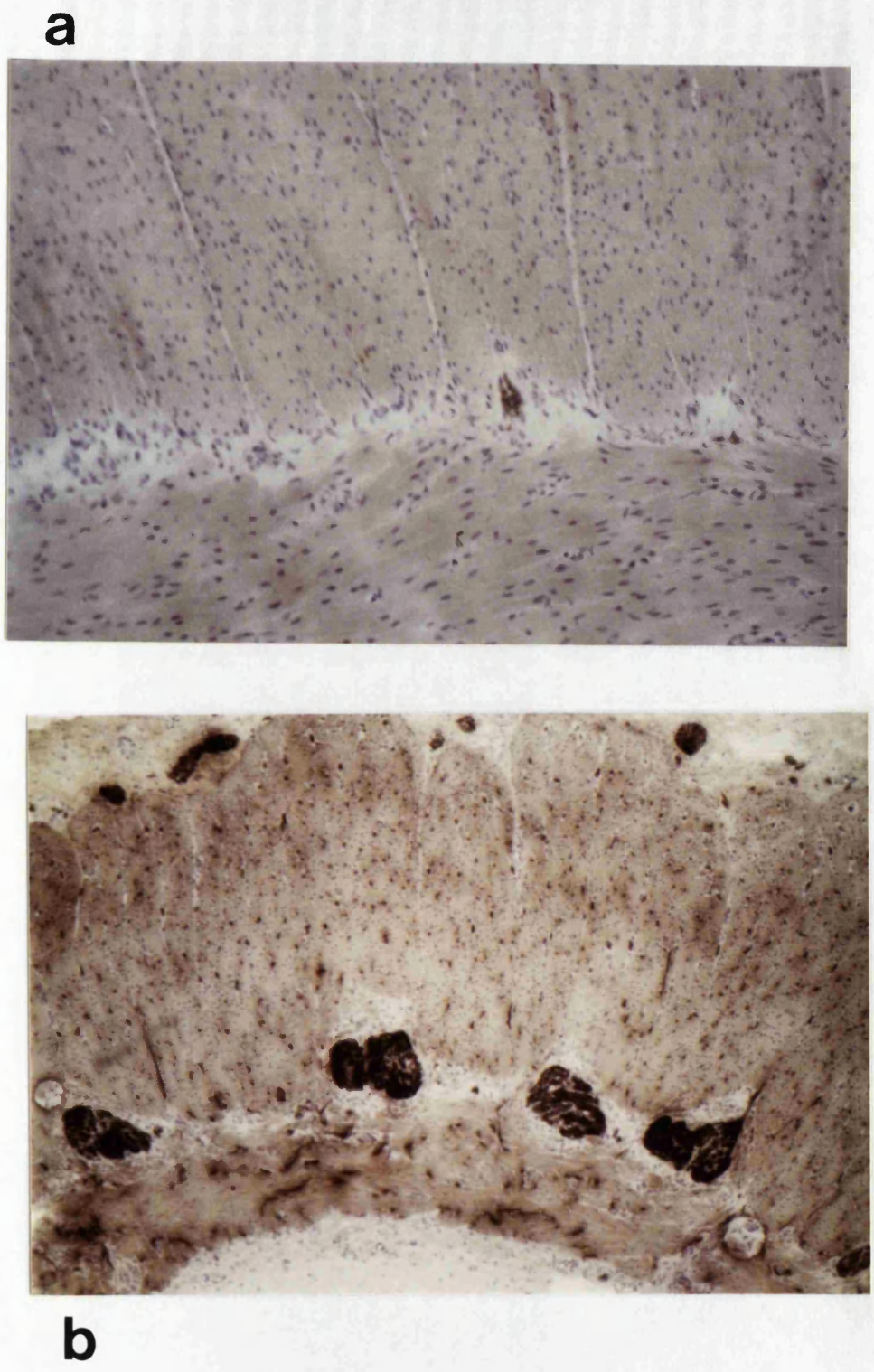


Figure 10. (a) The second sample of ileum from the patient with inflammatory plexopathy showing only remnants of AChE-positive elements in the myenteric plexus. No AChE-positive nerves are identified in the muscularis propria. Magnification x160. (b) AChE-activity in normal bowel. Note numerous nerves especially in the circular muscle. Magnification x80

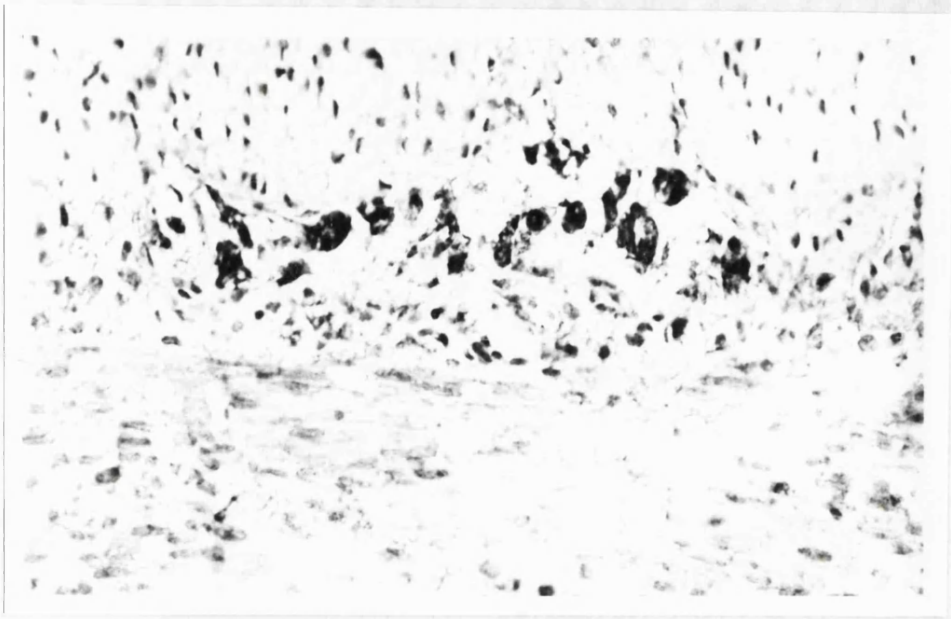


Figure 11. Serum (at a titre of 1:640) from the patient with inflammatory plexopathy reacting with myenteric neurons of newborn piglet ileum. Magnification x340

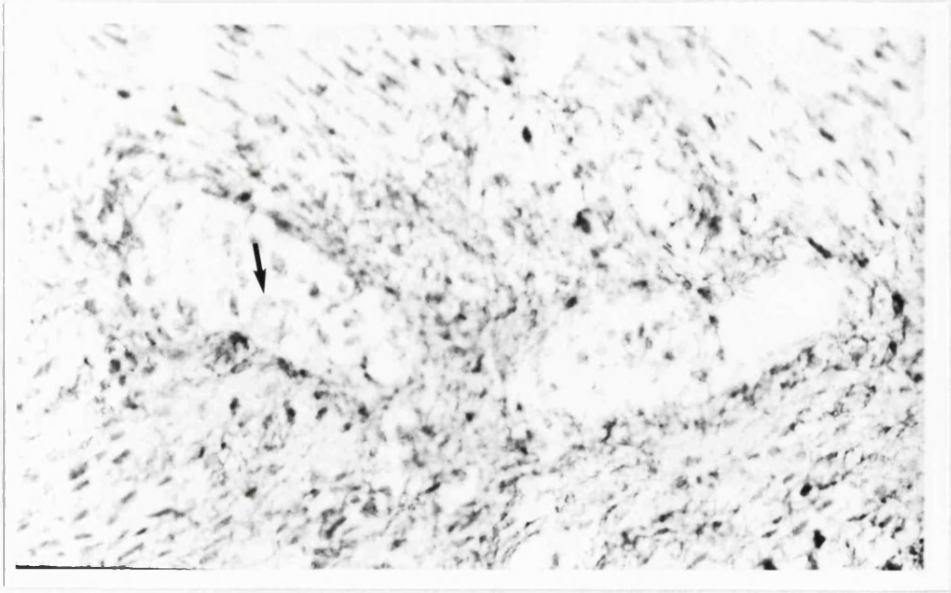


Figure 12. Control serum applied neat to section of piglet ileum shows no immunoreactivity in the myenteric neurons (arrow). Magnification x340.

Table 6. Innervation of the circular muscle in hypoganglionosis and aganglionosis: Comparison of AChE, N-CAM and NF

Patients	AChE	N-CAM	NF
GB	R	N	Inc (RT97,N52)
LS	N	N	N
Han	A	N	N
FH	A	A	N
Sa	A	A	Inc (N52)
HD	N/Inc	N/Inc	Inc

R	Reduced	AChE	acetyl cholinesterase
A	Absent	N-CAM	neural cell adhesion molecule
N	Normal	NF	neurofilament
Inc	Increased	HD	Hirschsprung's disease

Morphometry of N-CAM in Muscularis Propria

N-CAM immunostaining using Eric1 was ideal for morphometric analysis by the automated system since background immunoreactivity was low and the immunopositive fibres were sharply defined. In the majority of the 18 samples analysed morphometrically from patients with CIIP (myopathic, neuropathic or undetermined) no obvious variability in the number of nerve and glial fibres in the circular muscle was shown, and the values did not greatly differ from the control data (Table 5). In two patients the numbers of N-CAM-positive nerve and glial fibres were significantly different (raised in one and virtually absent in the other), but these morphometric differences were clearly evident on simple microscopy (see above) so

that the time-consuming morphometric analysis had no additional benefits.

Microscopy of the Myenteric plexus in CIIP

The assessment of immunostaining for N-CAM in the myenteric plexus revealed no specific differences in the various forms of CIIP other than those detected by general morphological techniques or enzyme histochemistry (AChE). However, in the patient (described above) with aganglionosis due to inflammatory plexopathy and virtually absent AChE activity in the myenteric plexus, N-CAM positive fibres were surprisingly well preserved (Fig.13 compared with Fig.8) despite the absence of such fibres in the muscularis (see above). Positive immunostaining was also seen for S-100 protein indicating the glial nature for this immunoreactivity.

N-CAM Expression in Smooth Muscle Cells in CIIP

In the rectum from two patients with CIIP, one with electrophysiological and morphological evidence of an intestinal myopathy and the other with normal electrophysiology and morphology, the myocytes of the muscularis propria appeared to show punctate N-CAM immunostaining.

8.3.3.3 N-CAM Immunostaining in Developing Intestinal Smooth Muscle

In all the fetal samples (12 to 23 weeks gestation) the smooth muscle cells in the developing muscularis propria showed positive punctate immunostaining for N-CAM.

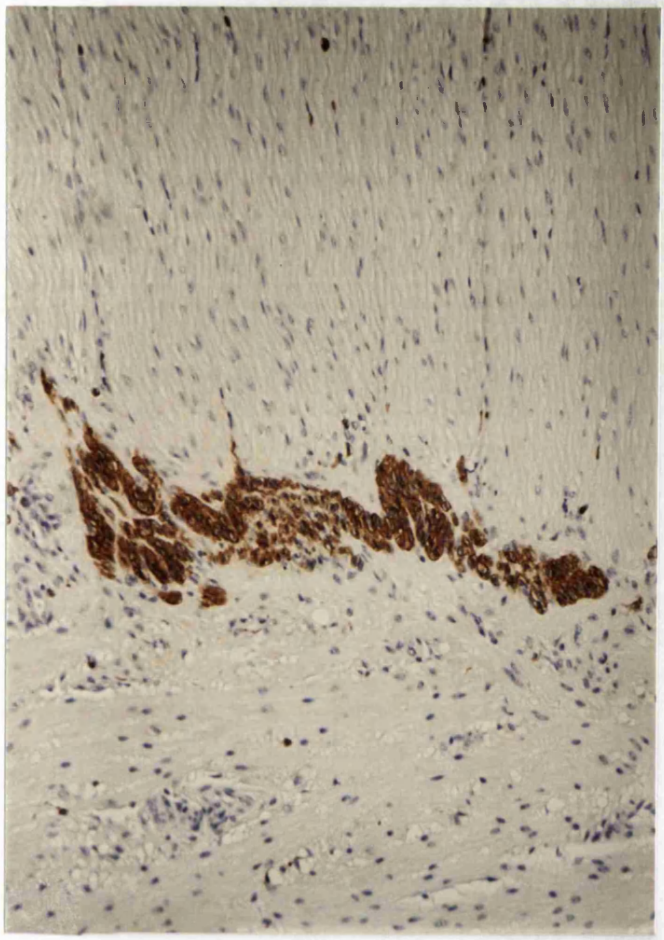


Figure 13. Immunostaining for N-CAM in the second ileal sample from the patient with inflammatory plexopathy showing preservation of positive (glial) elements in the myenteric plexus. (Compare normal AChE-activity in Fig. 10 and N-CAM in Fig. 8). Vacuolated nerve tracts and lack of positive fibres is seen in the muscularis. Magnification x160

8.3.4 Muscarinic Receptor Immunostaining

8.3.4.1 Muscarinic Receptor Immunostaining of Control Bowel

In the normal bowel stippled M35 immunostaining was seen in the perikarya of some of the enteric neurons and along the neuronal processes (Figs. 14 and 15). A variable degree of immunostaining was detected on the smooth muscle cells, most of which appeared moderately to strongly positive (Figs.14 and 15). In longitudinally cut myocytes there was variability in immunostaining along the length of the muscle fibres perhaps reflecting the differences in receptor density.

Smooth muscle in the blood vessel walls was also immunoreactive. Generally the background was acceptably low but a few fibroblasts showed evidence of immunoreactivity. As with all immunoperoxidase methods analysis of the fibre content of the lamina propria was unreliable due to the many cells with endogenous peroxidase. Blocking this seriously compromised the specific immunostaining.

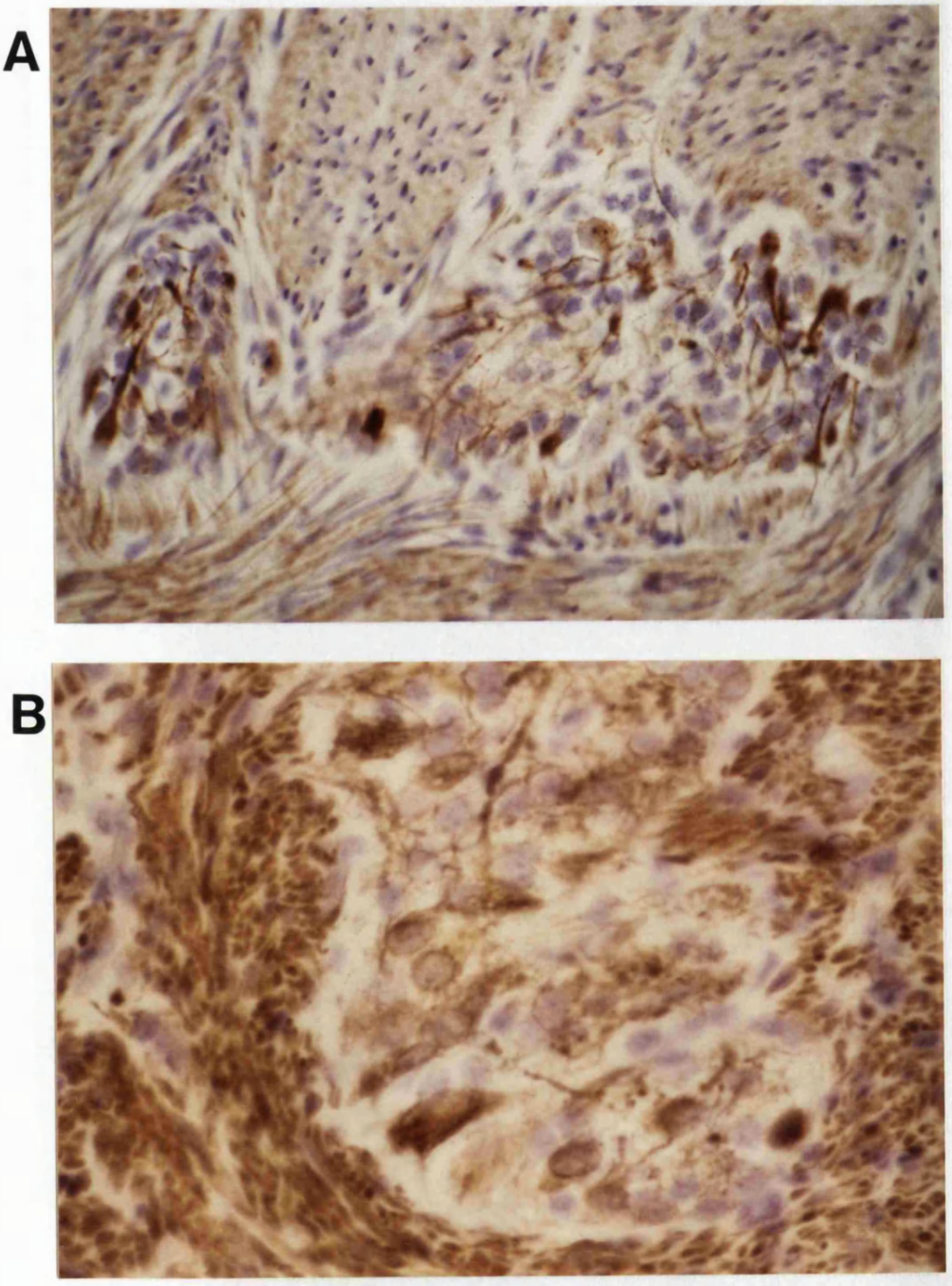


Figure 14. Normal intestine. Immunostaining for muscarinic receptors (M35) in the myenteric plexus showing reactivity in the axons and cell bodies. Note positive immunostaining of the smooth muscle cells. Magnification (a) x400 (b) x640

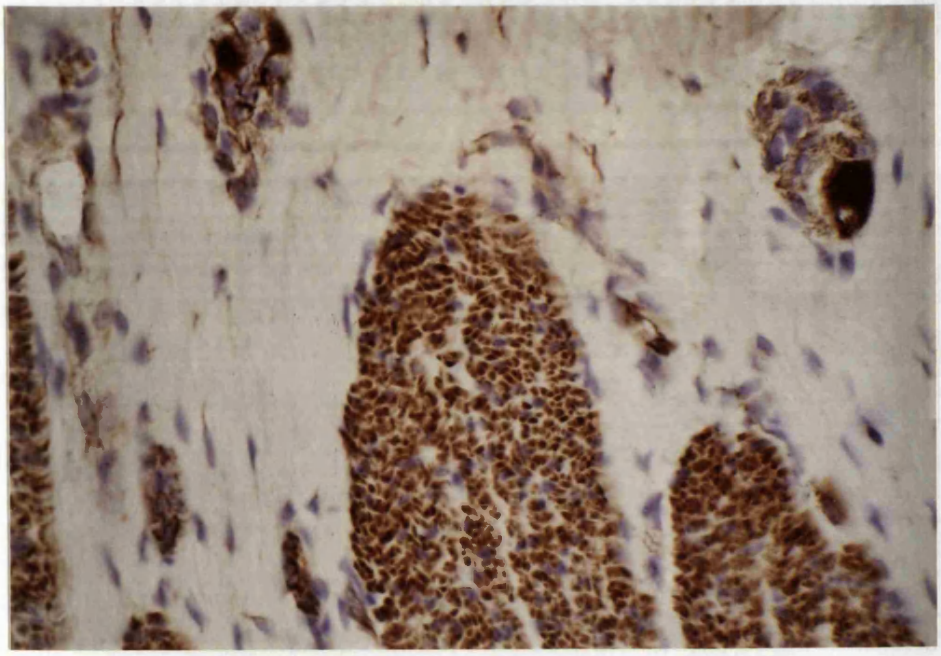


Figure 15. Normal intestine. Positive immunoreactivity for muscarinic receptors in the neurons of the submucosal plexus and the inner circular muscle layer. Magnification x400

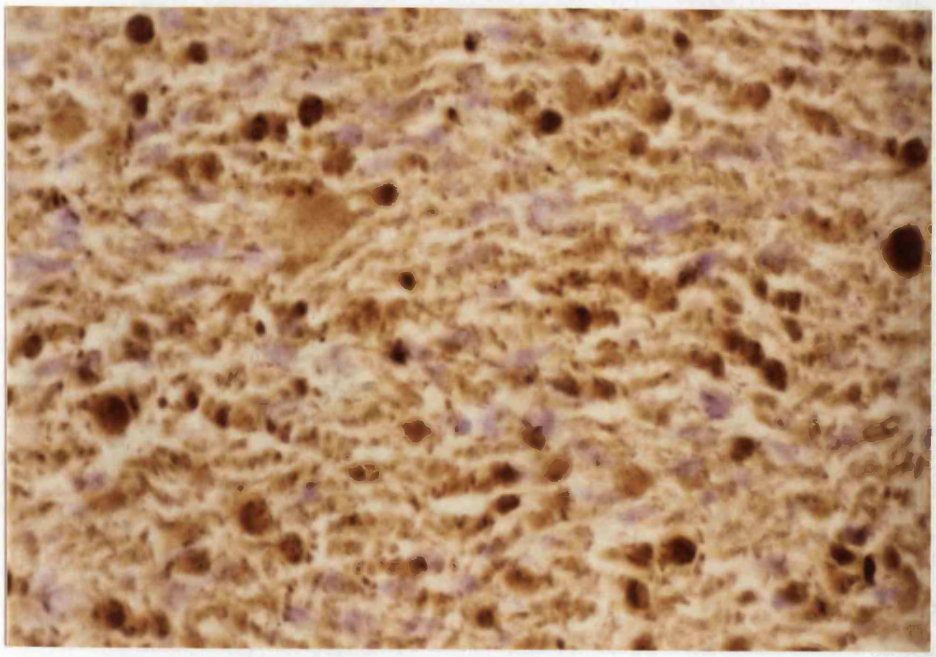


Figure 16. Immunostaining of muscarinic receptors in the circular muscle of a patient with intestinal myopathy showing enlarged dense smooth muscle cells with strong immunoreactivity among other more normal looking myocytes. Magnification x640

8.3.4.2 Muscarinic Receptor Immunostaining in CIIP

Immunostaining of the circular muscle (longitudinal muscle and muscularis mucosae) in patients with CIIP was very similar to controls. The smooth muscle cells were immunoreactive in all samples, but a patchy distribution was noted in one patient with intestinal myopathy and in one other myopathic patient bloated, strongly positive myocytes were seen particularly in the circular muscle (Fig.16). In two neuropathic patients (one of whom had IND) the area adjacent to the myenteric plexus showed considerably weaker staining than the rest of the circular muscle. Both myopathic patients with autophagic activity showed a grossly abnormal receptor distribution, particularly adjacent to the myenteric plexus. Although smooth muscle cells were immunoreactive it appeared that some atrophic myocytes were totally negative and much of the staining was due to the presence of brushlike, tangled proliferating nerve fibres in these specimens. In the rest of the samples examined immunostaining did not differ from the controls. The enteric plexuses showed no obvious differences from the controls.

8.4 Discussion

Although the original aim of this part of the study was to identify abnormalities in patients with neuropathic disease, the most striking and unexpected finding was the increase in fine, tangled, NF-positive, brush-like nerve fibres in the circular muscle of patients with myopathic disease. This increase was greatest with antibodies to phosphorylated heavy and medium NF and was seen in 13 of the 15 patients with electrophysiological and/or histological evidence of intestinal myopathy, but was not encountered in the neuropathic patients nor in controls. The only exception was one patient with neuropathic electrophysiology in whom the colon displayed some tangled

fibres in the circular muscle with just one of the NF antibodies (RT97). In all cases the tangled fibres were confirmed to be neural by ultrastructural examination.

The significance of the increase in 'tangled' NF-positive nerve fibres remains speculative. Although the innervation of skeletal muscle is different from that of smooth muscle, it is possible that some analogies can be drawn. Neurocladism, that is sprouting of terminal axonal endings to produce fine tangles barely visible on routine microscopy even using an oil-immersion objective, has been observed in primary skeletal myodegenerative disease. It has been postulated to result from a disturbance in the normal relationship between the terminal axons and atrophied muscle fibres (Coers and Woolf 1959). Disruption of neurotransmitter release at the skeletal neuromuscular junction can also result in atrophy and degeneration of myocytes with sprouting terminal axonal endings (Narahashi 1974). Although there is no single nerve-to-myocyte interaction in smooth muscle, it has been proposed that interstitial cells of Cajal (ICC) may act as the intermediaries between nerves and the syncytium of smooth muscle cells (Rumessen and Thuneberg 1982, 1991a,1991b; Rumessen et al. 1982, 1992, 1993; Thuneberg 1982, 1989; Thuneberg et al 1982; Daniel and Berezin 1992). It is possible that with a defect in the process by which enteric nerve fibres interact with interstitial or smooth muscle cells, the nerve fibres denied their target cells might ramify to produce the tangled fibres identified in this study in the circular muscle of myopathic bowel wall. Further support for this suggestion is severely hampered by difficulties in identifying ICCs. ICCs share many ultrastructural features with smooth muscle cells and fibroblasts by which they can be identified by electron microscopy, and the problems with ultrastructural artifacts in surgically resected intestine have already been described in Chapter 6.

With regard to the patients with neuropathic disease, some increase in nerve fibres stained by NF antibodies in the circular muscle coat was identified in the patients with HD, those with IND and in seven of the patients with neuropathic electrophysiology, as well as in two patients with undetermined CIIP. However, this increase was in short, linear nerve fibres, the appearances of which were quite different from the tangled, brush-like aggregates in the myopathic patients (compare Figs.1 and 2).

Previous reports of NF immunostaining using two antibodies (2F11 and 3G6) on paraffin sections (Kluck et al. 1986; Tibboel et al. 1987) showed no reactivity in axons of myenteric axon bundles in CIIP compared with strong immunostaining in controls. Immunoreactivity in the neuronal perikarya was negative in both CIIP and controls. The present study has given quite different results in that both axons and many neuronal cell bodies were positive with NF antibodies, the number of nerve cell bodies labelled varying with the different antibodies used. More intense perikaryal reactivity was recorded with antibodies to dephosphorylated antibodies whilst with antibodies to phosphorylated NF the majority of neurons were negative. In general, but not exclusively, the neuronal perikarya thus appeared to be weakly phosphorylated and axons phosphorylated.

The differences between this study and those quoted almost certainly relate to the material examined (Kluck et al. 1986, used paraffin sections whilst here acetone-fixed cryostat sections of snap-frozen intestine were employed). In addition the antibodies to NF were different: Kluck et al. (1986) used 2F11 and 3G6, neither of which were employed in this study.

The demonstration here of strongly phosphorylated heavy NF in axons and occasional cell bodies also conflicts with the original findings of Eaker et al. (1990). However, it is now known that a low degree of NF phosphorylation is usual in mature intestine but that strongly phosphorylated NF are found in the myenteric plexus of newborn rat (Eaker et al.1992). Thus in infancy and childhood, nerve cell immunostaining for strongly phosphorylated NF is not surprising. However, it is harder to explain immunoreactivity with antibodies to phosphorylated NF in the intestine from the three adult patients examined. The disparity in the results is likely to be a reflection of the sensitivities of the methods employed, i.e. the less sensitive indirect immunofluorescence used by Eaker et al. (1990), and the more sensitive ABC method applied here.

N-CAM immunostaining was assessed in nerve fibres and associated glia in the circular muscle coat of specimens from 35 patients with neuropathic, myopathic and undetermined forms of CIIP and compared with controls (n=9). In the majority of patients no differences from the controls were noted, but in two patients N-CAM-positive elements were increased, while in two others they were virtually absent. These subjective observations were confirmed by morphometric analysis in 22 patients (including one with increased and another with virtually absent N-CAM immunostaining) and four controls (Table 5).

Of the four patients with abnormal N-CAM immunostaining, one with aganglionosis showed a virtual absence of N-CAM-positive nerves and glia in the muscularis propria (Table 6; Sa). AChE activity in the muscle coats was also depleted and there was no increase in AChE-positive nerves in the lamina propria. Another patient with

no obvious N-CAM-positive fibres in the muscularis propria had inflammatory plexopathy which had resulted in intestinal denervation (Table 6; FH). The remaining two patients had elevated rather than reduced numbers of positive fibres in the muscle coats. Both were patients with myopathic disease associated with autophagic activity already identified in Chapter 6 as having distinctly different morphological characteristics. The presence of increased N-CAM positive nerve and glial fibres in the muscularis propria thus provides an additional marker of this subset of myopathic CIIP.

In most cases N-CAM immunostaining in the circular muscle gave very similar results to those obtained with AChE histochemistry. However, in two patients with hypoganglionosis showing reduced or absent AChE activity in the circular muscle, N-CAM immunostaining was normal (Table 6; patients Han and GB).

A similar close correlation between N-CAM immunostaining and AChE histochemistry was also noted in the myenteric plexus. However in the patient with inflammatory plexopathy in whom damage to the enteric plexuses had resulted in denervated intestine, considerable N-CAM reactivity was seen in the myenteric plexus despite the absence of neurons and AChE-positive elements. The N-CAM reactivity was associated with glial elements, also identified by immunostaining for S100 protein. Tew et al. (1992) have shown that implants of myenteric plexus into the brain encourage axonal sprouting in the surrounding neurons and postulated that enteric glia may have a neurotropic effect. It is possible, therefore, that the preservation of glial cells identified by N-CAM immunostaining represents an attempt to stimulate a similar process following damage to enteric neurons.

N-CAM immunostaining was seen in the smooth muscle cells in all the fetal samples which is in keeping with observations in the fetal rat (Akeson et al. 1988). However, immunostaining in the smooth muscle was detected in only two of the samples, both of rectum, from infants and children with CIIP. The persistence of a normally transiently expressed protein in the smooth muscle in these two patients may be of importance as an expression of intestinal neuromuscular immaturity. One of these patients had myopathic electrophysiology and morphologically identifiable myopathic features in the rectum, however, a normal EGG was obtained in the other patient and rectal morphology was also normal. If persistent N-CAM staining could, as suggested, be a marker of immaturity, this might be of relevance to the functional abnormality demonstrated, but it is difficult to be more specific on the basis of the data available.

No clear abnormalities in M35 (muscarinic receptors) immunostaining could be detected either in the myenteric plexus or in the circular muscle in patients with CIIP. Some deviations from 'normality' were noted but their significance is hard to specify and may be related to alterations in receptor density in contracted muscle. The choice of a practically pan-muscarinic receptor antibody could explain the lack of differences. Antibodies specific to muscarinic receptor subtypes may reveal abnormalities, especially if an antibody to M3 was used (it is not clear if M3 is recognised by M35). With immunohistochemical methods quantification of receptor density is difficult and to detect mildly altered density it may be necessary to employ radioligands to quantify these differences.

Chapter 9 Conclusions and the Future

9.1 Conclusions

With the exception of Hirschsprung's disease (HD) primary intestinal neuromuscular disease (chronic intestinal pseudo-obstruction; CIIP) is uncommon, and significant series of affected patients have seldom been studied systematically. The pathological changes identified in non-HD CIIP are often subtle, and frequently elude detection by conventional histological techniques. Exceptions to this general rule involve isolated reports of familial neuropathies and myopathies where the intestinal involvement is often only part of a generalised neuromuscular disorder (Schuffler et al.1981; Schuffler 1989; Lowsky 1993).

In this thesis surgically resected samples of intestine have been examined from 35 patients, mainly children, with various forms of CIIP using standard histopathological methods as well as morphometric, silver staining, ultrastructural, enzyme and immunohistochemical techniques. In an additional 15 patients routine light microscopical, enzyme histochemical and ultrastructural investigations were made without immunohistochemical investigations. Post-mortem specimens of intestine from patients without gastrointestinal symptoms or pathology were used as controls. The contribution that these various examinations offer to the elucidation of CIIP has been analysed critically and additional new immunohistochemical approaches have also been assessed.

Most patients with CIIP have symptoms from birth or early childhood (Milla 1991) but their clinical features allow little opportunity for distinguishing even major

categories such as those with primary neural, as opposed to smooth muscle disease. This distinction is best made by electrophysiological studies as detailed in Chapter 2. In neuropathic disease small intestinal manometry characteristically reveals non-propagating bizarre MMCs, while the amplitude of contractions in smooth muscle disease is low (Devane et al. 1992a). By surface electrogastrography there is no dominant frequency in the electrical control activity in myopathic disease whilst in neuropathic disease tachy- or bradygastria is present (Devane et al 1992b).

Myopathic CIIP

With regard to the myopathic forms of CIIP, conventional histopathological examination of the intestine using paraffin wax-embedded tissue has little to offer, although a minority of patients exhibit fibrosis, atrophy and vacuolation of smooth muscle cells not found in neuropathic disease. Even electron microscopy is of limited value, but seemingly specific markers of myopathic disorder include mild degrees of fibrosis and ultrastructural myocyte vacuolation. However, such changes were not seen in every patient with intestinal myopathy. Many apparent ultrastructural abnormalities in the intestinal muscle coats, such as subsarcolemmal blebbing, are shown to be artifacts, probably related to tissue handling and preparation since they are seen equally in neurogenic disorders and in controls and can be induced in animal experiments.

The most notable contributions of this thesis relate to myopathic CIIP and include the following:

(1) Abnormalities in the content of contractile proteins (particularly actins) in smooth muscle cells identified immunohistochemically were found in the intestine from seven

patients with intestinal myopathy and may well explain their intestinal motor dysfunction. Aberrations in immunostaining were seen even in the absence of morphological myopathic changes by light and/or electron microscopy (fibrosis, atrophy and central vacuolation). In one patient negative alpha smooth muscle actin immunostaining was clearly confined to the bulk of the circular muscle coat in which normal immunoreactivity was seen for myosin, tropomyosin, filamin, caldesmon and desmin. Immunostaining in all other intestinal muscle layers, including the innermost layer of the circular muscle, was the same as in controls with all the antibodies employed including the antibody 1A4 that is specific for alpha smooth muscle actin. An embryological developmental defect is postulated for this abnormality. Immunohistochemical aberrations in the other six patients consisted of absent or reduced immunoreactivity in both layers of the muscularis propria and muscularis mucosae for alpha and gamma smooth muscle isoactins using the selective antibody CGA7. In three patients this was accompanied by a deficiency in immunostaining with the specific antibody to alpha smooth muscle actin.

(2) An increase in tangled, brush-like collections of nerves identified by neurofilament protein immunostaining was observed in the circular muscle coat of a most patients with intestinal myopathy, but was not seen in controls or neuropathic disorders.

(3) Two unrelated patients with apparently specific morphological findings indicating a distinct subset of intestinal myopathic disease were identified. These changes comprised profound fibrosis of the muscularis propria, gross atrophy of the myocytes which showed focal acid phosphatase activity, and an ultrastructurally distinct and previously unrecorded appearance in the smooth muscle cells indicating autophagic

activity (active, dilated lysosomes containing electron-dense degradation products). These appearances were accompanied by a prominant proliferation of nerve fibres in the circular muscle identified by their acetyl cholinesterase (AChE)-activity, as well as by neurofilament protein and neural cell adhesion molecule immunostaining.

Neuropathic CIIP

With regard to the neuropathic forms of CIIP, some useful conclusions were also possible, as summarised below:

(1) Previously described entities such as hyper- and hypoganglionosis were critically evaluated by morphometric methods as follows:

(a) Baseline data for myenteric neuron density in childhood were established in order to assess myenteric hypo- or hyperganglionosis more objectively. The normal neuron density in the myenteric plexus was shown not to be age-related.

(b) Comparison of myenteric neuron density in patients identified as having hyper- and hypoganglionosis against the baseline data revealed that elevated or reduced density correlated with intestinal motility disturbances.

(2) The importance of examining the myenteric plexus, which is ultimately concerned with intestinal motility, was emphasised in this study, and reservations were expressed about the diagnostic use of mucosal biopsies that sample only the submucosal plexus concerned mainly with secretory and sensory functions of the ENS.

(a) All patients identified as having IND (on the basis of myenteric hyperganglionosis, frequent large submucosal ganglia, heterotopic neurons, an increase in vertically aligned AChE-positive nerve fibres in the lamina propria and

increased AChE-positive fibres in the circular muscle) also had HD. In these patients IND was identified in a variable length of intestine proximal to the aganglionic segment and all had symptoms of disturbed intestinal motility after attempted surgical correction of HD.

(b) Although it is accepted that both isolated hypo- and hyperganglionosis may exist in the absence of HD, their diagnosis by mucosal biopsy alone remains controversial. The frequency with which isolated IND in particular is diagnosed varies considerably in different centres and this clearly relates to the diagnostic criteria applied. With regard to mucosal biopsy specimens, a spectrum of criteria applied by workers reporting a high incidence of IND (Borchard et al. 1991) were used for reassessing a series of rectal suction biopsies previously reported as normal at this hospital. Even with these criteria, only 11% showed changes supposed to suggest IND, and one important change, namely hyperplasia of the submucosal plexus, appeared to represent an age-related phenomenon. The clinical significance of the findings of Borchard et al. (1991) remain unproven, since many patients so diagnosed show a return to normality of both symptoms and pathological changes with time. Thus further careful clinical evaluation is required to settle this issue.

(3) Some of the pathological changes described in IND may also occur in a minority of patients with neurofibromatosis (Fuller and Williams 1991) and multiple endocrine neoplasia syndrome (MEN 2B). Gastrointestinal manifestations may be the first indication of these conditions (Fuller and Williams 1991; D'Amore et al. 1991; Feinstat et al. 1984), but often there is poor correlation between the severity and extent of pathological aberrations in intestinal innervation and any functional abnormalities present. Nevertheless, it is important that neurofibromatosis and MEN

2B are excluded in patients apparently presenting with isolated IND.

(4) It is generally agreed that a diagnosis of hypoganglionosis requires examination of the myenteric plexus (Borchard et al. 1991; Scharli 1992). A decreased neuron density compared with the baseline data was associated with abnormal enteric motor function in five patients described in this thesis. However, when making the diagnosis of hypoganglionosis based on neuronal counts, there are important pitfalls to be avoided. For example, the distal rectum normally has a reduced neuronal density (Aldridge and Campbell 1968) and biopsies that include this region are not suitable for assessment. In addition, neuron density in the jejunum and ileum is normally about half of that of the colon and suitable control data are required when the small intestine is considered. Enteric hypoganglionosis can also be an acquired phenomenon; secondary hypoganglionosis in childhood is rare but in one such patient described here it appeared to result from an autoimmune process.

(5) Silver staining of the childhood myenteric plexus was shown to be unrewarding and failed to identify significant abnormalities compared with normal control data. Moreover, the absence of argyrophilic neurons was identified as a normal feature of the developing myenteric plexus and was common in neonates and infants, and in some cases up to the age of 18 months.

(6) In neuropathic CIIP, an immunohistochemical approach using antibodies to NF, N-CAM and muscarinic receptors to identify subtle neural abnormalities in the myenteric plexus was largely unhelpful.

9.2 The Future

9.2.1 Contractile Proteins

This study has shown that one particularly significant area needing further investigation is the content of contractile protein (particularly actin) isoforms in intestinal smooth muscle. Defects of this nature may be the basis of some forms of intestinal myopathy in which conventional histopathology and ultrastructural examination fail to demonstrate morphological abnormalities. There is a need to obtain further isoform-specific antibodies to assess the isoform profile of a variety of contractile proteins involved in intestinal smooth muscle contraction. The amino acid sequences of many isoforms are known, specific peptides can be synthesised and monoclonal antibodies to these could be raised. Such antibodies could be used to study the ontogeny of isoforms of contractile proteins and irregularities in the isoform profile might identify further patients in whom abnormalities of this type underlie their intestinal smooth muscle dysfunction. This could also open the way to more fundamental studies at the genetic and molecular levels.

9.2.2 Neurotransmitters and their Receptors

The study of neurotransmitters controlling smooth muscle contraction or relaxation is another interesting area touched on only briefly in this thesis. In particular the inhibitory non-cholinergic non-adrenergic (NANC) nerves are central to intestinal motility. These are proposed to include peptidergic and purinergic nerves, and it has been suggested that the recently identified neurotransmitter, nitric oxide (NO) is one of the final mediators of the (NANC) inhibition of the intestinal circular muscle contraction (Milla 1992; Stark et al. 1993). Nitric oxide synthase (NOS) catalyses the production of NO from L-arginine and the distribution of the multifunctional NOS can

be studied immunohistochemically and by enzyme histochemical methods for NADPH-dehydrogenase (Hope et al. 1991; Dawson et al. 1991). Both methods show identical localisation in the brain, myenteric plexus and smooth muscle, and carefully applied, the simple enzyme histochemical method can be used to map NOS. An abnormality in the content of NADPH-dehydrogenase-positive nerves in the circular muscle has been shown in the pylorus of infants with hypertrophic pyloric stenosis (Vanderwinden 1992). Identification of the presence or absence of NANC nerves could thus be helpful in CIIP.

Investigation into receptors of neurotransmitters such as the muscarinic receptor by application of antibodies specific to a particular receptor type (M1, M2, M3) might also improve our understanding of the pathophysiology of some forms of CIIP. Muscarinic receptors were studied by immunostaining with a pan-muscarinic receptor antibody, but abnormalities were not identified in the patients studied. This may have been due to the lack of antibody specificity for individual muscarinic receptor types. It might also be that receptor density is altered in CIIP so that techniques allowing quantification using radioligands may be required.

Intestinal motor activity develops in broadly similar ways in most species, with difference only in the timing of specific events in pre- or postnatal development. These patterns in the experimental animal and in the human small intestine in fetal, perinatal and adult life have been documented (Ruckebusch 1986; Weisbrodt 1987; Bisset 1988a,1988b). It has been shown that 5HT activates the peristaltic reflex (Ruckebusch and Bardon 1984; Ruckebusch 1986) and that the application of 5HT antagonists reverts the mature intestinal motor pattern to the fetal form (Ruckebusch

1986). The ontogeny of 5HT or its receptors would be interesting and could underlie some abnormalities in intestinal motor patterns.

9.2.3 Interstitial Cells of Cajal (ICC)

Another area for future research into nerve-to-smooth muscle connections is the role of the interstitial cells of Cajal (ICC). These cells are thought to control intestinal motor function by modulating neurotransmission of NANC inhibitory activity (Thuneberg 1982,1989; Thuneberg et al. 1982; Daniel and Berezin 1992; Rumessen and Thuneberg 1982, 1991; Rumessen et al. 1982,1992,1993). The major drawback to studying ICCs is the lack of a reliable means for their identification. Currently ICCs are recognised only by their selective staining characteristics and their specific ultrastructural appearances, and are often difficult to identify especially in surgically resected intestine. When a specific marker is found, a systematic examination and morphometric analysis of ICCs in intestine from patients with CIIP may reveal abnormalities not recognised to date.

9.2.4 Developmental Control Genes

CIIP in the majority of children is a congenital and probably developmental disorder, so that study of the mechanisms controlling nerve cell migration and differentiation, as well as smooth muscle development, will increase our understanding of diseases of intestinal motility.

The body plan of many organisms is controlled by highly conserved developmental control genes (Redline 1992) including the homeobox (hox) genes, first identified in

Drosophila and recently confirmed in vertebrates. It is suggested that the vertebrate hox genes, like those of Drosophila, specify the positional identity of structures along the rostral-caudal axis. It is conceivable that homeobox genes, which are first expressed during gastrulation, have a specified role in the ordered, critically timed sequences operating during the development of the tissues in the intestinal tract. The order in which the smooth muscle layers are laid down is probably predetermined and the migration of neuroblasts from the neural crest preprogrammed.

In the mouse and man there are 38 homeobox genes (Scott 1992), which appear in overlapping homologous clusters (in mouse hoxa, hoxb, hoxc, hoxd; in man HOXA, HOXB, HOXC and HOXD) on the DNA of four different chromosomes. In man these clusters are located on chromosomes 7, 17, 12 and 2 respectively. Wolgemuth and colleagues (1989) produced transgenic mice overexpressing the mouse Hox-1.4 (now known as hoxa-4) gene, and noticed that these mice developed congenital megacolon. I had an opportunity to examine sections of distal large intestine from these mice and found them to be hypoganglionic. A more detailed study of the expression of different hox genes using in situ hybridisation, in the normal developing gastrointestinal tract and in transgenic animals could be an exciting field. This might be extended to examine the expression of these genes in the intestine from patients with CIIP where over- or underexpression of the gene may be responsible for developmental abnormalities that might not be detectable by other means.

9.2.5 Establishment of Tissue and Clinical Data Banks of Patients with CIIP

CIIP is fortunately uncommon but for the affected individual it is a devastating and debilitating affliction. The subtlety or lack of the histopathological abnormalities does

not lessen the severity of the symptoms. It is important that detailed clinical information is obtained from all patients investigated. These patients should be studied by electrophysiological techniques and when surgical intervention becomes necessary, full-thickness intestinal tissue should be taken for histological studies. The intestine must be preserved in many different ways to facilitate any investigation that may become necessary, including routinely processed tissue for paraffin sections and electron microscopy, and snap-frozen intestine for histochemical investigation. Ideally in vitro physiological studies should be included but these are available only in specialist centres and tissue cannot be preserved for the later performance of these investigations. Since CIIP is uncommon, systematic compilation of tissue and clinical data banks will be required if further advances in the recognition of specific entities in the spectrum of intestinal neuromuscular disease is to be achieved.

References

Aherne WA. (1967) Methods for counting discrete tissue components in microscopical sections. J Roy Mic Soc 87, 493

Aherne WA and Dunnill MS. (1982) Morphometry, Edward Arnold, London

Akeson RA, Wujek JR, Roe S, Warren SL and Small SJ. (1988) Smooth muscle cells transiently express N-CAM. Brain-Res 464, 107-120

Aldridge RT and Campbell PE. (1968) Ganglion cell distribution in the normal rectum and anal canal. A basis for the diagnosis of Hirschsprung's disease by anorectal biopsy. J Pediatr Surg 3, 475-490

Alstead EM, Murphy MN, Flanagan AM, Bishop AE and Hodgson HJF. (1988) Familial autonomic visceral myopathy with degeneration of muscularis mucosae. J Clin Pathol 41, 424-429

Andre C, Guillet JG, De Backer J-P, Vanderheyden P, Hoebeke J and Strosberg AD. (1984) Monoclonal antibodies against the native or denatured forms of muscarinic acetylcholine receptors. EMBO J 3, 17-21

Andre C, Marullo S, Guillet J-G, Convents A, Lauwereys M, Kaveri S, Hoebeke J and Strosberg AD. (1987) Immunochemical studies of the muscarinic acetylcholine receptor. J Receptor Research 7, 89-103

Anuras S, Mitros FA, Nowak TV, Ionasescu VV, Gurll NJ, Christensen J and Green JB. (1983) Familial visceral myopathy with external opthalmoplegia and autosomal recessive transmission. Gastroenterology 84, 346-353

Anuras S, Mitros FA, Milano A, Kuminsky R, Decanio R and Green JB. (1986a) A familial visceral myopathy with dilatation of the entire gastrointestinal tract. Gastroenterology 90, 385-390

Anuras S, Mitros FA, Soper RT, Pringle KC, Maves BV, Younoszai MK, Franken EA Jr and Whitington P. (1986b) Chronic intestinal pseudo-obstruction in young children. Gastroenterology 91, 62-70

Anuras S, Anuras J and Bozeman T. (1990) Small intestinal manometric studies in patients with familial visceral myopathies. J Gastrointes Mot 2, 190-193

Ariel I, Hershlag A, Lernau OZ, Nissan S and Rosenmann E. (1985) Hypoganglionosis of the myenteric plexus with normal Meissner's plexus: A new variant of colonic ganglion cell disorders. J Pediatr Surg 20, 90-92

Armoury RA, Fellows RA, Goodwin CD, Hall RT, Holder TM and Ashcroft KW. (1977) Megacystis-microcolon-intestinal hypoperistalsis syndrome:a cause of intestinal obstruction in the newborn period. J Pediatr Surg 12, 1063-1065

Bagwell CE, Filler RM, Cutz E, Stringer D, Ein SH, Shandling B, Stephens CA and Wesson DE. (1984) Neonatal Intestinal pseudo-obstruction. J Pediatr Surg 19,

732-739.

Bannister R and Hoyes AD. (1981) Generalised smooth muscle disease with defective muscarinic receptor function. BMJ 282, 1015-1018

Barth O. (1870) Hochgradige Kothstauung in Folge einer durch zu langes Mesocolon zu Stande gekommenen Darmverlagerung. Arch d Heilkunde 11, 119-124

Bayliss WM and Starling EH. (1899) The movement and innervation of the small intestine. J Physiol 24, 99-143. In: Wingate DL (1981) Backwards forwards with the migrating complex. Dig Dis Sci 26, 641-664

Beaumont W. (1833) Experiments and observations on the gastric juice and the physiology of digestion. FP Allen, Plattsburgh. In: Wingate DL. (1981) Backwards forwards with the migrating complex. Dig Dis Sci 26, 641-664

Belai A, Schmidt HHHW, Hoyle CHV, Hassall CJS, Saffrey MJ, Moss J, Forstermann U, Murad F and Burnstock G. (1992) Colocalization of nitric oxide synthase and NADPH diaphorase in the myenteric plexus of the rat. Neuroscience Lett 143, 60-64

Bentley JFR. (1966) Seminar on pseudo- Hirschsprung's disease and related disorders. Arch Dis Childh 41, 143-154

Berdon WE, Baker DH, Blanc WA, Britt G, Santulli TV and Donovan C. (1976).

Megacystis-microcolon-intestinal hypoperistalsis syndrome: A new cause of intestinal obstruction in the newborn. Report of radiologic findings in five newborn girls. Am J Roentgenol Radium Ther Nucl Med 126, 957-964

Berseth CL. (1989) Gestational evolution of small intestine motility in preterm and term infants. J pediatrics 115, 646-651

Bindl L, Emons D, Haverkamp F, Fahnenstich H, Kovalewski S and Meier-Ruge W. (1989) Das Megazystis-mikrokolon-intestinale hypoperistaltik syndrom: eine Neuropathie? Z Kinderchir 44, 249-252

Bisset WM. (1988a) The development of motor control systems in the gastrointestinal tract of preterm infant. In: Disorders of gastrointestinal motility in childhood. Milla PJ (ed). John Wiley, Chichester, p 17-27

Bisset WM. (1988b) Intestinal motor activity in the preterm infant. In: Disorders of gastrointestinal motility in childhood. Milla PJ (ed). John Wiley, Chichester, p 29-37

Bisset WM, Watt JB, Rivers RPA and Milla PJ. (1988) Ontogeny of fasting small intestinal motor activity in the human infant. Gut 29, 483-488

Bisset WM, Watt J, Rivers RPA and Milla PJ. (1989) Postprandial motor response of the small intestine to enteral feeds in preterm infants. Arch Dis Childh 64,1356-1361

Bodian M, Stephens F D and Ward B C H. (1949) Hirschsprung's Disease and idiopathic megacolon. Lancet 1, 6-15

Boldyreff V. (1902) Periodic wave phenomena in the secretory function of the digestive tract. Gaz Hop Botkine 34, 1529-1542 (Russian). In: Wingate DL. (1981) Backwards forwards with the migrating complex. Dig Dis Sci 26, 641-664

Boldyreff W. (1911) Einige neue Seiten der Tatigkeit des Pankreas. Der ubertritt des Pankreassaftes und anderer darmsekrete in den Magen. Die physiologische un klinische Bedeutung dieser erscheinung. Ergebnisse der Physiologie 11, 121-217 In: Wingate DL. (1981) Backwards forwards with the migrating complex. Dig Dis Sci 26, 641-664

Borchard F, Meier-Ruge W, Wiebecke B, Briner J, Munterfering H, Fodisch HF, Holschneider AM, Schmidt A, Enck P and Stolte M. (1991) Innervationsstorungen des Dickdarmes - Klassifikation und Diagnostik, Patologe 12, 171-174

Bretano A. (1904) Uber einen Fall von Hirschsprung'scher Krankheit. Verhandl d deutsch Gesellsch f Chir 33, 265-268

Briner J, Oswald HW and Hirsig J. (1986) Neuronal intestinal dysplasia - clinical and histochemical findings and its association with Hirschsprung's disease. Z Kinderchir 41, 282-286

Bristowe JS. (1885) The consequences of long continued constipation. Brit M J 1,

1085-1888

Burns GA, Karcher LP and Cummings JF. (1990) Equine myenteric ganglionitis: a case of chronic intestinal pseudo-obstruction. Cornell Vet 80, 53-63

Bryant J. (1924) Observations upon growth and length of the human intestine. Am J Med Sci 167, 499-520

Bughaighis AG and Emery JL. (1971) Functional obstruction of the intestine due to neurological immaturity. Prog Ped Surg 3, 37-52

Busch W. (1858) Beitrag zur physiologie der verdauungsorgene. Virchow's Arch F Path Anat 14, 140-186. In: Wingate DL (1981) Backwards forwards with the migrating complex. Dig Dis Sci 26, 641-664

Bywater RAR and Taylor G. (1986) Non-cholinergic excitatory and inhibitory junction potentials in the circular smooth muscle of the guinea pig ileum. J Physiol 374, 153-164

Bywater RAR, Taylor GS and Furukawa K. (1987) The enteric nervous system in control of motility and secretion. Dig Dis 5, 193-211

Bywater RAR, Small RC and Taylor GS. (1989) Neurogenic slow depolarizations and rapid oscillations in the membrane potential of circular muscle of mouse colon. J Physiol 413, 505-519

Cameron JAM. (1928) On the etiology of Hirschsprung's disease. Arch Dis Child 3, 210-211

Christensen J, Dent J, Malagelada J-R and Wingate DL. (1990) Pseudo-obstruction. Gastroenterology International 3, 107-119

Christensen J, Stiles M J, Rick G A and Sutherland J. (1984) Comparative anatomy of the myenteric plexus of the distal colon in eight mammals. Gastroenterology 86, 706-713

Coers and Woolf. (1959) The innervation of muscle. A biopsy study, Blackwell Scientific Publications, Oxford

Coflenski JT, Adler KB, Woodcock-Mitchell J, Mitchell J and Evans JN. (1988) Proliferative changes in the pulmonary arterial wall during short term hyperoxic injury to the lung. Am J Pathol 132, 563-573

Cohen MC, Moore SW, Neveling U and Kaschula ROC. (1993) Acquired aganglionosis following surgery for Hirschsprung's disease: a report of five cases during a 33 year experience with pull-through procedures. Histopathology 22, 163-168

Colemont LJ and Camilleri M. (1989) Chronic intestinal pseudo-obstruction: diagnosis and treatment. Mayo Clin Proc 64, 60-70

Dajani OM, Slim MS and Mansour A. (1986) Acquired hypoganglionosis after Soave endorectal pull-through procedure. A case report. Z Kinderchir 41, 248-249

Dalla Valla A. (1920) Richerche istologiche su di un caso di megacolon congenito. Pediatrica (Barcelona) 28,740-752

D'Amore ESG, Manivel JC, Pettinato G, Niehans GA and Snover DC. (1991) Intestinal ganglioneuromatosis: Mucosal and transmural types. A clinicopathologic and immunohistochemical study of six cases. Human Pathology 22, 276-286

Daniel EE and Berezin I. (1992) Interstitial cells of Cajal: are they major players in control of gastrointestinal motility? J Gastrointest Mot 4, 1-24

Daniel EE and Sarna S. (1978) The generation and conduction of activity in smooth muscle. Annu Rev Pharmacol Toxicol 18, 145-166

Dawson TM, Bredt DS, Fotuhi M, Hwang PM and Snyder SH. (1991) Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissues. Proc Natl Acad Sci 88, 7797-7801

Desa DJ. (1991) Alimentary Tract. In: Text book of fetal and perinatal pathology. Volume 2. Wigglesworth JS & Singer DB (eds). Boston, Blackwell Scientific Publications, Oxford, p 903-979

Devane SP, Coombes R, Smith VV, Bisset WM, Booth IW, Lake BD and Milla PJ.

(1992a) Persistent gastrointestinal symptoms after correction of malrotation. Arch Dis Childh 67, 218-221

Devane SP, Ravelli.AM, Bisset WM, Smith VV, Lake BD and Milla PJ. (1992b) Gastric antral dysrhythmias in children with chronic intestinal pseudo-obstruction (CIIP). Gut 33, 1477-1481

Dieler R, Schroder JM, Skopnik H, Steinau G. (1990) Infantile hypertrophic pyloric stenosis: myopathic type. Acta-Neuropathol (Berl) 80, 295-306

Duhamel B. (1966) Seminar on pseudo-Hirschsprung's disease and related disorders. Arch Dis Childh 41, 143-154

Dyer NH, Dawson AM, Smith BF and Todd IP. (1969) Obstruction of bowel due to lesion in the myenteric plexus BMJ 1, 686-689

Eaker EY, Shaw G and Sninsky CA. (1990) Neurofilament immunoreactivity in myenteric neurons differs from that found in the central nervous system. Gastroenterology 99, 1364-1371

Eaker EY and Sallustio JE. (1992) Developmentally acquired differences in the neurofilaments in myenteric neurons studied in primary culture and neonatal intestine. Gastroenterology 101, A127

Ebers L. (1836) Geschichte eines seltenen Falles von Ileus. Hufelands J. 83/2, 62.

In: Griffith C. (1899) Congenital idiopathic dilatation of the colon. Am J Med Sci 118, 283-297

Ehrenpreis Th, Bentley JFR, Nixon HH, Spencer B, Lister J, Duhamel B, Pages R and Katz A. (1966) Seminar on pseudo-Hirschsprung's disease and related disorders. Arch Dis Childh 41, 143-154

Elema JD, de Vries JA and Vos LJM. (1973) Intensity and proximal extension of acetyl cholinesterase activity in the mucosa of the rectosigmoid in Hirschsprung's disease. J Pediatr Surg 8, 361-368

Emanuel B, Gault J and Sanson J. (1967) Neonatal intestinal obstruction due to absence of intestinal musculature: A new entity. J Pediatr Surg 2, 332-335

Erdohazi M. (1974) Retarded development of the enteric nerve cells. Developmental Medicine and Child Neurology 16, 365-368

Fadda B, Maier WA, Meier-Ruge W, Scharli A and Daum R. (1983) Neuronale intestinale Dysplasie. Eine kritische 10-Jahres-Analyse klinischer und bioptischer Diagnostik. Z Kinderchir 38, 305-311

Fadda B, Pistor G, Meier-Ruge W, Hofmann-von Kapp-herr S, Muntefering H and Espinoza R. (1987) Symptoms, diagnosis and therapy of neuronal intestinal dysplasia masked by Hirschsprung's disease. Pediatr Surg Int 2, 76-80

Fatigati V and Murphy RA. (1984) Actin and tropomyosin variants in the smooth muscles. Dependence on tissue type. J Biol Chem 259, 14383-14388

Faulk DL, Anuras S, Gardner GD, Mitros FA, Summers RW and Christensen J. (1978) A familial visceral myopathy. Ann Intern Med 89, 600-606

Feinstat T, Tesluk H, Schuffler M D, Krishnamurthy S, Verlenden L, Gilles W, Frey C and Trudeau W. (1984) Megacolon and neurofibromatosis: A neuronal intestinal dysplasia. A case report and review of the literature. Gastroenterology 86, 1573-1579

Fenton T, Harries JT and Milla PJ. (1983) Disordered small intestinal motility; a rational basis for toddler diarrhoea. Gut 24, 897-903

Finney JMT. (1908) Congenital idiopathic dilatation of the colon. Surg Gynec & Obst 6, 624-643

Foucar E, Lindholm J and Anuras S. (1985) A kindred with dysplastic nevus syndrome associated with visceral myopathy and multiple basal cell carcinomas. Lab Invest 52, 23A

Fuller CE and Williams GT. (1991) Gastrointestinal manifestations of type I neurofibromatosis (von Recklinghausen's disease). Histopathology 19, 1-11

Furness JB and Costa M. (1987) The enteric nervous system. Churchill Livingstone, Edinburgh

Furness JB, Bornstein JC, Smith TK, Murphy R and Pompolo S. (1989) Correlated functional and structural analysis of enteric neural circuits. Arch Histol Cytol 52, 161-166

Furness JB and Costa M. (1987) Cytoarchitectonics of the enteric nervous system. In: The enteric nervous system. Furness JB and Costa M (eds), Churchill Livingstone, Edinburgh, p 26-54

Furukawa K, Taylor GS and Bywater. (1986) An intracellular study of myenteric neurons in the mouse colon. J Neurophysiol 55, 1395-1406

Gabella G. (1981) Structure of smooth muscles. In: Smooth muscle: an assessment of current knowledge. Bulbring E, Brading AF, Jones AW and Tomita T. (Eds), Edward Arnold, London, p 1-46

Gabella G. (1987a) Structure of muscles and nerves in the gastrointestinal tract. In: Physiology of the Gastrointestinal Tract. Johnson LR (Ed). Raven Press, New York, p 335-381

Gabella G. (1987b) Dynamic aspects of the morphology of the intestinal muscle coat. In: Cellular physiology and clinical studies of gastrointestinal smooth muscle. Szurszewski JH (Ed). Elsevier Publishers B.V. (Biochemical Division), Amsterdam, p 5-31

Gabella G. (1987c) The number of neurons in the small intestine of mice, guinea pig

and sheep. Neuroscience 22, 737-752

Gabella G. (1988) Structure of smooth muscle. In: Gastrointestinal motility. Kumar D and Gustavsson S (Eds), John Wiley & Sons, Chichester, p 33-45

Gabella, G. (1989a). Structure of intestinal musculature. In: Handbook of physiology. Section 6, Part 1,vol 1, The gastrointestinal system. Motility and circulation, Schultz, SG, Wood, JD and Rauner, BB (Eds). American Physiological Society, p 103-139

Gabella G. (1989b) Fall in the number of myenteric neurons in ageing guinea pigs. Gastroenterology 96, 1487-1493

Gabella, G. (1990a). General aspects of the fine structure of smooth muscles. In: Ultrastructure of smooth muscle. Motta PM (Ed). Kluwer Academic Publishers, London, p 1-21

Gabella, G. (1990b). Smooth muscle in the gut and airways. In: Ultrastructure of smooth muscle. Motta PM (Ed). Kluwer Academic Publishers, London, p 137-151

Gabella G. (1990c) Development of smooth muscle: ultrastructural study of the chick embryo gizzard. Anat Embryol 180, 213-226

Gabella G. (1990d) Hypertrophy of visceral smooth muscle. Anat Embryol 182, 409-424

Garrett J R and Howard E R. (1981) Myenteric plexus of the hind gut: developmental abnormalities in humans and experimental studies. In: Development of the autonomic nervous system. Ciba Foundation symposium 83. Pitman Medical, London, p 326-354

Gaunt ST and Singh PB. (1990) Homeogene expression patterns and chromosomal imprinting. Perspectives 6, 208-212

Gee S. (1884) Idiopathic dilatation of the large intestine. St Barthol Hosp Rep 20, 19-21

Glukhova MA, Frid MG and Koteliansky VE. (1990) Developmental changes in expression of contractile and cytoskeletal proteins in human aortic smooth muscle. J Biol Chem 265, 13042-13046

Gottlieb AI, Langille BL, Wong MKK and Kim DW. (1991) Biology of disease. Structure and function of the endothelial cytoskeleton. Lab Invest 65, 123-137

Gown AM, Vogel AM, Gordon D and Lu PL. (1985) A smooth muscle specific monoclonal antibody recognizes smooth muscle actin isozymes. J Cell Biol 100, 807-813

Goyal RK. (1989) Muscarinic receptor subtypes. New Engl J Med 321, 1022-1029

Griffith C. (1899) Congenital idiopathic dilatation of the colon. Am J Med Sci 118, 283-297

Hartshorne DJ. (1987) Biochemistry of the contractile process in smooth muscle. In: Physiology of the gastrointestinal tract. Volume 1, 2nd edition. Johnson LR (Ed). Raven Press, New York, p 423-482

Headon MP, Sloper JJ, Hiorns RW and Powell TPS. (1985) Sizes of neurons in the primate lateral geniculate nucleus during normal development. Dev Brain Res 18, 51-56

Hirschsprung H. (1888) Stuhltragheit Neugeborener in Folge von Dilatation und Hypertrophie des Colons. Jahrbuch f. Kinderheilkunde NF 27, 1-7

Hirschsprung H. (1900) Fortsatte erfaringer om den medfodte dilatation og hypertrofi af tyktarmen. Hospitalstidende 8, 165-178

Hollenbeck PJ. (1989) The transport and assembly of the axonal cytoskeleton. J Cell Biol 108, 223-227

Hope BT, Michael GJ, Knigge KM and Vincent SR. (1991) Neuronal NADPH diaphorase is nitric oxide synthase. Proc Natl Acad Sci 88, 2811-2814

Houropian DS and Kim Y. (1982) Encephalomyeloneuropathy with ganglionitis of the myenteric plexus in the absence of cancer. Am Neurol 11, 628-631

Hsu S M, Raine L and Fanger H. (1981) Use of avidin biotin peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabled

antibody (PAP) procedures. J Histochem Cytochem 29, 577-580

Humphry A, Mancer K and Stephens CA. (1980) Obstructive circular-muscle defect in the small bowel in a one-year-old child. J Pediatr Surg 15, 197-199

Husain AN, Hong HY, Gooneratne S, Muraskas J and Black PR. (1992) Segmental absence of small intestinal musculature. Pediatr Pathol 12, 407-415

Hyman PE, McDiarmid SV, Napolitano J, Abrams CE and Tomomasa T. (1988) Antroduodenal motility in children with chronic intestinal pseudo-obstruction. J Pediatr 112, 899-905

Ikeda K, Goto S, Nagasaki A and Taguchi T. (1988) Hypogenesis of intestinal ganglion cells: a rare cause of intestinal obstruction simulating aganglionosis. Z Kinderchir 43, 52-53

Jacobi A. (1869) On some important causes of constipation in infants. Am J Obstet 2, 96-113

Jacobs E, Ardichvili D, Perissino A, Gottignies P and Hanssens J-F. (1979) A case of familial visceral myopathy with atrophy and fibrosis of the longitudinal muscle layer of the entire small bowel. Gastroenterology 77, 745-750

Kamijo K, Hiatt RB and Kolle GB. (1953) Congenital megacolon, a comparison of the spastic and hypertrophied segments with respect to cholinesterase activities and

sensitivities to acetyl choline, DFP and barium ions. Gastroenterology 24, 173-185

Kapila L, Haberkorn S and Nixon HH. (1975) Chronic adynamic bowel simulating Hirschsprung's disease. J Pediatr Surg 10, 885-892

Kaschula ROC, Cywes S, Katz A and Louw JH. (1987) Degenerative leiomyopathy with massive megacolon. Myopathic form of chronic idiopathic intestinal pseudo-obstruction occurring in indigenous Africans. Perspect Pediatr Pathol 11, 193-213

Katz A. (1966) Seminar on pseudo-Hirschsprung's disease and related disorders. Arch Dis Childh 41, 143-154

Kedinger M, Simon-Assmann P, Bouziges F, Arnold C, Alexandre E and Haffen K. (1990) Smooth muscle actin expression during rat gut development and induction in fetal skin fibroblastic cells associated with intestinal embryonic epithelium. Differentiation 43, 87-97

Kelly KA. (1981) Motility of the stomach and gastroduodenal junction. In: Physiology of the gastrointestinal tract. Johnson LR (Ed). Raven Press, New York, p 393-410

Kessel M & Gruss P. (1990) Murine developmental control genes. Science 249, 374-379

Kirtane J, Talwalker V and Dastur DK. (1984) Megacystis, microcolon, intestinal hypoperistalsis syndrome: possible pathogenesis. J Pediatr Surg 19, 206-208

Kluck P, Tibboel D, Leendertse-Verloop K, van der Kamp AWM, ten Kate FJW and Molenaar JC. (1986) Diagnosis of congenital neurogenic abnormalities of the bowel with monoclonal anti-neurofilament antibodies. J Pediatr Surg 21, 132-135

Krishnamurthy S, Schuffler M D, Rohrmann C A and Pope C E. (1985) Severe idiopathic constipation is associated with distinctive abnormality of the colonic myenteric plexus. Gastroenterology 88, 26-34

Krishnamurthy S, Schuffler MD, Belic L and Schweid AI. (1986) An inflammatory axonopathy of the myenteric plexus producing rapidly progressive intestinal obstruction. Gastroenterology 90, 754-758

Krishnamurthy S and Schuffler MD. (1987) Pathology of neuromuscular disorders of the small intestine and colon. Gastroenterology 93, 610-639

Kuroda M. (1985) Change of actin isomers during differentiation of smooth muscle. Biochim Biophys Acta 843, 208-213

Lake BD, Puri P, Nixon HH and Claireaux AE. (1978) Hirschsprung's disease. An appraisal of histochemically demonstrated acetyl cholinesterase activity in suction rectal biopsy specimens as an aid to diagnosis. Arch Path Lab Med 102, 244-247

Lake BD. (1988) Observations on the pathology of pseudo-obstruction. In: Disorders of gastrointestinal motility in childhood. Milla PJ (Ed). John Wiley, Chichester, p 81-90.

Lake BD. (1989) Hirschsprung's disease and related disorders. In: Gastrointestinal and oesophageal pathology. Whitehead R (Ed). Churchill Livingstone, Edinburgh, p 257-268

Lake, BD. (1990) The diagnosis of Hirschsprung's disease and pseudo-obstruction. In: Histochemistry in pathology, Filipe MI and Lake BD (Eds). Churchill Livingstone, Edinburgh, p 211-220

Lehman W, Sheldon A and Madonia W. (1987) Diversity in smooth muscle thin filament composition. Biochim Biophys Acta 914, 35-39

Leigh PN, Dodson A, Swash M, Brion J-P and Anderton BH. (1989) Cytoskeletal abnormalities in motor neuron disease. Brain 112, 521-535

Lennander KG. Fall av medfodd dilatation och hypertrofi av flexura sigmoidea hos ett barn (maladie de Hirschsprung) Nord Med Ark 1900. In: Hirschsprung's disease. (1970) Ehrenpreis T (Ed.). Year Book Medical Publishers Inc, Chicago

Lennon VA, Sas DF, Busk MF, Scheithauer B, Malagelada JR, Camilleri M and Miller LJ. (1991) Enteric neuronal autoantibodies in pseudo-obstruction with small cell lung carcinoma. Gastroenterology 100, 137-142

Lewis TD, Daniel EE, Sarna SK, Waterfall WE and Marzio L. (1978) Idiopathic intestinal pseudo-obstruction. Report of a case, with intralumenal studies of mechanical and electrical activity, and response to drugs. Gastroenterology 74,

107-111

Lewis W & Gonzalez B. (1990) Actin isoform mRNA alterations induced by Doxorubicin in cultured heart cells. Lab Invest 62, 69-76

Li YF, Bowers RL, Haley-Russell D, Moody FG and Weisbrodt NW. (1990) Actin and myosin isoforms in gallbladder smooth muscle following cholesterol feeding in prairie dogs. Gastroenterology 99, 1460-1466

Lister J. (1966) Seminar on pseudo-Hirschsprung's disease and related disorders. Arch Dis Childh 41, 143-154

Loeb A. (1976) Space structures. Their harmony and counterpoint. Addison-Wesley, Reading

Lowsky R, Davidson G, Wolman S, Jeejeebhoy KN and Hegele RA. (1993) Familial visceral myopathy associated with a mitochondrial myopathy. Gut 34, 279-283

MacKenzie JM & Dixon MF. (1987) An immunohistochemical study of the enteric neural plexi in Hirschsprung's disease. Histopathology 11, 1055-1066

Malagelada JR. (1990) Clinical aspects of Gastro-duodenal motor coordination. In: Gastro-pyloro-duodenal coordination. van Nueten JM, Schuurkes JHJ and Akkermans LMA (Eds), Wrightson, Petersfield, p 229-243

Malone PS, Ransley PG and Kiely EM. (1990) Preliminary report: the antigrade continence enema. Lancet 336, 1217-1218

Martin JE, Swash M, Kamm MA, Marher K, Cox EL and Gray A. (1990) Myopathy of internal anal sphincter with polyglucosan inclusions. J Pathol 161, 221-226

Matsuyama T, Luiten PGM, Spencer DG Jr and Strosberg AD. (1988) Ultrastructural localisation of immunoreactive sites for muscarinic acetylcholine receptor proteins in the rat cerebral cortex. Neurosci Res Communications 2, 69-76

McDonald GB, Schuffler MD, Kadin ME and Tytgat GNJ. (1985) Intestinal pseudo-obstruction caused by diffuse lymphoid infiltration of the small intestine. Gastroenterology 89, 882-889

McKusick VA. (1992) Mendelian inheritance in man. 10th edition, vol 2. John Hopkins, Baltimore and London. Item 249210 p 1521-1522

Meier-Ruge W. (1967) Zur Pathologie und bioptischen Diagnostik des Morbus Hirschsprung in Relation zum Megacolon acquisitum und functionale. Verh dtsch Ges Path 51, 323-328

Meier-Ruge W. (1968) Das Megacolon seine Diagnose un Pathologhysiologie. Virchows Arch Abt A Path Anat 344, 67-85

Meier-Ruge W, Morger R and Rehbein F. (1970) Das hypoganglionaere Megakolon

als Begleitkrankheit bei Morbus Hirschsprung. Z Kinderchir 8, 254-264

Meier-Ruge W. (1971) Uber ein Erkrankungsbild des Colon mit Hirschsprung-Syptomatik. Verh Dtsch Ges Pathol 55, 506-510

Meier-Ruge W. (1972) Enzymhistochemische Schnellmethoden. In: Die intra-operative Schnellschnittuntersuchung. Hermanek P and Bunte H (Eds). Urban and Schwarzenberg, Munchen. p 48-62, 294-301

Meier-Ruge W. (1974) Hirschsprung's disease: Its aetiology, pathogenesis and differential diagnosis. Current topics in pathology 59, 131-179

Meier-Ruge W. (1985) Angeborene Dysganglionosen des Colon. Kinderarzt 16, 151-164

Meier-Ruge W, Kaufeler RE and Bronnimann P. (1992) Classification of inborn malformation of distal gut innervation. In: Inflammatory bowel disease and Morbus Hirschsprung. Hadziselimovic F and Herzog B (Eds). Kluwer Academic Publishers, London, p 177-201

Milla PJ, Lake BD, Spitz L, Nixon HH, Harries JT and Fenton TR. (1983) Chronic Idiopathic Intestinal Pseudo-obstruction in Infancy: a Smooth Muscle Disease. In: Gastrointestinal motility. Labo G and Bortolotti M (Eds). Cortinal International, Verona, p 125-131

Milla PJ. (1991) Chronic intestinal pseudo-obstruction. In: Gastrointestinal transit: pathophysiology and pharmacology. Kamm M A and Lennard-Jones J E (Eds). Wrightson, Petersfield, p 157-168

Milla PJ. (1992). Gastric-outlet obstruction in children. New Engl J Med 327, 558-559

Moummi C, Magous R, Strosberg and Bali J-P. (1988) Muscarinic receptors in isolated smooth muscle cells from gastric antrum. Biochemical Pharmacology 37, 1363-1369

Morat JP. (1893) Sur quelques particularites de l'innervation motrice de l'estomac et de l'intestin. Arch de Physiol Norm et Path. 5:142-153. In: Wingate DL. (1981). Backwards forwards with the migrating complex. Dig Dis Sci 26, 641-664

Munakata K, Okabe I and Morita K. (1978) Histologic studies of rectocolic aganglionosis and allied diseases. J Pediatr Surg 13, 67-75

Munakata K, Morita K, Okabe I and Sueoka H. (1985) Clinical and histologic studies of neuronal intestinal dysplasia. J Pediatr Surg 20, 231-235

Munakata K, Okabe I and Morita K. (1992) Hypoganglionosis. Pediatr Surg Int 7, 8-11

Murphy RA. (1992) Do cytoplasmic and muscle specific isoforms of actin and myosin

heavy and light chains serve different functions in smooth muscle? Japanese J Pharmacol 58, 67P-74P

Narahashi T. (1974) Chemical as tools in the study of excitable membranes. Physiol Reviews 54, 813-889

Navarro J, Sonsino E, Boige N, Nabarra B, Ferkadji L, Mashako LMN and Cezard JP. (1990) Visceral neuropathies responsible for chronic intestinal pseudo-obstruction syndrome in pediatric practice:analysis of 26 cases. J Ped Gastroenterol and Nutr 11, 179-195

Niemi M, Kouvalainen K and Hjelt L. (1961) Cholinesterases and monoamine oxidase in congenital megacolon. J Path Bact 82, 363-366

Nixon HH. (1966) Seminar on pseudo-Hirschsprung's disease and related disorders. Arch Dis Childh 41, 143-154

Nixon RA and Sihag RK. (1991) Neurofilament phosphorylation: a new look at regulation and function. TINS 14, 501-506

Nonaka M, Goulet O, Arahan P, Fekete C, Ricour C and Nezelof C. (1989) Primary intestinal myopathy, a cause of chronic idiopathic intestinal pseudo-obstruction syndrome (CIPS): clinicopathological studies of seven cases in children. Pediatr Pathol 9, 409-424

Nybroe O, Linnemann D and Bock E. (1988) NCAM biosynthesis in brain. Neurochem Int 12, 251-262

Otey CA, Kalnoski MH and Bulinski JC. (1987) Identification and quantification of actin isoforms in vertebrate cells and tissues. J Cell Biochem 34, 113-124

Owens GK and Thompson MM. (1986) Developmental changes in isoactin expression in the rat aortic smooth muscle cells in vivo. J Biol Chem 261, 13373-13380

Pages R. (1966) Seminars on pseudo-Hirschsprung's disease and related disorders. Arch Dis Childh 41, 143-154

Parry CH. (1825) Singular and fatal accumulation of faeces. In: collections from the unpublished medical writings of the late C H Parry, London, Underwoods, vol 2. p 380-386

Patel K, Moore SE, Dickson G, Rossell RJ, Beverley PC, Kemshead JT and Walsh FS. (1989) Neural cell adhesion molecule (NCAM) is the antigen recognised by monoclonal antibodies of similar specificity in small-cell lung carcinoma and neuroblastoma. Int J Cancer 44, 573-578

Perry S V and Grand R J A. (1979) Mechanisms of contraction and the specialised protein components of smooth muscle. Br Med Bull 35, 219-226

Pistor G. (1989) Functional colonic ultrasonography; Normal findings of colonic

motility and follow-up in neuronal intestinal dysplasia. Progress in Pediatric Surgery 24, 155-164

Puri P, Lake BD, Nixon HH, Mishalany H and Claireaux AE. (1977a) Neuronal colonic dysplasia: an unusual association of Hirschsprung's disease. J Pediatr Surg 12, 681-685

Puri P, Lake BD and Nixon HH. (1977b) Adynamic bowel syndrome. Report of a case with disturbance of the cholinergic innervation. Gut 18, 754-759

Puri P, Lake BD, Gorman F, O'Donnell B and Nixon HH. (1983) Megacystis-microcolon-hypoperistalsis syndrome: a visceral myopathy. J Pediatr Surg 18, 64-69

Ravitch MM (1966) p 153 In: Bentley JFR, Nixon HH, Ehrenpreis Th, Spencer B, Lister J, Duhamel B, Pages R and Katz A. Seminar on pseudo-Hirschsprung's disease and related disorders. Arch Dis Childh 41, 143-154

Reddy SN, Di Lorenzo C, Tomomasa T, Snape WJ Jr and Hyman PE. (1992) Is electrogastrography (EGG) a substitute for manometry in the study of gastrointestinal motility disorders in children? Gastroenterology 102, A229

Redline RW, Neish A, Holmes LB and Collins T. (1992) Biology of Disease. Homeobox genes and congenital malformations. Lab Invest 66, 659-670

Retzlaff K. (1920) Zur Hirschsprung'schen Krankheit. Berl klin Wehnschr 57, 319-

Rinecker H, Chaussy C and Brendel W. The propagation of contractile waves from duodenum to jejunum. Pfugers Arch 1969;305:210-218. In: Wingate DL. (1981). Backwards forwards with the migrating complex. Dig Dis Sci 26, 641-664

Rintala R, Rapola J and Louhimo I. (1989) Neuronal intestinal dysplasia. Progress in Pediatric Surgery 24, 186-192

Robertson HE and Kernohan JW. (1938) The myenteric plexus in congenital megacolon. Proc Staff Meet Mayo Clin 13, 123-125

Rode H, Moore SW, Kaschula ROC, Brown RA and Cywes S. (1992) Degenerative leiomyopathy in children. A clinico pathological study. Pediatr Surg Int 7, 23-29

Rodrigues CA, Shepherd NA, Lennard-Jones JE, Hawley PR and Thompson HH. (1989) Familial visceral myopathy: a family with at least 6 involved members. Gut 30, 1285-1292

Rossowski WJ, Ertan A, Rice J, Ozden A, Covington S and McCord R. (1988) Alterations in cholinergic muscarinic and somatostatin binding sites in a patient with idiopathic intestinal pseudo-obstruction. Am J Med Sci 296, 399-405

Ruckebusch Y and Laplace JP. (1967) La motricite intestinale chez le mouton: phenomines mecaniques et electrique. C R Soc Biol 161, 2517-2523. In: Wingate DL.

(1981) Backwards forwards with the migrating complex. Dig Dis Sci 26, 641-664

Ruckebusch Y and Bardon T. (1984) Involvement of serotonergic mechanisms in initiation of small intestinal cyclic motor events. Dig Dis Sci 29, 520-527

Ruckebusch Y. (1986) Development of digestive motor patterns during perinatal life: mechanism and significance. J Pediatr Gastroenterology Nutr 5, 523-536

Frederici Ruyschii. (1691) Observationum Anatomico-Chirurgicarum Centuria. Amstelodami. In: Hirschsprung's disease (1970), Ehrenpreis T (Ed). Year Book Medical Publishers Inc, Chicago p 15-18

Rumessen JJ and Thuneberg L. (1982) Plexus muscularis profundus and associated interstitial cells. 1. Light microscopical studies of mouse small intestine. Anat Rec 203, 115-127

Rumessen JJ, Thuneberg L and Mikkelsen HB. (1982) Plexus muscularis profundus and associated interstitial cells. 2. Ultrastructural studies of mouse small intestine. Anat Rec 203, 129-146

Rumessen JJ and Thuneberg L. (1991) Interstitial cells of Cajal in human small intestine. Ultrastructural identification and organisation between the main smooth muscle layers. Gastroenterology 100, 1417-1431

Rumessen JJ, Mikkelsen HB and Thuneberg L. (1992) Ultrastructure of interstitial

cells of Cajal associated with deep muscular plexus in human small intestine. Gastroenterology 102, 56-68

Rumessen JJ, Mikkelsen HB, Qvortrup K and Thuneberg L. (1993) Ultrastructure of interstitial cells of Cajal in circular muscle of human small intestine. Gastroenterology 104, 343-350

Sacher P, Briner J and Stauffer UG. (1991) Unusual cases of neuronal intestinal dysplasia. Pediatr Surg Int 6, 225-226

Sams VR, Bobrow LG, Happerfield L and Keeling J. (1992) Evaluation of PGP9.5 in the diagnosis of Hirschsprung's disease. J Pathol 168, 55-58

Sawtell NM & Lessard JL. (1989) Cellular disruption of smooth muscle actins during mammalian embryogenesis: Expression of the alpha vascular but not the gamma enteric isoform in differentiating striated myocytes. J Cell Biol 109, 2929-2937

Scharli AF and Meier-Ruge W. (1981) Localized and Disseminated Forms of Neuronal Intestinal Dysplasia Mimicking Hirschsprung's Disease. J Pediatr Surg 16, 164-170

Scharli AF and Meier-Ruge W. (1986) Hirschsprung's-related disorders of intestine. Proceedings of plenary session on hundred years of Hirschsprung's disease (1886-1986) 8th Congress, Asian Association of Paediatric surgeons p 29-35

Scharli AF. (1992) Neuronal intestinal dysplasia. Pediatr Surg Int 7, 2-7

Schlaepfer WW. (1987) Neurofilaments: structure, metabolism and implications in disease. J Neuropath Exp Neurol 46, 117-129

Schmidt JE. (1909) Uber Hirschsprung'sche Krankheit, inbesondere ihre chirurgische Behandlung. Beitr z klin Chir 61, 682-724

Schofield DE and Yunis EJ. (1991) Intestinal neuronal dysplasia. J Pediatr Gastroenterol Nutr 12, 182-189

Schuffler MD, Lowe MC and Bill AH. (1977a) Studies of idiopathic intestinal pseudo-obstruction. 1.Hereditary hollow visceral myopathy:clinical and pathological studies. Gastroenterology 73, 327-338

Schuffler MD and Pope CE. (1977b) Studies of idiopathic intestinal pseudo-obstruction. 2.Hereditary hollow visceral myopathy: Family studies. Gastroenterology 73, 339-344

Schuffler MD, Bird TD, Sumi SM and Cook A. (1978) A familial neuronal disease presenting as intestinal pseudo-obstruction. Gastroenterology 75, 889-898

Schuffler MD, Rohrmann CA, Chaffee RG et al. (1981) Chronic intestinal pseudo-obstruction. A report of 27 cases and review of the literature. Medicine 60, 173-196

Schuffler MD and Jonak Z. (1982) Chronic idiopathic intestinal pseudo-obstruction caused by a degenerative disorder of the myenteric plexus: the use of Smith's method to define the neuropathology. Gastroenterology 82, 476-486

Schuffler MD, Leon SH and Krishnamurthy S. (1985) Intestinal pseudo-obstruction caused by a new form of visceral neuropathy: palliation by radical small bowel resection. Gastroenterology 89, 1152-1156

Schuffler MD, Pagon RA, Schwartz R and Bill AH. (1988a) Visceral myopathy of the gastrointestinal and genitourinary tracts in infants. Gastroenterology 94, 892-898

Schuffler MD. (1988b) Chronic idiopathic intestinal pseudo-obstruction. In: Gastrointestinal motility. Kumar D and Gustavsson S (Eds). John Wiley, Chichester, p 383-399

Schuffler MD. (1989) Neuromuscular abnormalities of small and large intestine. In: Gastrointestinal and Oesophageal Pathology. Whitehead R (Ed). Churchill Livingstone, Edinburgh, p 329-353

Schwartzenberg C. (1849) Die peristaltische Bewegung des Dunndarms. Ztschr f Rationelle Med 7, 311-331. In: Wingate DL. (1981) Backwards forwards with the migrating complex. Dig Dis Sci 26, 641-664

Scott PM. (1992) Vertebrate homeobox gene nomenclature. Cell 71, 551-553

Shaw A, Shaffer H, Teja K, Kelly T, Grogan E and Bruni C. (1979) A perspective for pediatric surgeons: chronic idiopathic intestinal pseudo-obstruction. J Pediatr Surg 14, 719-727

Shires AK and Rubenstein PA. (1989) Non uniform behaviour of multiple isoactins in the same cell is a cell dependant phenomenon. Cell Motility and Cytoskeleton 14, 263-270

Simpser E, Kahn E, Kenigsberg K, Duffy L, Markowitz J and Daum F. (1991) Neuronal intestinal dysplasia: Quantitative diagnostic criteria and clinical management. J Pediatr Gastroenterol Nutr 12, 61-64

Skalli O, Ropraz P, Trzeciak A, Benzonana G, Gillessen D and Gabbiani G. (1986) A monoclonal antibody against alpha smooth muscle actin. A new probe for smooth muscle differentiation. J Cell Biol 103, 2787-2796

Smith Blanca. (1968) Pre- and postnatal development of the ganglion cells of the rectum and its surgical implications. J Pediatr Surg 3, 386-391

Smith Barbara. (1967) Myenteric plexus in Hirschsprung's disease. Gut 8, 308-312

Smith Barbara. (1968a) The myenteric plexus in drug-induced neuropathy. J Neurol Neurosurg Psychiat 30, 506-510

Smith Barbara. (1968b) Effect of irritant purgatives on the myenteric plexus in man

and the mouse. Gut 9, 139-143

Smith Barbara. (1982) The neuropathology of pseudo-obstruction of the intestine. Scandinavian Journal of Gastroenterology (suppl) 71, 103-109

Smith JA, Hauser SC and Madara JI. (1982) Hollow visceral myopathy. A light- and electron microscopic study. Am J Surg Pathol 6, 269-275.

Smout AJPM, de Wilde K, Kooyman CD and Ten Thije OJ. (1985) Chronic idiopathic intestinal pseudo-obstruction. Coexistence of smooth muscle and neuronal abnormalities. Dig Dis Sci 30, 282-287

Spencer B. (1966) Seminar on pseudo-Hirschsprung's disease and related disorders. Arch Dis Childh 41, 143-154

Stanciu C and Bennett JR. (1975) The general pattern of gastroduodenal motility: 24 hour recordings in normal subjects. Revista Medico-chirurgicala a Societatea de Medici si Naturalist din Iasi 79, 31-36

Stanguellini V, Camilleri M and Malagalada JR. (1987) Chronic idiopathic pseudo-obstruction clinical and intestinal manometric findings. Gut 28, 5-12

Stark ME, Bauer AJ, Sarr MG and Szurszewski JH. (1993) Nitric oxide mediates inhibitory nerve input in human and canine jejunum. Gastroenterology 104, 398-409

Summers RW, Anuras S and Green J. (1983) Jejunal manometric patterns in health, partial intestinal obstruction and pseudo-obstructions. Gastroenterology 85, 1290-1301

Szurszewski JH. (1969) A migrating electric complex of the canine small intestine. Am J Physiol 217, 1757-1763. In: Wingate DL. (1981) Backwards forwards with the migrating complex. Dig Dis Sci 26, 641-664

Tanner MS, Smith B and Lloyd JK. (1976) Functional intestinal obstruction due to deficiency of argyrophilic neurones in the myenteric plexus: familial syndrome presenting with short bowel, malrotation and pyloric hypertrophy. Arch Dis Childh 51, 837-841

Taylor GS and Bywater RAR. (1988) Intrinsic control of the gut. Bailliere's clinical gastroenterology. 2, 1-22

Tew, EMM., Anderson, PN. and Burnstock, G. (1992) Implantation of the myenteric plexus into the corpus striatum of adult rats: survival of the neurons and glia and interactions with host brain. Restorative Neurology and Neuroscience 4, 311-321.

Thuneberg L. (1982) Interstitial cells of Cajal: intestinal pacemaker cells? Adv Anat Embryol Cell Biol 71, 1-130

Thuneberg L, Rumessen JJ and Mikkelsen HB. (1982) The interstitial cells of Cajal: intestinal pacemaker cells? In: Motility of the digestive tract. Wienbeck M (Ed). Raven Press, New York, p 115-122

Thuneberg L. (1989) Interstitial cells of Cajal. In: Handbook of physiology, Section 6, Part 1,vol 1, The gastrointestinal system. Motility and circulation. Schultz SG, Wood JD and Rauner BB (Eds). American Physiol Soc. p 349-386

Tibboel D, Meijers JHC, Kluck P, van der Kamp AWM, ten Kate FWJ, van Haperen-Heuts ICCM and Molenaar JC. (1987) Monoclonal antibodies for diagnosis and research in enteric nervous system pathology. Dev Neurosci 9, 133-143

Tiffin ME, Chandler LR and Faber HK. (1940) Localized absence of the ganglion cells of the myenteric plexus in congenital megacolon. Am J Dis Child 59, 1071-1082

Tittel K. (1901) Uber eine angeborene Missbildung des Dickdarmes. Wiener klinische Wochenschrift 14, 903-907

Tsukada T, Tippens D, Gordon D, Ross R and Gown AM. (1987) HHF35, a muscle actin specific monoclonal antibody. 1. Immunocytochemical and biochemical characterization. Am J Pathol 126, 51-60

Van der Schee EJ and Grashuis JL. (1987) Running spectrum analysis as an aid in the representation and interpretation of electrogastrographic signals. Medical and biological Engineering and computing 25, 57-62

Vanderwinden J-M0, Mailleux P, Schiffmann SN, Vanderhaeghen J-J and De Laet M-H. (1992). Nitric oxide synthase activity in infantile hypertrophic pyloric stenosis. New Engl J Med 327, 511-515

Vaos GC. (1989) Quantitative assessment of the stage of neuronal maturation in the developing human fetal gut- a new dimension in the pathogenesis of developmental anomalies of the myenteric plexus. J Pediatr Surg 24, 920-925

Vargas JH, Sachs P and Ament ME. (1988) Chronic intestinal pseudo-obstruction syndrome in pediatrics. Results of national survey by members of the North American Society of Pediatric Gastroenterology and Nutrition. J Pediatr Gastroenterol Nutr 7, 323-332

Venizelos ID, Shousha S, Bull TB and Parkins RA. (1988) Chronic intestinal pseudo-obstruction in two patients. Overlap of features of systemic sclerosis and visceral myopathy. Histopathol 12, 533-540

Walsh FS, Moore SE and Lake BD. (1987) Cell adhesion molecule N-CAM is expressed by denervated myofibres in Werdnig-Hoffman and Kugelberg-Welander type spinal muscular atrophy. J Neurol Neurosurg Psychiatr 50, 439-442

Walsh FS. (1988) The NCAM gene is a complex transcriptional unit. Neurochem Int 12, 263-267

Weisbrodt NW. (1981) Motility of the small intestine. In: Physiology of the gastrointestinal tract. Johnson LR (Ed). Raven Press, New York, p 411-443

Weisbrodt NW. (1987) Motility of the small intestine. In: Physiology of the gastrointestinal tract. Johnson LR (Ed). Raven Press, New York, p 631-663

Wells T R, Landing B H, Ariel I, Nadorra R and Garcia C. (1987) Normal anatomy of the myenteric plexus of infants and children. Perspect pediatr pathol 11, 152-174

Wiswell TE, Rawlings JS, Wilson JL and Pettett G. (1979) Megacystis-microcolon-intestinal hypoperistalsis syndrome. Pediatrics 63, 805-808

Wolgemuth DJ, Behringer RR, Mostoller MP, Brinster RL and Palmiter ERD. (1989) Transgenic mice overexpressing the mouse homeobox-containing gene Hox-1.4 exhibit abnormal gut development. Nature 337, 464-467

Wood JD. (1972) Excitation of intestinal muscle by atropine, tetrodotoxin and xylocaine. Am J Physiol 222, 118-125

Wood JD. (1981) Physiology of the enteric nervous system. In: Physiology of gastrointestinal tract. Johnson LR (Ed). Raven Press, New York, p 1-37

Wood, JD. (1988a). Order and disorder in the little brain of the gut: An introduction to the enteric nervous system, part 1. Motility 3, 11-13

Wood, JD. (1988b). Order and disorder in the little brain of the gut: An introduction to the enteric nervous system, part 2. Motility 4, 10-11

Wood, JD. (1989a). Order and disorder in the little brain of the gut: An introduction to the enteric nervous system, part 3. Motility 5, 12-13

Wood, JD. (1989b). Order and disorder in the little brain of the gut: An introduction to the enteric nervous system, part 4. Motility 6, 11-13

Wood JD. (1990) Neural mechanisms of gastro-duodenal co-ordination. In: Gastro-pyloro-duodenal co-ordination. van Nurten JM, Schuurkes JAJ and Akkermans LMA (Eds). Wrightson, Petersfield, p 35-66

Wozniak ERT, Fenton TR and Milla PJ. (1984) Fasting small intestinal motor activity in chronic idiopathic intestinal pseudo-obstruction. Pediatr. Res. 18, 1060

Yamagiwa I, Ohta M, Obata K and Washio M. (1988) Intestinal pseudo-obstruction in a neonate caused by idiopathic muscular hypertrophy of the entire small intestine. J Pediatr Surg 23, 866-869

Yanagihara J, Shimotake T, Deguchi E and Iwai N. (1991) Histologiacal investigation of the myenteric plexus of the entire gut in an infant with hypogenesis of the intestine. Eur J Pediatr Surg 2, 114-117

Appendix. Methods

Acid Phosphatase

Incubation medium: 31.5 mg of sodium beta glycerophosphate (Sigma)

5 ml 0.1 M acetate buffer pH 5.0

5 ml 0.008 M lead acetate (BDH)

1. Fix cryostat sections (5-7μm thick) in formol calcium 1-3 minutes.
2. Wash in tap water, rinse in distilled water.
3. Incubate at 37°C for 30 minutes.
4. Wash in tap water and treat in dilute ammonium sulphide (BDH; 2ml in 250 ml of tap water) for 30 seconds.
5. Wash in tap water, counterstain nuclei in Carazzi's haematoxylin for 1 minute, wash well and mount in glycerine jelly.

Acetyl Cholinesterase (AChE)

Incubation medium: A:

5 mg acetylthiocholine iodide (Koch Light)

6.5 ml 0.1 M acetate buffer pH 6.0

0.5 ml 0.1 M sodium citrate (BDH)

1.0 ml 30 mM Copper sulphate (BDH)

1.0 ml distilled water

0.2 ml 4 mM Iso-ompa (iso-octamethyl pyrophosphoramide (Koch Light)

B: 1.0 ml 5 mM potassium ferricyanide (BDH)

Solutions A and B can be made in bulk and stored in aliquots at -20°C Mix 9 ml of

A with 1 ml of B just before incubation.

1. Fix 10 μm thick cryostat sections in formol calcium 30 seconds.
2. Wash in distilled water and incubate in freshly mixed A+B for 60 minutes at 37°C
3. Wash in distilled water and incubate at room temperature in 0.05% para-phenylene diamine dihydrochloride (BDH) in 0.1 M phosphate buffer pH 6.8 for 45 minutes.
4. Wash in distilled water and treat with 2% osmium tetroxide (Johnson Matthey) at room temperature for 10 minutes.
5. Wash, counterstain nuclei lightly in Carazzi's haematoxylin (45 seconds) and wash well before dehydrating in absolute alcohol, clearing in xylene and mounting in DPX.

Celloidinised Periodic Acid Schiff (PAS)

Schiff reagent: dissolve 1 g pararosaneline hydrochloride (Sigma) in 100 ml of boiled distilled water, stir well, cool to 60°C and add 2 g potassium metabisulphite (BDH) and 20 ml 1M hydrochloric acid (BDH). Stopper flask well and leave in the dark for overnight.

Add 1 teaspoonful of activated charcoal, shake well, filter and store in +4°C under a layer of xylene to maintain the sulphur dioxide concentration.

1. Protect cryostat sections (5-7μm thick) with 0.25% celloidin in absolute alcohol, dry in positive air.
2. Treat with 0.5% periodic acid for 8 minutes.
3. Wash well in tap water and rinse in distilled water.
4. Treat with Schiff reagent 15 minutes.
5. Rinse in distilled water and wash well (at least 5 minutes) in tap water.

6. Counterstain nuclei in Carazzi's haematoxylin 4 minutes, wash well, dehydrate in absolute alcohol, clear in xylene and mount in DPX.

Haematoxylin and Eosin (H&E)

1. Rinse cryostat sections (5-7μm thick) in absolute alcohol.

2. Wash in tap water and fix in formol calcium for 1 minute.

3. Stain in Harris' haematoxylin for 1 minute.

4. Blue in running tap water.

5. Stain in 1% aquous eosin for 1-2 minutes.

6. Dehydrate in absolute alcohol, clear in xylene and mount in DPX.

Harris' Haematoxylin

A 2.5 g haematoxylin in 50 ml absolute alcohol

B 50 g potassium alum in 500 ml distilled water

Mix A and B and heat to boiling

Add 1.5 g yellow mercuric oxide

Cool rapidly

Add 20 ml glacial acetic acid

final pH 2.3

Carazzi's Haematoxylin

Haematoxylin 1 g

Sodium iodate 0.2 g

Potassium alum 50 g

Glycerol 200 ml

Distilled water 800 ml

Dissolve haematoxylin and alum separately in water.

Mix and leave overnight.

Add iodate and glycerol and leave overnight then ready to use.

Gomori Trichrome

Staining solution:

0.6 g chromotrope 2R

0.3 g fast green fcs

0.6 g phosphotungstic acid

1 ml glacial acetic acid

make up to 100 ml with distilled water

adjust pH to 3.4 with 1M NaOH

1. Stain nuclei with Harris' haematoxylin for 5 minutes.
2. Wash in distilled water.
3. Stain with trichrome solution 10 minutes.
4. Rinse in two changes of 0.2% acetic acid.
5. Dehydrate in absolute alcohol, clear in xylene and mount in DPX.

Picro-Sirius

1. Fix 10 μm thick cryostat sections in formol calcium 10-30 minutes.
2. Wash in tap water and rinse in distilled water.

3. Stain sections in 0.1% sirius red in saturated aqueous picric acid 10-30 minutes.

4. Dehydrate in absolute alcohol, clear in xylene and mount in DPX.

Silver Staining

Fixative:

10% formol saline:

8.5 g sodium chloride (BDH)

100 ml 40% formaldehyde

900 ml distilled water

Cajal's formol ammonium bromide:

40% Formaldehyde (BDH) 15 ml

ammonium bromide (BDH) 2 g

distilled water 85 ml

Alcoholic pyridine:

Absolute alcohol 50 ml

Distilled water 40 ml

Pyridine (BDH) 10 ml

Ammonical silver solution:

20% silver nitrate into which is added .880 ammonia solution until the precipitate first formed is just dissolved.

10% formalin:

40% formaldehyde	100 ml
tap water	900 ml
marble chips	10-20

Carbol Xylene:

Molten anhydrous phenol	25 ml
xylene	75 ml
or	
phenol white crystalline	100 g
xylene	300 ml

1. Treat 50μm thick sections cut on a freezing microtome tangentially at the plain of the myenteric plexus with Cajal's formol ammonium bromide for 24 hours.
2. Rinse in two changes of distilled water and transfer to the alcoholic pyridine for 1 hour at 37°C.
3. Rinse in two changes of distilled water and transfer sections to 20% silver nitrate at 37°C for 1 hour. Use a flat glass petri dish and ensure that sections are flat and not overlapping with each other.
6. Take sections through three changes of 10% formalin in tap water.
7. Transfer sections to 2% formalin in tap water, then two changes of distilled water.
8. Treat sections with ammonical silver solution with constant agitation for 1 minute.
9. Transfer sections through three changes of 1% formalin in tap water (agitate rapidly in each) to distilled water.
10. Treat with 5% sodium thiosulphate for 5 minutes.

11. Wash thoroughly.

12. Pick sections singly on glass slides teasing out creases. Dry and blot sections well before dehydrating in absolute alcohol and clearing in carbol xylene first and then in xylene.

Electron Microscopy

Araldite mixture:

10 ml araldite 212 (BioRad)

10 ml DDSA (dodecenyl succinic anhydride; Agar)

0.2 ml BDMA (N,N-dimethylbenzylamine; Sigma)

0.25 ml dibutylthalate (Koch Light)

Mix well

Lead citrate:

Dissolve 1.33 g of lead nitrate in 30 ml of boiled, cooled distilled water. Add 1.76 g sodium citrate-$2H_2O$. Shake vigorously for 1 minute then intermittently for 30 minutes. Add 0.8 ml 10 M NaOH, shake and dilute to 50 ml with boiled, cooled distilled water. Ensure the pH is pH12. Keep in +4°C and centrifuge before use.

1. Fix full-thickness samples of opened intestine at room temperature in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 and containing 2.5mM calcium chloride for 2 hours. Take smaller full-thickness blocks (measuring approximately 3x3mm) of the outer fixed surface of the gross samples and continue fixation for at least 12 hours.

2. Transfer blocks into 0.1 M cacodylate buffer pH 7.4 and containing 2.5 mM

calcium chloride for 15 minutes at room temperature.

3. Post-osmicate at +4°C for 60 minutes in 1% osmium tetroxide in 0.1 M cacodylate buffer containing 5mM calcium chloride.

4. Wash briely (10 minutes) in 0.1 M cacodylate buffer at pH 7.4 containing 2.5mM calcium chloride.

5. Follow by two changes of 70% alcohol overall excess of 15 minutes.

6. Dehydrate in two changes of acidified dimethoxypropane (1 drop of concentrated hydrochloric acid in 50 ml of dimethoxypropane) for total of 5 minutes.

7. Discard the last change of dimethoxypropane and replace with the araldite mixture.

8. Place unstoppered vials in 60°C for 15-30 minutes. Recap and impregnate rotating at room temperature for 18 hours.

9. Block out in fresh araldite and polymerise at 60°C 24-48 hours.

10. Cool blocks and cut sections to reveal the muscularis propria and the myenteric plexus.

11. Pick ultrathin sections up on copper grids, contrast in 10% uranyl acetate in absolute alcohol for 10 minutes, rinse in absolute alcohol and further treat for 10 minutes with lead citrate. Grids are then rinsed in 0.02 M sodium hydroxide and distilled water before viewing with a transmission electron microscope.

ABC method for Immunohistochemistry

Phosphate buffered saline (PBS): made from PBS tablets 10 per litre.

10 μm cryostat sections of snap frozen intestine fixed in acetone at -20°C for 10 minutes and air dried.

1. Apply the primary antibody at an appropriate titre (previously determined) in phosphate buffered saline (PBS) for 1-24 hours at room temperature in a moist chamber.

2. Wash in PBS for 2 minutes.

3. Apply the secondary antibody, biotinylated rabbit anti mouse IgG +IgM subclass antibody (monoclonal primary antibody) at 1:150 or biotinylated swine antirabbit IgG + IgM subclass (polyclonal primary antibody) 1:200. For muscarinic receptor use a biotinylated rabbit anti mouse IgM subclass antibody at titre of 1:150. The secondary antibody is applied to the sections for 30-60 minutes at room temperature.

4. Wash in PBS for 2 minutes.

5. Treat with avidin biotin peroxidase complex (prepared at least 30-60 minutes in advance by mixing 10μl of avidin with 10 μl of biotin in 1 ml of PBS) for 30-60 minutes.

6. Wash in PBS 2 minutes.

7. Demonstrate the peroxidase with 1 ml of 5% diamino benzidine in dimethyl formamide in 100 ml of PBS adding 100 μl of 30% hydrogen peroxide immediately prior to incubation at room temperature for 10 minutes.

8. Wash, counterstain nuclei with Carazzi's haematoxylin, wash, dehydrate in alcohol, clear in xylene and mount in DPX.

Blocking for Endogenous Peroxidase

A. 0.3% hydrogen peroxide in absolute methanol

Treat sections prior to primary, secondary or tertiary antibody in the ABC method with A at room temperature 10-30 minutes and follow with the ABC method.

B. Periodic Acid-Borohydride

1. treat sections prior to primary, secondary or tertiary antibody in the ABC method first with 2.5% periodic acid for 5 minutes.

2. rinse in distilled water.

3. treat with 0.2% sodium -or potassium borohydride 2 minutes and wash in water and follow with the ABC protocol.

C. Glucose-glucoseoxidase

1. treat sections prior to primary, secondary or tertiary antibody in the ABC method with :

10mM glucose

1mM sodium azide

100 units of glucose oxidase (Sigma G6891) in PBS at 37°C for 1 hour then follow with the ABC method.

Immunohistochemistry for Autoantibodies:

1. Apply patient's serum (doubly diluted from neat to 1:640 with PBS) on 10μm thick cryostat sections of full-thickness piglet intestine for 60 minutes at room temperature in a moist chamber.

2. Wash in PBS.

3. Apply polyclonal antihuman IgG (Boehringer) at a titre of 1:600 in PBS for 30 minutes at room temperature.

4. Wash in PBS.

5. Follow the polyclonal immunostaining protocol from step 3 onwards as described above for the ABC method.

Formol calcium:

100 ml 40% formaldehyde

15.8 g dried calcium acetate

make up to 1 l with distilled water

18
Isolated intestinal neuronal dysplasia: a descriptive histological pattern or a distinct clinicopathological entity?

V. V. SMITH

INTRODUCTION

Intestinal neuronal dysplasia (IND), an abnormality of intestinal innervation associated with pseudo-obstructive dysmotility, was described in 1971 by Meier-Ruge[1]. He identified morphological abnormalities best recognized histochemically, which included hyperplasia of both the intermuscular and submucosal plexuses and an increase in acetylcholinesterase (AChE)-positive fibres in the lamina propria. The condition may occur as an isolated abnormality or in association with aganglionosis (Hirschsprung's disease; HD). Since it was first recognized there has been some confusion about the criteria on which the diagnosis is based, and this has resulted in widely differing reported frequencies for this disorder. For example, at the Hospital for Sick Children, Great Ormond Street, we have identified only seven cases (0.3%) amongst 2420 patients biopsied between 1975 and 1991, whilst Scharli[2] reported 54 cases (62%) in a series of 115 patients; other authors[3-6] have quoted incidences of 18–30%, which is similar to that of HD.

In most centres the histological and histochemical investigation of gut motility disorders is based on tissue biopsies of rectal mucosa and submucosa. In an attempt to clarify the diagnostic criteria for IND in such specimens a working party laid down guidelines in 1991[7]. These included two obligatory criteria, namely hyperplasia of the submucosal plexus and increased AChE-positive nerve fibres around submucosal blood vessels. Two other features, although not essential for the diagnosis, were: heterotopic ganglion cells in the lamina propria (neuronal heterotopia) and increased AChE-positive nerve fibres in the lamina propria.

To date, in making the diagnosis of IND we have placed special emphasis on the increase in AChE-positive nerve fibres in the lamina propria (Figs 1 and 2). These fibres run vertically from the bottom to the top of the lamina propria, and are finer than the coarse fibres, which often run in both the horizontal and vertical planes, seen in biopsies of older babies with HD

Figure 1 Section of rectal mucosa from a patient with IND showing marked increase in fine vertical AChE-positive nerve fibres in the lamina propria. Note no increase in these fibres is seen in the muscularis mucosae (×120)

(Fig. 3). No increase in AChE-positive fibres is seen in the muscularis mucosae in IND (Fig. 1) in contrast to HD where a gross and diffuse increase in thick, knotted fibres in the muscularis mucosae is virtually diagnostic of the disease (Fig. 3).

Whilst an occasional neuron in the lamina propria is not, in itself, diagnostic of IND, in the majority of our cases several such heterotopic neurons were noted in the lamina propria in addition to the increased AChE-positive fibres.

Hyperplasia of the submucosal plexus is an important diagnostic feature, and incorporates firstly an increased frequency of ganglion cell clusters and sometimes the presence of large ganglia defined by Scharli[2] as containing in excess of seven ganglion cells (Fig. 4). The frequent ganglion cell clusters have been described as having a 'button-like' appearance[3,5,7] and are associated with thickened sprouting AChE-positive nerve processes (Fig. 5).

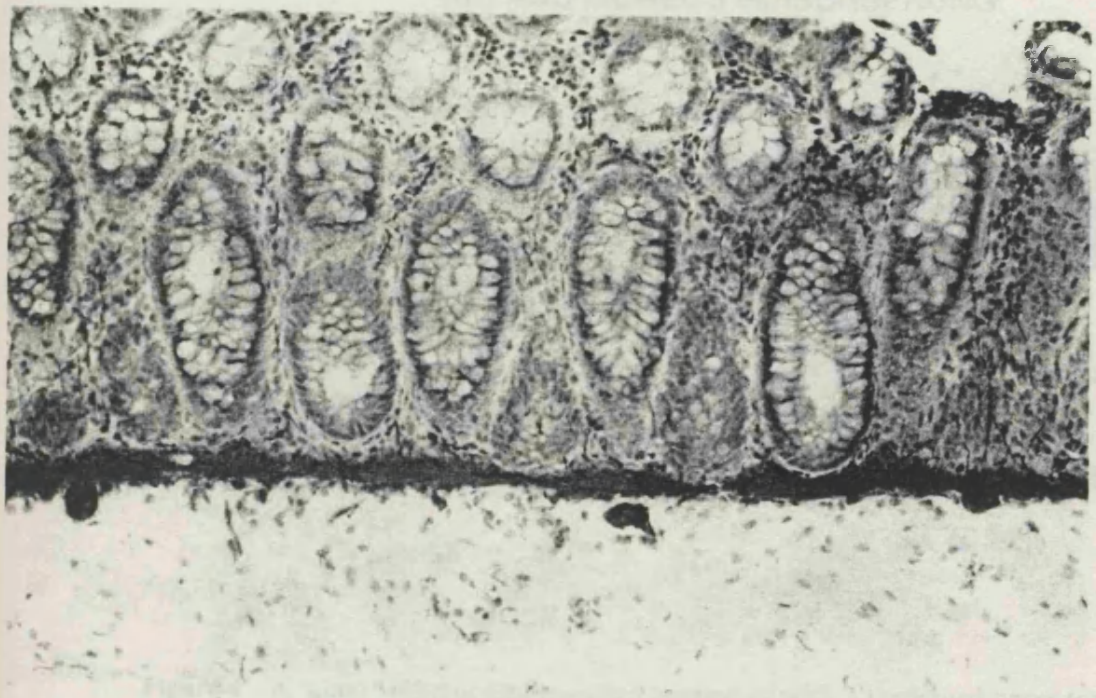


Figure 2 AChE pattern in normal rectal mucosa showing the almost complete lack of positive fibres in the lamina propria or muscularis mucosae (× 120)

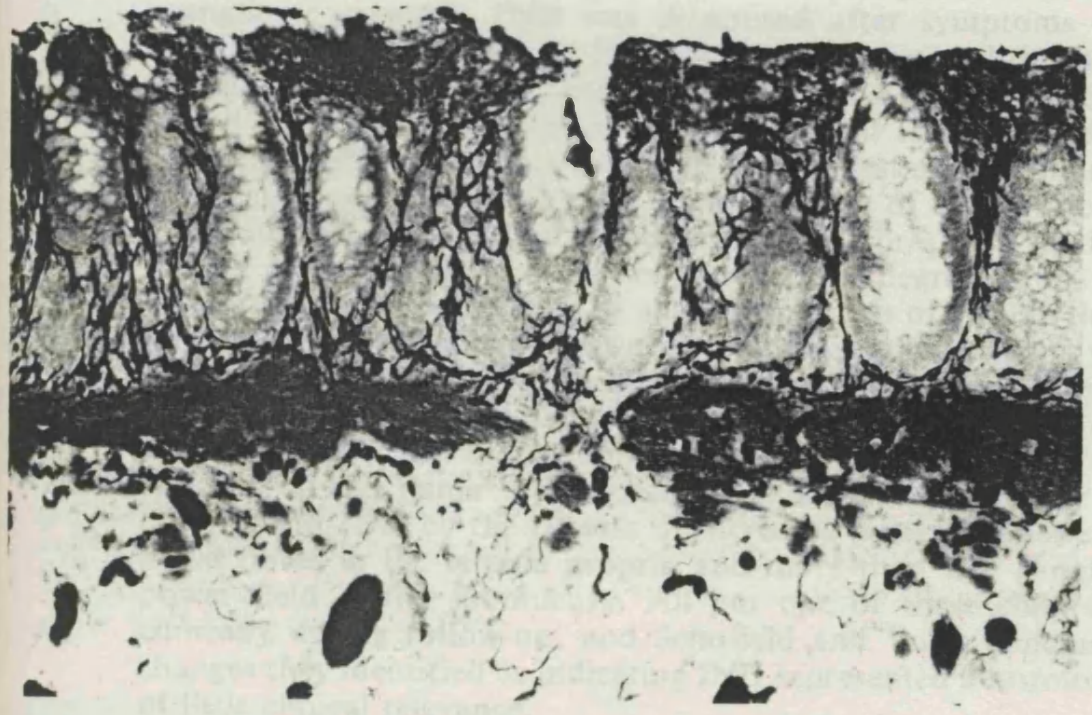


Figure 3 Rectal mucosa from a patient with HD showing marked increase in thick coarse AChE-positive fibres, which run in both vertical and horizontal planes in the lamina propria. Note thick knotted fibres are also prominent within the muscularis mucosae (× 120)

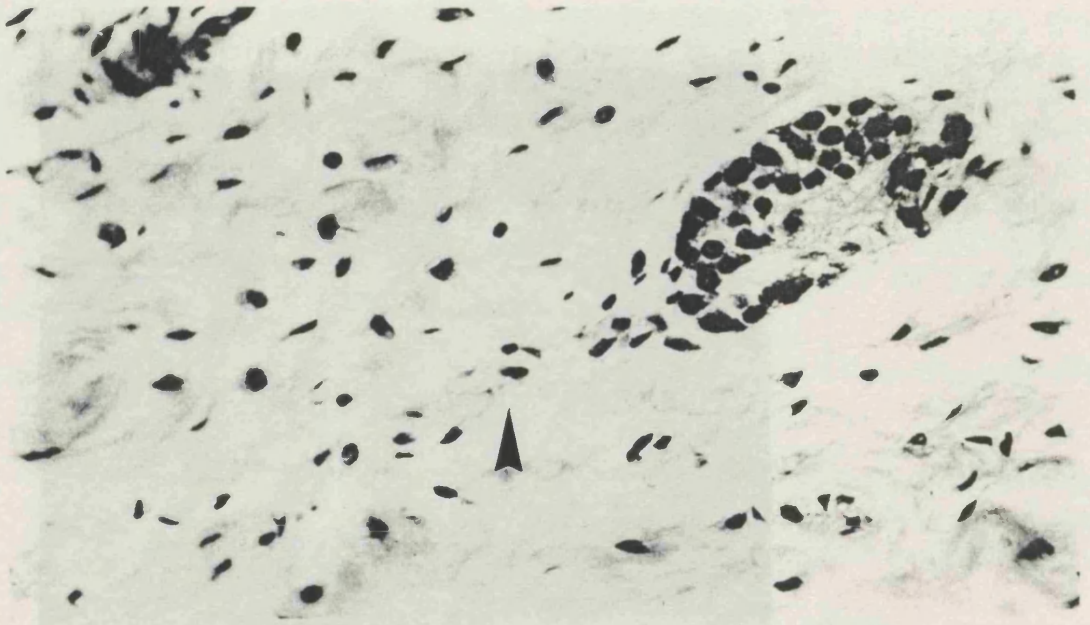


Figure 4 A 'giant' submucosal ganglion associated with a thickened nerve (arrowhead) from a patient with IND. (Haematoxylin and eosin, ×480)

An increase in AChE-positive nerve fibres around the submucosal blood vessels, whilst noted in some of our patients, was not included as a significant discriminator for the diagnosis of IND in our material.

Since 1975 we have identified only seven patients with IND, all of whom had concomitant HD with IND affecting bowel proximal to the aganglionic segment. IND was diagnosed after symptoms of disturbed intestinal motility continued following a corrective pull-through operation for aganglionosis. Lamina proprial increase in AChE-positive nerve fibres is said not to be present in older children with IND[7]. However, biopsies from each of our patients exhibited an unquestionable increase in these fibres in biopsies taken between 2 and 10 years of age. Although we have noted some increase in AChE-positive nerve fibres in a small number of suction rectal biopsies from other patients, the degree of this abnormality was insufficiently convincing to allow a diagnosis of IND in the absence of other diagnostic features. However, the absence of patients with isolated IND in our series, and the overall much lower frequency of this diagnosis in our patients compared with other reports, indicates a marked difference in interpretation of the biopsy appearances.

Schofield and Yunis[8] investigated 456 children in whom they identified 38 (8.3%) with IND by the criteria of increased acetylcholinesterase-positive nerve fibres in the lamina propria and more than five ganglia per high-power field in the submucosa. All but one of these children improved clinically during follow-up, and Schofield and Yunis concluded that the changes they identified as indicating IND represented a histological pattern of little clinical relevance.

Whilst few would doubt the existence of IND as a cause of intestinal pseudo-obstruction in children, it is clear that the diagnostic criteria, particularly when applied to rectal suction biopsy specimens, vary in different

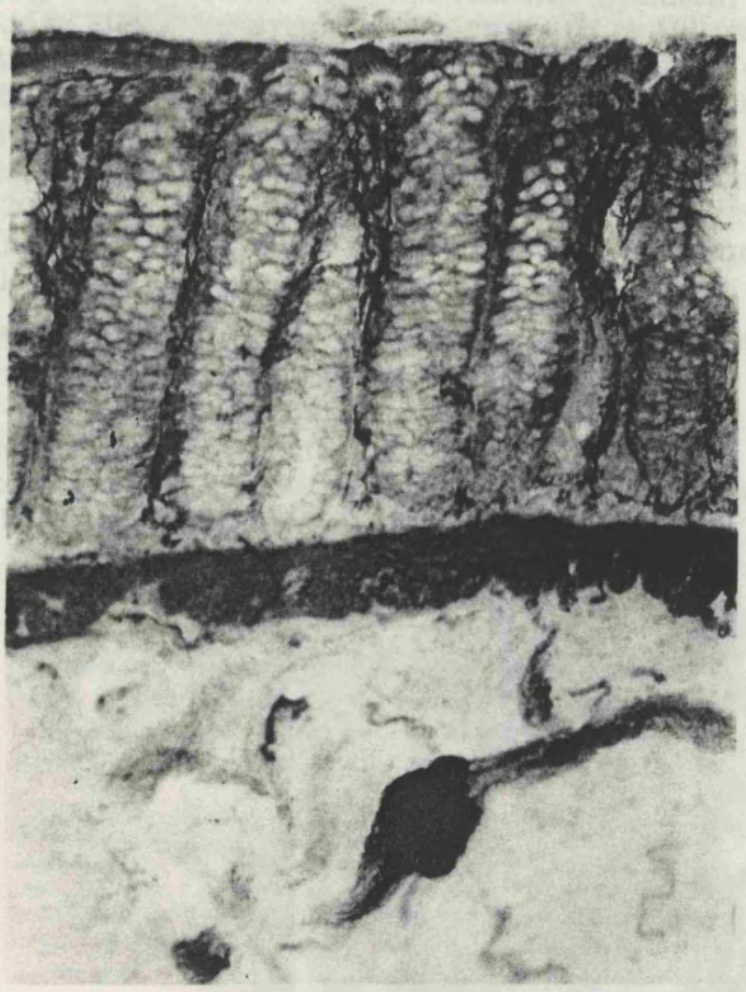


Figure 5 'Button-like' ganglion in the rectal submucosa from a patient with IND. Note increased AChE-positive nerves in the lamina propria but not in the muscularis mucosae (× 120)

centres and their significance in terms of clinical outcome is not well founded. The results of Schofield and Yunis[8] conflict with much of the other data reported in the literature[2-6], and for this reason we have embarked on a similar study of our own material.

Our investigation is in two parts. The first, reported here, has involved the reassessment of a series of suction rectal biopsies taken from patients admitted to the Hospital for Sick Children, Great Ormond Street, during the investigation of apparent pseudo-obstruction, applying, with minor modifications, the diagnostic criteria used by Borchard *et al.*[7].

A careful blind clinical follow-up of these patients, without knowledge of the biopsy findings, will then be performed to assess any clinical significance of the biopsy findings.

METHODS

Of a consecutive series of 548 infants and young children submitted to suction rectal biopsy during the investigation of suspected intestinal dysmotility

during 1989–91 at the Hospital for Sick Children, 137 (25%) were diagnosed as HD. Of the remaining biopsies from 411 patients, 326 (59%) were excluded from the present study because the amount of submucosa included in the biopsy, whilst adequate for confirming or excluding HD, was insufficient for assessing changes indicative of IND. Thus biopsies from 85 children aged 2 days to 10 years (median 11 weeks) remained. Of these children 16 (20%) were aged 1 week or less. All of these specimens had been regarded as 'normal' when originally reported, but were reassessed for the present study noting the following criteria:

1. Any apparent increase in AChE activity in the lamina propria, including minor and focal increases (Fig. 6).
2. Neuronal heterotopia.
3. Hyperplasia of the submucosal plexus in terms of the frequency and size of the ganglia and the association of ganglia with thickened nerves (budding). Ganglia were classified as 'infrequent' when examination of

Figure 6 Suction rectal biopsy showing mild focal increase in AChE-positive nerve fibres in the lamina propria. This change hitherto was not regarded as abnormal (× 120)

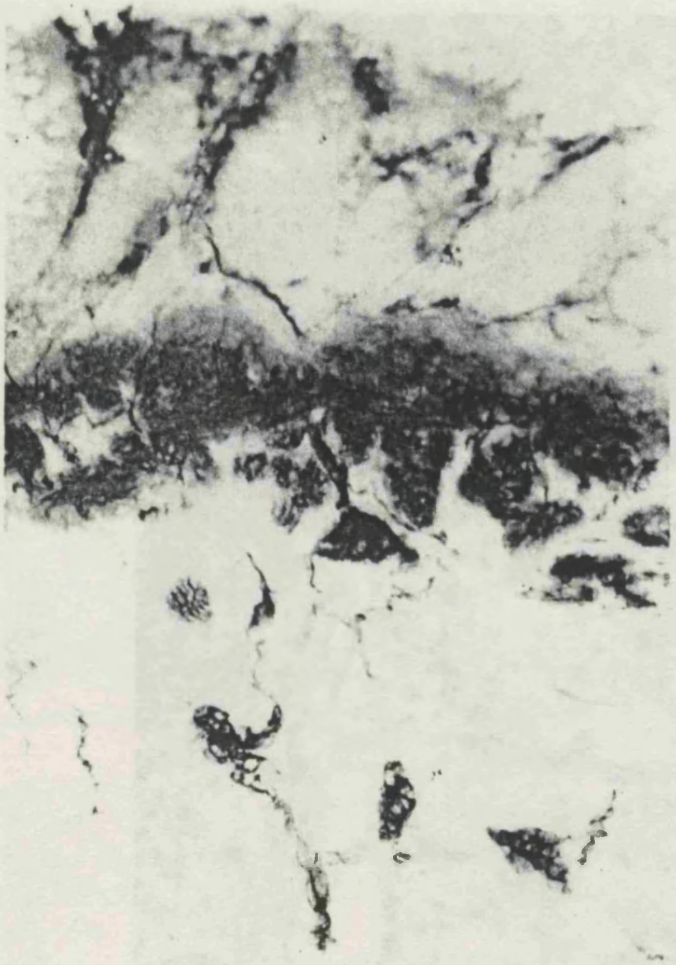


Figure 7 AChE activity in a suction rectal biopsy from a neonate showing frequent submucosal ganglia in 'button-like' association with nerves. Note also a mild increase in lamina proprial AChE-positive fibres (×160)

several sections was necessary to identify a single submucosal neuron or cluster. If ganglia were readily found without an excessive search in most sections their frequency was termed 'average'. Ganglia were classified as 'frequent' when all the sections examined showed several neurons or clusters (Figs 7 and 8) in every field examined with a ×10 objective. The sizes of the ganglia were assessed by counting neurons in a number of ganglia, and the term 'giant' ganglion was applied if more than seven neurons were identified in a cluster.

4. Increased number of AChE-positive nerve fibres around submucosal blood vessels (Fig. 9).

RESULTS

In none of the biopsies were changes seen sufficient to diagnose IND by our original criteria and, in particular, the marked diffuse increase in

Figure 8 Haematoxylin and eosin stained section of rectal mucosa from a neonate showing frequent submucosal ganglia (arrowheads) (×200)

AChE-positive fibres in the lamina propria, which we previously regarded as a key factor, was lacking. In this study an increase was recorded when it was of much lesser degree and often focal in distribution. By the reduced diagnostic thresholds described above, 60 patients (71%) were identified in whose biopsies some features of IND were detected.

In biopsies from 15 patients an increase in AChE-positive nerve fibres in the lamina propria was noted.

Neuronal heterotopia was not a feature in the biopsies examined, occurring in only one specimen in which it was not accompanied by any lamina proprial increase in AChE-positive nerve fibres.

Ganglia in the submucosa were frequent in biopsies from 38 patients (45%), average in 31 (36%) and infrequent in 16 (19%); giant ganglia were present in only four patients (5%). As shown in Fig. 10 a relationship existed between the frequency of submucosal ganglia and the patient's age

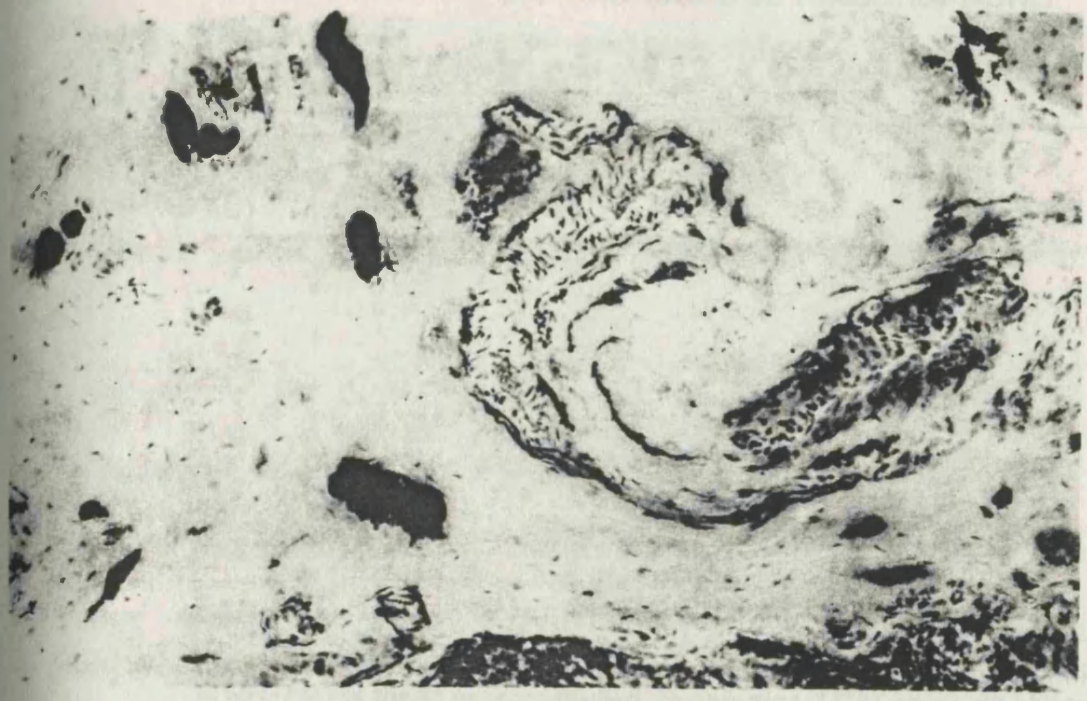


Figure 9 AChE activity around submucosal blood vessels considered to be increased (× 120)

at biopsy. Frequent ganglia occurred in 22 (73%) of the 30 patients biopsied under the age of 4 weeks, but in only 16 (29%) of the 55 patients biopsied after the age of 4 weeks. On the other hand, all 16 patients with infrequent ganglia were biopsied after 4 weeks of age. Comparing younger (aged less than 4 weeks) with older children (aged more than 4 weeks) significant differences between those with 'frequent', 'average' and 'infrequent' ganglia were established (chi-square = 18.28; $p < 0.01$).

In most biopsies only occasional isolated AChE-positive nerve fibres around submucosal blood vessels were seen, but biopsies from 20 patients showed several prominent positive fibres.

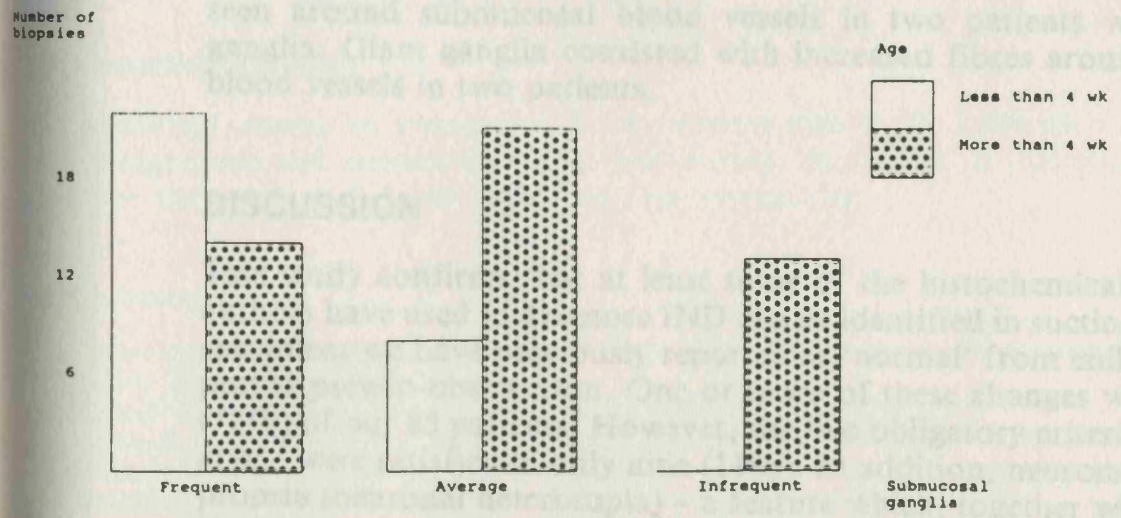


Figure 10 Relationship between frequency of submucosal ganglia (frequent, average or infrequent) and age (more or less than 4 weeks) at biopsy

Frequent ganglia (38)	AChE positive fibres SM vessels (20)	AChE positive fibres LP (15)	Giant ganglia (4)	
+	+	+	-	6
+	+	-	-	3
+	-	+	+	2
+	-	-	-	28
-	-	+	+	1
-	-	-	+	1
-	+	-	-	11
-	-	+	-	8
			Total	60 (71%)

Figure 11 Various histological features seen in IND, one or more of which were encountered in 60 (71%) of the 85 patients studied. In only nine (11%) were both obligatory criteria for IND of Borchard *et al.*[7] met (see box)

All four criteria together were not seen in any one patient (Fig. 11), but frequent ganglia and increased nerve fibres around the submucosal blood vessels coexisted in nine patients, six of whom also had some increases in lamina proprial nerve fibres. It is of interest that of these nine patients with biopsies showing both obligatory criteria for the diagnosis of IND required by Borchard *et al.*[7], eight were aged more than 4 weeks, and five were aged more than 2 years at biopsy. An increase in AChE-positive nerve fibres around submucosal blood vessels was also seen in biopsies with average frequency of ganglia (nine patients), three of whom also showed lamina proprial increase in positive nerve fibres. Prominent nerve fibres were also seen around submucosal blood vessels in two patients with infrequent ganglia. Giant ganglia coexisted with increased fibres around submucosal blood vessels in two patients.

DISCUSSION

This study confirms that at least some of the histochemical criteria other authors have used to diagnose IND can be identified in suction rectal biopsy specimens we have previously reported as 'normal' from children with suspected pseudo-obstruction. One or more of these changes were seen in 60 (71%) of our 85 patients. However, the two obligatory criteria of Borchard *et al.*[7] were satisfied in only nine (11%). In addition, neurons in the lamina propria (neuronal heterotopia) - a feature which, together with an obvious increase in vertical AChE-positive fibres in the lamina propria, we have hitherto regarded as important for the diagnosis of IND - were absent from all the biopsies studied.

In our previous experience we have diagnosed IND in only seven patients, all of whom had concomitant HD. Whilst these patients had undoubted persistent intestinal motor dysfunction even after surgical resection of the aganglionic segment, the histochemical abnormalities identified as IND were considerably more marked than those accepted by Borchard *et al.*[7], and in particular they had a marked diffuse increase in AChE-positive fibres in the lamina propria. The association between IND and aganglionosis is well recognized[9,10] and IND is also identified as an isolated entity independent of HD[1-7]. Whilst in many centres the reported incidence of IND is much higher than at Great Ormond Street, rising to as high as 80% in association with aganglionosis[10] it is agreed that most patients improve with conservative management[2-5,8,9,11-15] and that this improvement may be accompanied by reversion of the histochemical changes to normality[13,14].

The question therefore arises whether the changes identified as IND really depict a clinicopathological entity or whether they merely form part of the spectrum of normality. This problem is complicated by the complete lack, for obvious ethical reasons, of good, age-matched control biopsy material from children with normal intestinal function. It is of great interest, however, with regard to one important criterion for IND, namely hyperplasia of the submucosal plexus, that in this series 'frequent' ganglia were seen significantly more often in younger children aged less than 4 weeks at biopsy, and so could merely represent an age-related phenomenon.

It is true, nevertheless, that our previous dismissal of minor differences in the histochemical pattern observed in rectal suction biopsies may relate to the tertiary referral practice at our hospital. Detailed follow-up of our patients, except those with established HD, tends to be performed at the referring hospitals, which might blunt our awareness of residual problems of intestinal motility in those patients in whom aganglionosis was excluded. It is for this reason that we will now conduct a careful follow-up of all the patients described here, to establish any clinical significance of our findings.

Acknowledgements

I express my thanks to Professors R. A. Risdon and B. D. Lake for their encouragement and constructive criticism during the course of this study, and for their valued help in preparing this manuscript.

References

1. Meier-Ruge W. Uber ein Erkrankungsbild des Colon mit Hirschsprung-Syptomatik. Verh Dtsch Ges Pathol. 1971;55:506-10.
2. Scharli AF. Neuronal intestinal dysplasia. J Pediatr Surg Int. 1992;7:2-7.
3. Meier-Ruge W. Angeborene Dysganglionosen des Colon. Der kinderarzt. 1985;16:151-64.
4. Scharli AF, Meier-Ruge W. Localized and disseminated forms of neuronal intestinal dysplasia mimicking Hirschsprung's disease. J Pediatr Surg. 1981;16:164-70.
5. Fadda B, Maier WA, Meier-Ruge W, Scharli AF, Daum R. Neuronale intestinale Dysplasie. Eine kritische 10-Jahres-Analyse klinischer und bioptischer Diagnostik. Z Kinderchir. 1983;38:305-11.

6. Scharli AF, Meier-Ruge W. Hirschsprung's-related disorders of intestine. Proceedings of plenary session on hundred years of Hirschsprung's disease (1886-1986) 8th Congress, Asian Association of Paediatric Surgeons; 1986:29-35.
7. Borchard F, Meier-Ruge W, Wiebecke B, Briner J, Munterfering H, Fodish HF, Holschneider AM, Schmidt A, Enck P, Stolte M. Innervationsstorungen des Dicksarmes - Klassifikation und Diagnostik. Patologe 1991;12:171-4.
8. Schofield DE, Yunis EJ. Intestinal neuronal dysplasia. J Pediatr Gastroenterol Nutr. 1991;12:182-9.
9. Fadda B, Pistor G, Meier-Ruge W, Hofmann-von Kapp-herr S, Muntefering H, Espinoza R. Symptoms, diagnosis and therapy of neuronal intestinal dysplasia masked by Hirschsprung's disease. J Pediatr Surg Int. 1987;2:76-80.
10. Briner J, Oswald HW, Hirsig J. Neuronal intestinal dysplasia - clinical and histochemical findings and its association with Hirschsprung's disease. Z Kinderchir. 1986;41:282-6.
11. Sacher P, Briner J, Stauffer UG. Unusual cases of neuronal intestinal dysplasia. Pediatr Surg Int. 1991;6:225-6.
12. Munakata K, Morita K, Okabe I, Sueoka H. Clinical and histologic studies of neuronal intestinal dysplasia. J Pediatr Surg. 1985;20:231-5.
13. Rintala R, Rapola J, Louhimo I. Neuronal intestinal dysplasia. Prog Pediatr Surg. 1989;24:186-92.
14. Simpser E, Kahn E, Kenigsberg K, Duffy L, Markowitz J, Daum F. Neuronal intestinal dysplasia; quantitative diagnostic criteria and clinical management. J Pediatr Gastroenterol Nutr. 1991;12:61-4.
15. Pistor G. Functional colonic ultrasonography; Normal findings of colonic motility and follow-up in neuronal intestinal dysplasia. Prog Pediatr Surg. 1989;24:155-64.
16. Lake BD. The diagnosis of Hirschsprung's disease and pseudo-obstruction. In: Histochemistry in Pathology. Filipe MI, Lake BD, editors. New York: Churchill Livingstone; 1990;211-20.

FALK SYMPOSIUM 65

Paediatric Gastroenterology: Inflammatory Bowel Diseases and Morbus Hirschsprung

EDITED BY

F. Hadziselimovic and **B. Herzog**
Basler Kinderspital
Römergasse 8
CH-4058 Basel
Switzerland

Proceedings of the 65th Falk Symposium
(II. International Falk Symposium on Pediatric and Surgical Gastroenterology)
held in Basel, Switzerland, April 10 and 11, 1992

KLUWER ACADEMIC PUBLISHERS
DORDRECHT / BOSTON / LONDON

INTESTINAL NEURONAL DENSITY IN CHILDHOOD: A Baseline for the Objective Assessment of Hypo- and Hyperganglionosis

Virpi V. Smith, FIMLS □ Department of Histopathology, Hospitals for Sick Children, Great Ormond Street, London WC1N 3JH, UK

□ *In the absence of reliable baseline data for normal neuron density in the intestine, the diagnosis of hypo- and hyperganglionosis is purely subjective. This study has established the normal neuron density by neuron counts in paraffin sections taken both transversely (transverse sections, TS) and longitudinally (longitudinally sections, LS) in relation to the long axis of normal postmortem jejunum, ileum, and colon from 21 children (aged 4 weeks to 10 years). Intestine from two adults (aged 16 and 42 years) and colon alone from a further six adults (aged 16 to 83 years) were also studied. The mean density values in childhood were for jejunum 3.6/mm (TS), 3.7/mm (LS); for ileum 4.3/mm (TS, LS); and for colon 7/mm (LS), 7.7/mm (TS). The proximal margins of surgically resected colons from six patients with Hirschsprung's disease and one patient with suspected isolated hypoganglionosis were also analyzed and the neuron densities compared with the established postmortem data. Neuron density values outside two standard deviations from the postmortem mean were shown to correlate with continuing pseudo-obstructive symptoms in these patients.*

KEY WORDS: hypoganglionosis, hyperganglionosis, myenteric plexus, neuron density.

INTRODUCTION

Functional intestinal obstruction (pseudo-obstruction) in the absence of anatomical luminal occlusion is a result of defective gut motility, the causes of which can be broadly categorized as neurogenic or myopathic in origin (1). Of the neurogenic types, aganglionosis (Hirschsprung's disease) is the

I thank Professors R. Anthony Risdon and Brian D. Lake for their help and constructive comments in preparation of this article.

This work was funded in part by the Joint Research Board of the Hospitals for Sick Children and the Institute of Child Health.

This work was presented in part at the Annual Histochemistry Meeting of the Royal Microscopical Society January 1989. (Proc Royal Mic Soc 1989;24:A51).

0277-0938/93 $10.00 + .00

most commonly recognized. However, intestinal pseudo-obstruction may also occur in association with a paucity of ganglion cells in the myenteric plexus (so-called hypoganglionosis) and with intestinal neuronal dysplasia, a condition in which one of the morphological characteristics is an excess of intestinal neurons (hyperganglionosis) (2).

From a practical standpoint, aganglionosis is easily recognized, but the diagnosis of hyper- and hypoganglionosis is hampered by the lack of adequate studies of normal intestinal neuron densities and the wide range of results recorded where such measurements have been made (3–9). Furthermore, both hyper- and hypoganglionosis may affect segments of intestine (generally the colon) proximal to the distal aganglionic segment in Hirschsprung's disease (10–12). A short hypoganglionic segment is almost universal in this disease and is referred to as the "transitional zone," but occasionally the hypoganglionic segment may be more extensive. Whether this is regarded as hypoganglionosis in association with Hirschsprung's disease or as an abnormally long transitional zone is a matter of semantics. The practical point is that either an extensive hypoganglionic or hyperganglionic segment proximal to the aganglionic segment in Hirschsprung's disease may produce symptoms of disturbed intestinal motility that persist after a corrective pull-through operation with removal of the aganglionic segment (10–12).

This paper addresses the problem of recognizing cases of hyper- and hypoganglionosis concomitent with Hirschsprung's disease, defining normal neuron densities against which significant variations in neuron counts in the proximal resection margins of pull-through specimens can be identified. The surgical material usually available consists of formalin-fixed, paraffin-embedded blocks of colon, and for this reason hematoxylin and eosin-stained sections of such specimens were used in the present study.

Sections of colon obtained at postmortem examination from patients who died of non-gut-related diseases were examined, and in some instances the small intestine was also studied. Neuron counts were confined to the myenteric plexus because of difficulties in standardizing counts in the submucosal plexus and the fact that the latter were considerably more time-consuming, making them of limited practical value. Furthermore, hypoganglionosis appears to be confined to the myenteric plexus (12, 13).

Neuron counts were made in sections of blocks taken both transversely (TS) and longitudinally (LS) in relation to the long axis of the intestine. In addition, counts were made not only in children (the usual age group in which the diagnoses of hyper- and hypoganglionosis are made) but also in adults to investigate any effects of bowel lengthening with age on the neuron densities in the longitudinal sections and any other possible age-related influences.

MATERIALS AND METHODS

Materials

Controls. Sleeves of colon approximately 10 cm long, the distal margins of which were 2 cm proximal to the rectosigmoid junction, were removed at postmortem examination from patients who died of non-gut-related diseases (congenital heart disease, sudden infant death syndrome, pneumonia, metabolic liver disease, status asthmaticus, myocardial infarction, and road traffic accidents). These patients comprised 21 children ranging in age between 4 weeks and 10 years (group 1) and 8 adults aged between 16 and 83 years (group 2). Delay between death and necropsy ranged between 6 and 48 hours (median 24 hours).

Sleeves of small intestine of similar length, one taken from the jejunum with an upper limit 2 cm distal to the duodenojejunal flexure and one from the ileum with a lower limit 2 cm proximal to the ileocecal valve, were also taken from all the children in group 1 and two of the adults in group 2. For each specimen the bowel was opened, washed with saline, and fixed in 10% phosphate-buffered formalin. The circumference was measured and both longitudinal and transverse blocks measuring 0.5–2.8 cm were taken from the proximal jejunum, distal ileum, and distal descending colon and were processed routinely and embedded in paraffin wax. Sections (3 μm) were cut and stained by hematoxylin and eosin (H&E), omitting the differentiation step of acid alcohol after Harris's hematoxylin.

Surgical Tissue. Neurons were counted in longitudinal blocks from the proximal resection margins in four pull-through specimens from patients with Hirschsprung's disease. Neuron density was also analyzed in longitudinal sections of colon, from a patient in whom a subjective impression of isolated hypoganglionosis (small, sparse ganglia in the myenteric plexus) was noted. Two other patients had persistent pseudo-obstruction following pull-through operations for Hirschspring's disease with removal of the aganglionic segment. Second colonic resections showed evidence of intestinal neuronal dysplasia proximal to the aganglionosis diagnosed on adjacent blocks of frozen tissue that showed increased acetylcholinesterase-positive nerve fibers and heterotopic neurons in the lamina propria, with frequent, often large enteric ganglia in both myenteric and submucosal plexuses. All the above patients were children between 8 months and 10 years of age.

Methods

In both longitudinal and transverse sections of each specimen, the total number of neurons was counted in every tenth section from 60 serial sections

cut, and the density was expressed as the mean number of neurons per millimeter. The section length was measured with a calibrated eyepiece graticule. In a preliminary study using serial sections, counts of neurons identified by the H&E stain were compared with those obtained by analyzing sections in which neurons were demonstrated by immunohistochemical methods. A monoclonal antibody to neurofilament protein 200 kd (Amersham International plc, Aylesbury, UK) and polyclonal antibodies to protein gene product 9.5 (Ultraclone Ltd., Cambridge, UK) and neuron-specific enolase (Dako Ltd., High Wycombe, UK) were used. The standard avidin-biotin-peroxidase complex (ABC) method with diaminobenzidine (DAB) was employed (14). Biotinylated antimouse and antirabbit antibodies and the ABC complex were obtained from Dako (UK).

Neurons were recognized in H&E-stained sections by their large nucleus with its prominent nucleolus surrounded by the typical, ample, granular, amphoteric neuronal cytoplasm and were counted even when only part of the cell was included in the section (Fig. 1). The maximum diameter of neurons was also measured using a calibrated eyepiece graticule.

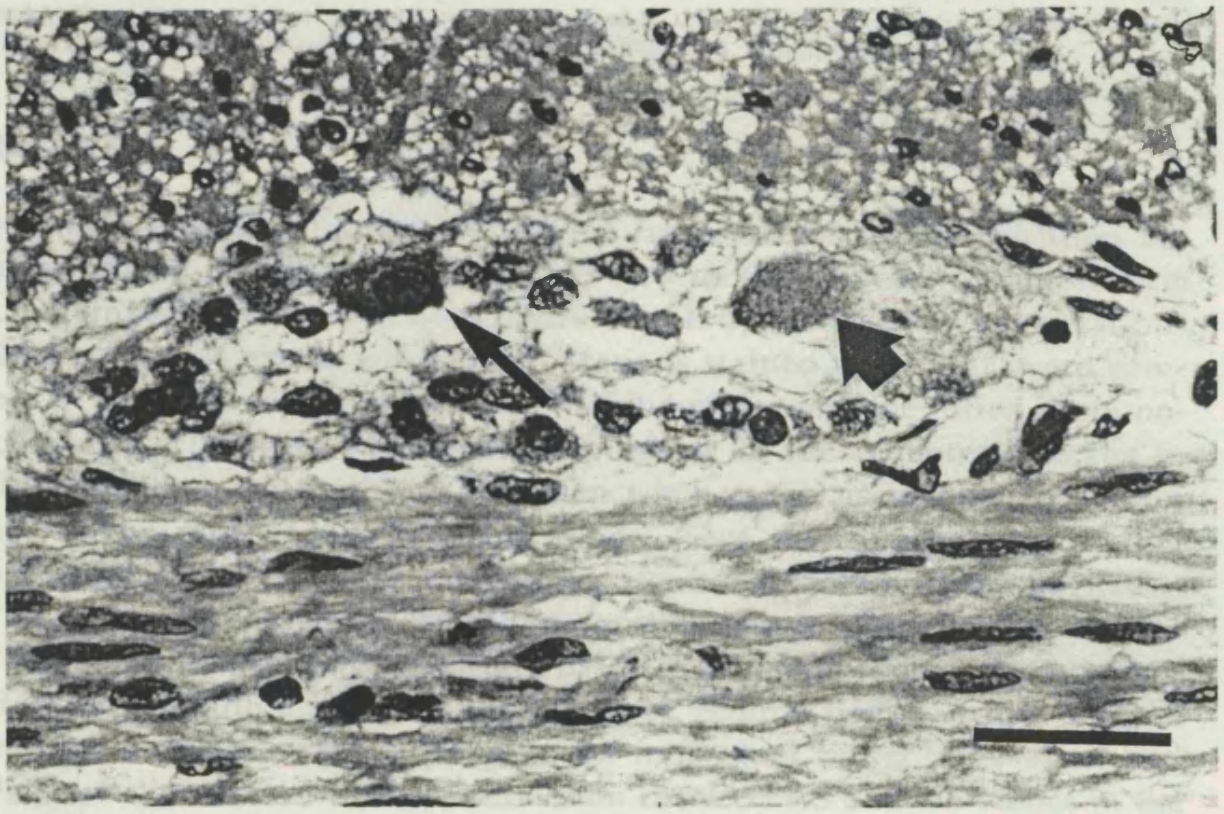


FIGURE 1. A section of jejunum showing the myenteric plexus containing one neuron with a nucleus and nucleolus (thin arrow) and a second neuron in which only cytoplasm is seen (thick arrow). Both appearances are included in the assessment of neuron density. The remaining cells comprise supporting cells easily identified by the absence of granular, amphoteric cytoplasm and/or the presence of compact nuclei. Scale mark 30 μm.

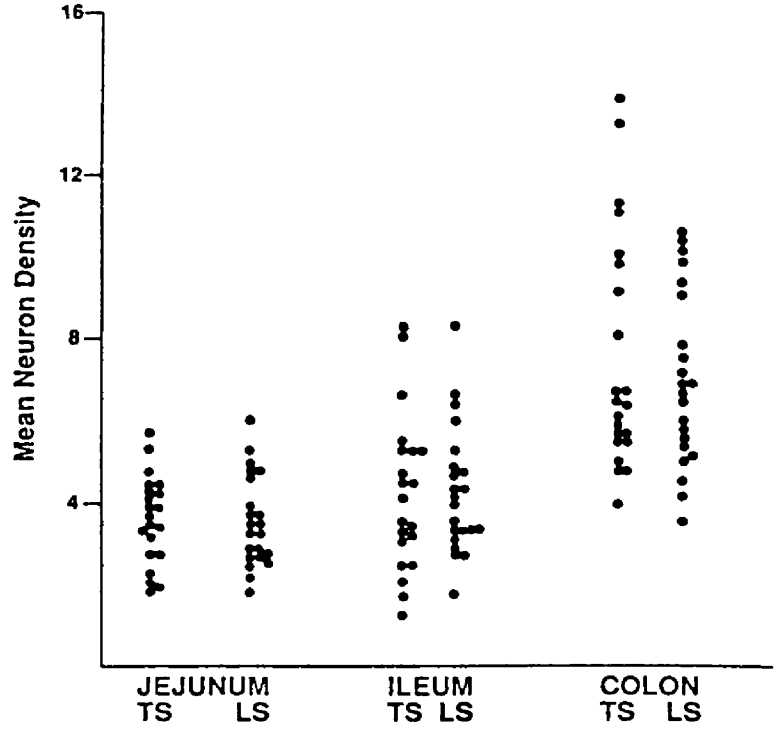


FIGURE 2. Neuron density in postmortem jejunum, ileum, and colon in childhood (4 weeks to 10 years) expressed as the mean number of neurons per mm counted at six levels 30 μm apart in a particular tissue block. Each dot represents one patient. TS, transverse sections; LS, longitudinal sections.

RESULTS

Supporting cells in the ganglia caused no problems in identification because the sparse cytoplasm of these cells was never granular or amphoteric and the compact nuclei were not confused with open vesicular neuronal nuclei. Moreover, the counts obtained in sections stained with H&E were comparable to those made on sections in which neurons were identified immunohistochemically. Neurons remained recognizable in the myenteric plexus in paraffin sections stained with H&E, even when autolysis had caused severe damage to the mucosa. Comparable counts were also obtained in cryostat sections of frozen tissue in the few samples examined, reflecting the negligible effect of fixation and paraffin processing on the neuron density.

The neuron density in postmortem tissue (Fig. 2) shows that the density is lowest in the jejunum and highest in the colon. Similar density values in both longitudinal and transverse sections in an individual bowel were seen, and Fig. 3 shows that there is a close correlation between LS and TS values in the same specimen ($y = 0.25 + 1.04x$; $r = .8$; $p > .001$). For group 1 the mean neuron density in the colon was 7.7/mm (TS), 7/mm (LS) and in the small intestine about 4/mm (ileum 4.3/mm in both TS and LS; jejunum 3.6/mm in TS and 3.7/mm in LS). In this group no significant variation could be shown in neuron density with age (Fig. 4). For group 2 the mean colonic neuron

density was 5.1/mm, perhaps indicating a slight fall in old age, although the numbers of patients were small and the result was not statistically significant (see Fig. 5).

The neuron density in the surgically resected colons is shown in Fig. 6. Neuron counts in the proximal blocks from two patients with Hirschsprung's disease (A and B) fell within two standard deviations from the control mean, but two others were outside this range, one with elevated density (C) and the other below this range (G), indicating evidence of hyper- and hypoganglionosis respectively in these two patients. Similarly, values were just at the level of two standard deviations below the control mean in patient F with a subjective impression of hypoganglionosis and well above two standard deviations in the two patients (D and E) in whom a diagnosis of intestinal neuronal dysplasia was established by histochemical criteria.

In children most neurons were about 20–23 μm in diameter with occasional nerve cells measuring 30 μm. Most of the neurons in the very young children were small (8–15 μm) and only occasional neurons were larger. In the

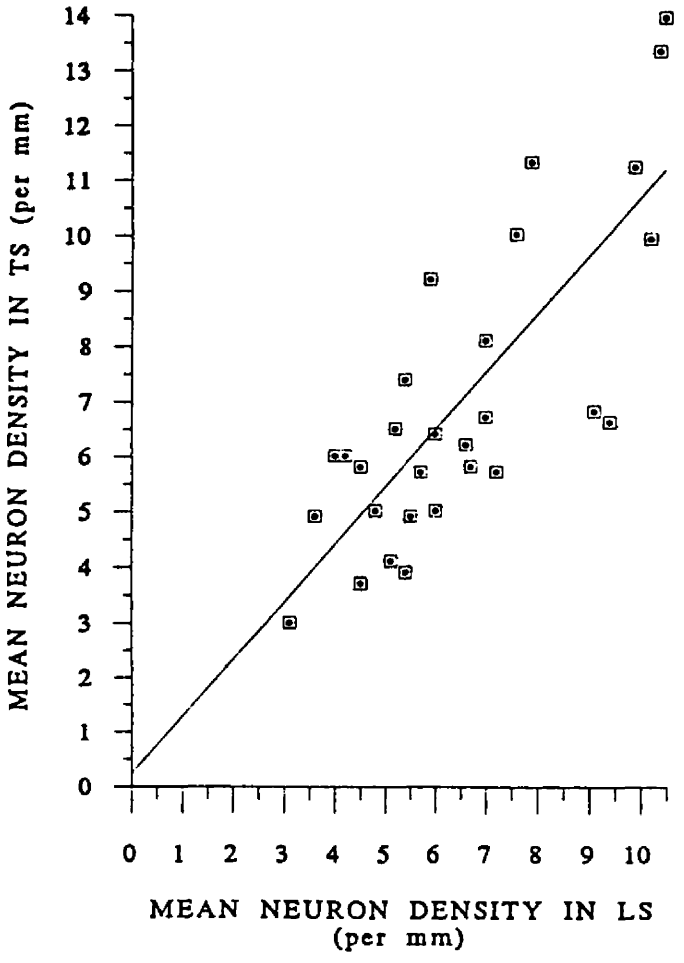


FIGURE 3. Neuron density (number of neurons per mm) compared in transverse sections and longitudinal sections from the same specimens of normal postmortem colon. There is a close linear relationship and significant correlation between the TS and LS counts. $y = 0.25 + 1.04x$; $r = .8$; $p < .001$.

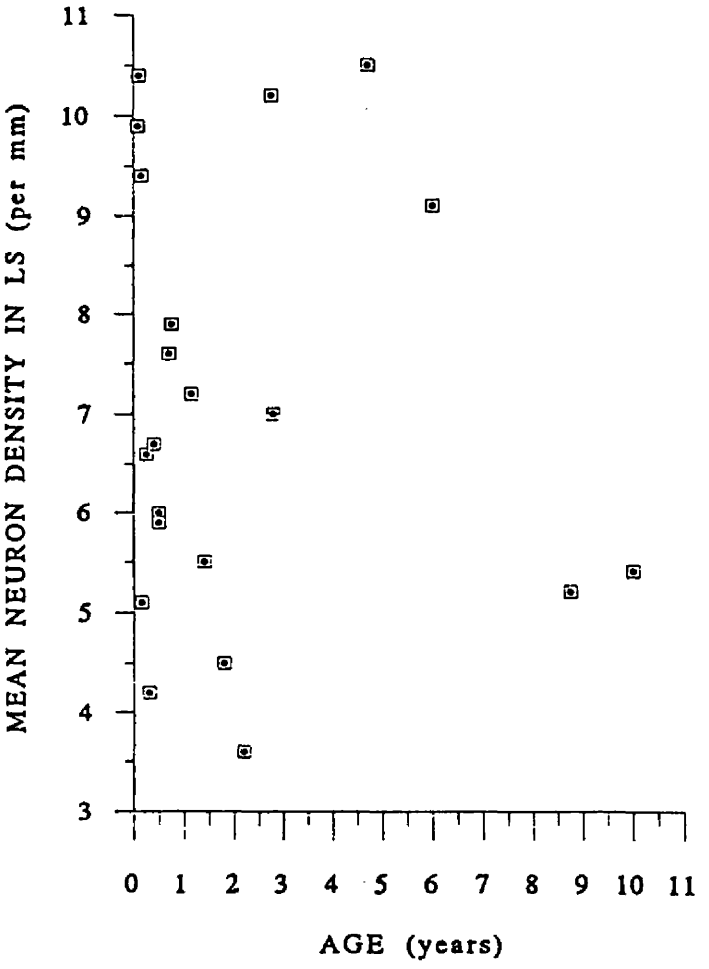


FIGURE 4. Neuron density, in LS of postmortem colon, plotted against age (4 weeks to 10 years).

adults, however, the largest nerve cell measured 40 μm in diameter and these larger cells constituted a considerable proportion of the neuron population.

In children the circumference of the postmortem colon ranged from 2 to 6 cm (mean 3.5 cm), of ileum 1 to 4 cm (mean 2 cm) and of jejunum 1.5 to 5 cm (mean 2.5 cm). In the fixed adult colon the circumference measured 3.5–7 cm (mean 5.5 cm). The circumferences of surgical specimens measured 3.5 to 5.5 cm.

DISCUSSION

Previous assessments of neuron densities in the myenteric plexus, mainly in adult intestine using various methods, have resulted in a wide range of normal values (Table 1). No consistent study using a standardized procedure has been employed in children.

The results presented here are 20 times higher than those of Schuffler and colleagues (4, 5). In both their studies and this one a seemingly similar approach was employed—counting each neuron only once using sections at ap-

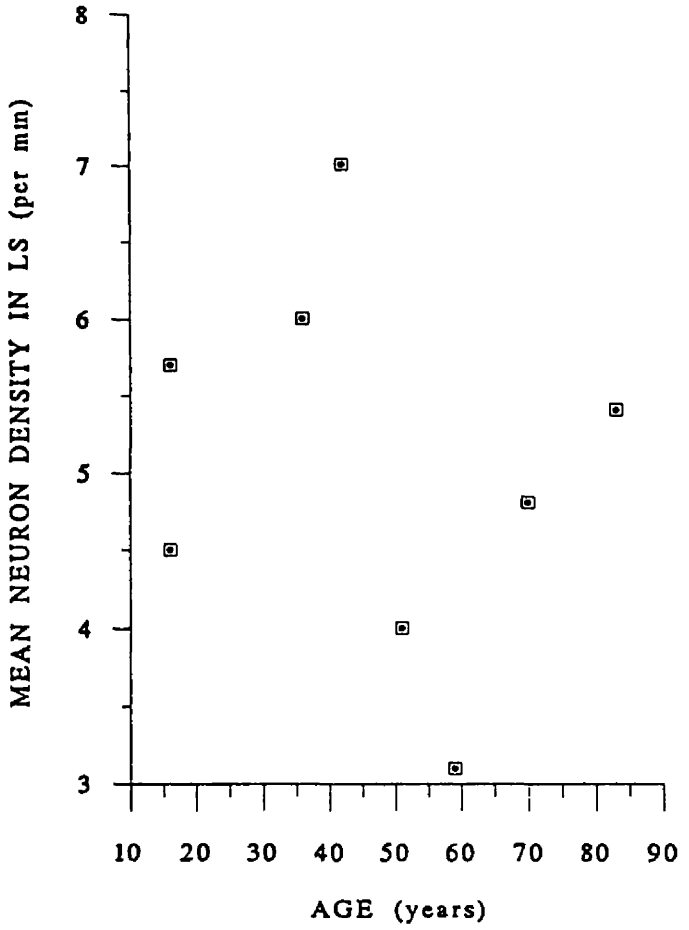


FIGURE 5. Neuron density in LS of normal postmortem colon plotted against age (16-83 years).

propriately spaced levels in paraffin blocks. Schuffler et al. (4, 5) counted only nucleated neurons, whereas in this study all neurons were counted regardless of the presence or absence of nuclei, identifying some by their characteristic cytoplasmic appearances alone. Different criteria for inclusion of neurons may in part explain the discrepancies. However, it is unlikely that these considerations would entirely explain such large differences. As the intestinal wall in children is thin, in this study blocks of colon mostly included the taenia. This may also contribute to the higher density values seen here, because neurons are more numerous in the myenteric plexus adjacent to taenia than elsewhere (M.D. Schuffler, 1988, personal communication).

At the other end of the scale, Meier-Ruge and colleagues (8) recorded neuron densities 10 times those encountered in this study and approximately 200 times those recorded by Schuffler et al. (4, 5). This is partly explained by the section thickness. The greater density recorded by Meier-Ruge et al. (8) is partly the result of using sections three times thicker than ours. However, it is unlikely that this could explain a 10-fold difference. Meier-Ruge et al. studied exclusively bowel resected for Hirschsprung's disease and some patients may have had intestinal neuronal dysplasia (hyperganglionosis) proximal to the

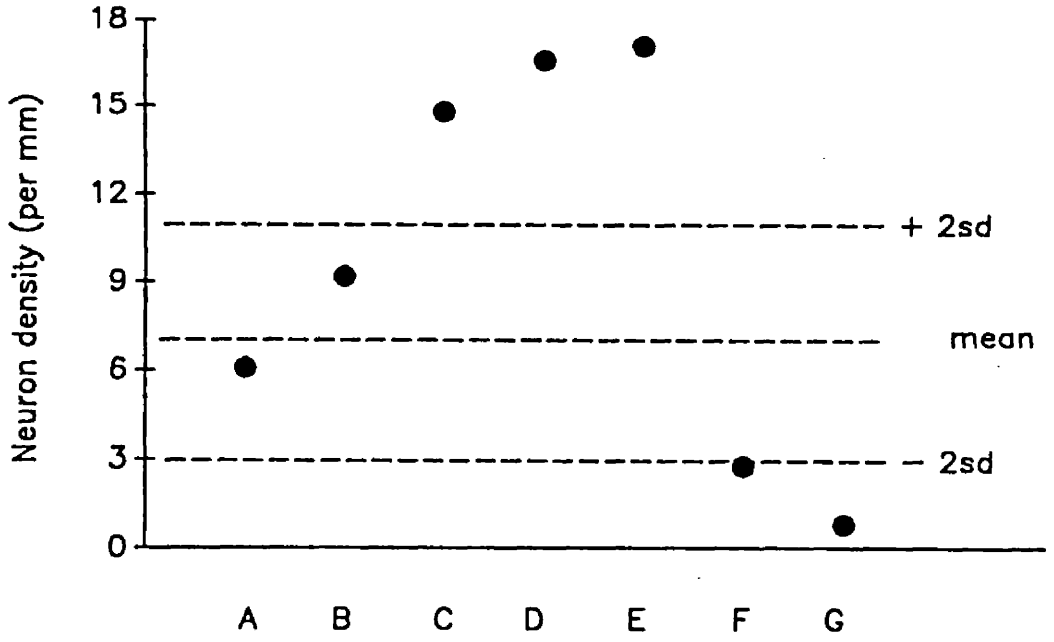


FIGURE 6. Neuron density in the most proximal block of the surgically resected colon from seven patients. Patients A, B, C, and G had Hirschsprung's disease and counts were made on the initial pull-through specimen. Patient C was later shown to have concomitent intestinal neuronal dysplasia. Patient G was regarded as having an unusually long hypoganglionic segment without glial cell proliferation. Patients D and E had persistent pseudo-obstructive symptoms following an initial pull-through operation for Hirschsprung's disease. Neuron density is that in a second pull-through intestine. On adjacent blocks of frozen tissue the diagnosis of intestinal neuronal dysplasia was made. Patient F had intestinal pseudo-obstruction and was observed to have microscopic features of isolated hypoganglionosis. Horizontal lines represent the mean control neuron density for colon in longitudinal sections in children: 7/mm ± 2 SD.

aganglionic segment, inflating the density recorded (W. Meier-Ruge, 1988, personal communication).

This study has looked systematically at neuron densities from specified sites in the normal intestine of children (aged 4 weeks to 10 years) and in eight adults (aged 16 to 83 years) by methods accessible to any histopathology laboratory. In contrast, other workers have analyzed adult intestinal neuron density alone (4, 5) or density in intestine from children with Hirschsprung's disease (8). In this study mean density values were highest for colon (TS 7.7/

TABLE 1. Comparison of Neuron Density in Different Studies of Normal Human Colon

Study	Density	Number of controls
Schuffler et al., 1978	27.8/100 mm	7
Schuffler and Jonak, 1982	42.9/100 mm	20
Meier-Ruge et al., 1970	756/10 mm	?
Ikeda et al., 1988	>30/10 mm	?
This study, 1992	7/mm	29

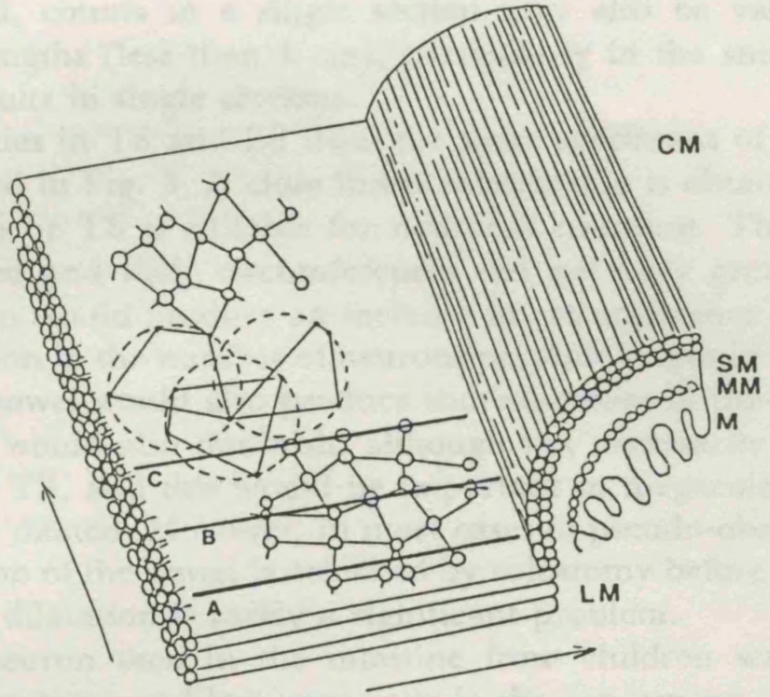


FIGURE 7. Diagrammatic representation of the myenteric plexus showing the concept of Dirichlet domains (circles). Random sections in planes indicated by lines A–C will miss some of the ganglia (B), all of the ganglia (A), or none of the ganglia (C) if short lengths of bowel are analyzed. LM, longitudinal muscle; CM, circular muscle; SM, submucosa; MM, muscularis mucosae; M, mucosa.

mm, LS 7/mm) and lowest for jejunum (3.6/mm TS and 3.7/mm LS), in keeping with observations of neural tissue volumes (15).

Routine H&E and immunohistochemical methods all allowed the identification of neurons. Almost identical density values were seen on serial sections in which different neuronal demonstration methods were employed. The H&E stain was preferred for its quickness and ease compared with the more cumbersome immunohistochemical methods.

The myenteric plexus is a three-dimensional network explained by organized spatial structures best fitting the concept of Dirichlet domains (15, 16). This network (see Fig. 7) consists of a set of polygons outlined by nerve trunks with ganglia at the apices of the polygons. Ganglia are spaced 1–5 mm apart in the colon (17) and farther apart in the small intestine (15). Any one section, particularly of a short length of intestine, may miss some or all ganglia (see Fig. 7). By analyzing at six levels in longitudinal or transverse blocks of intestine, the inclusion of a representative population of neurons is ensured, resulting in reliable and reproducible values of neuron density. To avoid counting each neuron more than once, every tenth section was chosen. This was based on the observation that the neuronal diameter in children never exceeded 30 μm.

The counts on paraffin sections are reliable. Provided that a sufficient

length is analyzed, counts in a single section may also be valid (Fig. 7). However, short lengths (less than 1 cm), particularly in the small intestine, may give false results in single sections.

Neuron densities in TS and LS from the same specimens of postmortem colon are compared in Fig. 3. A close linear relationship is obtained, indicating that either LS or TS is suitable for neuronal counting. The specimens were not distended and their circumferences did not vary greatly. Clearly, intestinal dilatation would produce an increase in circumference and a corresponding diminution in the number of neurons per unit length in TS. Because distention of the bowel would also produce some increase in the length, neuron counts in LS would also diminish, although not necessarily to the same extent as those in TS, and this would be important in megacolon, when the intestine is hugely dilated. However, in most cases of pseudo-obstruction surgical decompression of the bowel is achieved by colostomy before bowel resection, so intestinal dilatation is rarely a significant problem.

The largest neuron seen in the intestine from children was 30 μm in diameter and many measured less, especially in the very young. In the adults studied here a greater proportion of large neurons was seen, some measuring 40 μm in diameter. There are no reports, other than in the fetus (18), on neuron size in the human gut in relation to age, although growth in neuron volume has been shown in the monkey brain from infancy to adulthood (19). Observations of experimental animals by Gabella (20) also confirm that the average neuron size is significantly smaller in the newborn intestine than in the adult bowel, the difference being more marked in larger animals such as sheep and cattle than in smaller species such as the mouse and shrew.

Proximal blocks from resected intestine from two patients (Fig. 6, A and B) with Hirschsprung's disease showed density values within two standard deviations from the established postmortem mean; both of these patients appeared to be cured by the bowel resections. In contrast, of the patients with residual problems of intestinal dysmotility after pull-through operations, all were found to have density values outside two standard deviations from the control mean. Two of these patients (Fig. 6, D and E) had intestinal neuronal dysplasia (hyperganglionosis) diagnosed on the basis of other criteria, i.e., increased acetylcholinesterase-positive nerve fibers in the lamina propria, large ganglia in both submucosal and myenteric plexuses, and heterotopic neurons in the lamina propria. Moreover, patient C, with a high density value, was later shown by examination of frozen tissue to have intestinal neuronal dysplasia (hyperganglionosis) in addition to Hirschspring's disease distally. Thus neuron counts may be helpful in confirming or refuting a subjective impression of hyperganglionosis when only paraffin-embedded tissue is available for analysis.

The diagnosis of hypoganglionosis is based on the presence of sparse small

ganglia with scant neurons. In patient F, with symptoms of intestinal dysmotility and microscopic features of hypoganglionosis without concomitent Hirschsprung's disease, the neuron density was found to be just at two standard deviations from the mean, so the subjective impression of a relative paucity of neurons was supported but could not be diagnosed objectively as hypoganglionosis. In patient G, who had Hirschsprung's disease and appeared to have extensive hypoganglionosis without hypertrophied nerve trunks proximal to the aganglionic segment, however, a grossly reduced density value well below two standard deviations from the mean was seen.

The length of the intestine increases with increasing body length and, since it is assumed that neurons do not proliferate after birth, a reduction in neuron density would be expected with increasing age. However, this has been challenged by the following observations in animals. In the rat and chick there is an increase in neurons in the myenteric plexus during postnatal life, and tritiated thymidine uptake by neurons in the mouse duodenum can be seen at least 2 weeks after birth (20). At birth the human gut measures on average about 4 m (21) and in the adult about 7.5 m (21). The growth of the intestine is most rapid in the early years of childhood (21), and by 10 years of age both the small gut and colon have reached the adult lengths. Despite expectations, based on the reported higher neuron packing density in immature animals compared with adult animals (20), no obvious reduction in neuron density was encountered in this study in infants and children up to the age of 10 years, when the length of the child's intestine has reached the adult dimensions. In addition, no obvious reduction in neuron density was seen in the young adults, although in old age a slightly lower density was encountered (Fig. 5).

To explain this apparent discrepancy, it is possible that not all neurons were identified in the very young due to the presence of small nerve cells, whereas in the older children and adults these cells were more readily recognized. As the adult neurons are larger, it may also be possible that the same cell was counted twice. However, this would not be true in the intestine of the older children, where the largest neuron measured 30 μm. The small number of patients examined may also influence the results, as in all ages there are length variations in the gut (21). However, the possibility of neuron proliferation in childhood cannot be ruled out and warrants more extensive study.

REFERENCES

1. Christensen J, Dent J, Malagelada J-R, Wingate DL. Pseudo-obstruction. Gastroenterol Int 1990;3:107-19.
2. Garrett JR, Howard ER. Myenteric plexus of the hind gut: Developmental abnormalities in humans and experimental studies. In: Development of the Autonomic Nervous System (Ciba Foundation Symposium 83). London: Pitman Medical, 1981;326-54.

3. Munakata K, Okabe I, Morita K. Histologic studies of rectocolic aganglionosis and allied diseases. J Pediatr Surg 1978;13:67-75.
4. Schuffler MD, Bird TD, Sumi SM, Cook A. A familial neuronal disease presenting as intestinal pseudo-obstruction. Gastroenterology 1978;75:889-98.
5. Schuffler MD, Jonak Z. Chronic idiopathic intestinal pseudo-obstruction caused by a degenerative disorder of the myenteric plexus: The use of Smith's method to define the neuropathology. Gastroenterology 1982;82:476-86.
6. Feinstat T, Tesluk H, Schuffler MD, et al. Megacolon and neurofibromatosis: A neuronal intestinal dysplasia. A case report and review of the literature. Gastroenterology 1984;86:1573-79.
7. Krishnamurthy S, Schuffler MD, Rohrmann CA, Pope CE. Severe idiopathic constipation is associated with distinctive abnormality of the colonic myenteric plexus. Gastroenterology 1985;88:26-34.
8. Meier-Ruge W, Morger R, Rehbein F. The hypoganglionated megacolon as accompanying disease of Hirschsprung's disease. Z Kinderchir 1970;8:254-64 (in German).
9. Ikeda K, Goto S, Nagasaki A, Taguchi T. Hypogenesis of intestinal ganglion cells: A rare cause of intestinal obstruction simulating aganglionosis. Z Kinderchir 1988;43:52-3.
10. Fadda B, Pistor G, Meier-Ruge W, Hofmann-von Kapp-herr S, Muntefering H, Espinoza R. Symptoms, diagnosis and therapy of neuronal intestinal dysplasia masked by Hirschsprung's disease. J Pediatr Surg Int 1987;2:76-80.
11. Briner J, Oswald HW, Hirsig J. Neuronal intestinal dysplasia—clinical and histochemical findings and its association with Hirschsprung's disease. Z. Kinderchir 1986;41:282-6.
12. Borchard F, Meier-Ruge W, Wiebecke B, et al. Disorders of the innervation of the large intestine—classification and diagnosis: Results of a consensus conference of the Society of Gastroenterology 1 December 1990 in Frankfurt/Main (in German). Patologe 1991;12:171-4.
13. Scharli AF. Neuronal intestinal dysplasia. J Pediatr Surg Int 1992;7:2-7.
14. Hsu SM, Raine L, Fanger H. Use of avidin biotin peroxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 1981;29:577-80.
15. Wells TR, Landing BH, Ariel I, Nadorra R, Garcia C. Normal anatomy of the myenteric plexus of infants and children. Perspect Pediatr Pathol 1987;11:152-74.
16. Loeb A. Space Structures. Their Harmony and Counterpoint. Reading, MA: Addison-Wesley, 1976.
17. Bodian M, Stephens FD, Ward BCH. Hirschsprung's disease and idiopathic megacolon. Lancet 1949;1:6-15.
18. Vaos GC. Quantitative assessment of the stage of neuronal maturation in the developing human fetal gut—a new dimension in the pathogenesis of developmental anomalies of the myenteric plexus. J Pediatr Surg 1989;24:920-5.
19. Headon MP, Sloper JJ, Hiorns RW, Powell TPS. Sizes of neurons in the primate lateral geniculate nucleus during normal development. Dev Brain Res 1985;18:51-6.
20. Gabella G. Structure of muscles and nerves in the gastrointestinal tract. In: Johnson LR, ed. Physiology of the Gastrointestinal Tract. 2nd ed. New York: Raven Press, 1987;335-81.
21. Bryant J. Observations upon growth and length of the human intestine. Am J Med Sci 1924;167:499-520.

Received September 21, 1991
Accepted September 9, 1992

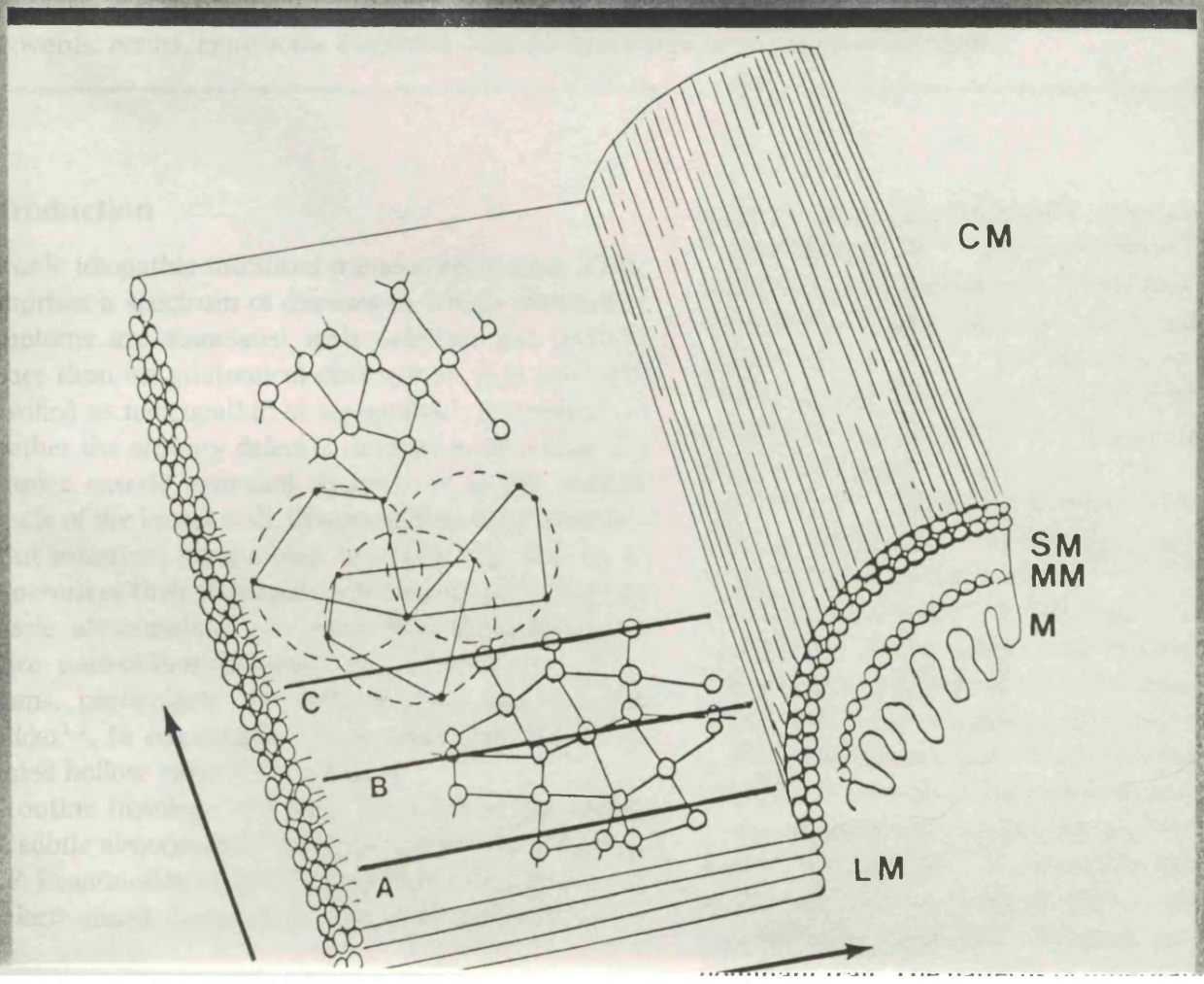
PPATDQ 13(2) 127–272, 1993
ISSN 0277-0938

Pediatric Pathology

Journal of the **Society for Pediatric Pathology**

Affiliated with the **International Paediatric Pathology Association**

Volume 13 Number 2 March–April 1993



Histopathology 1992, 21, 535–542

Intestinal pseudo-obstruction with deficient smooth muscle α-actin

V.V.SMITH, B.D.LAKE, M.A.KAMM* & R.J.NICHOLLS*
*Hospitals for Sick Children, Department of Histopathology, Great Ormond Street and *St. Mark's Hospital, City Road, London, UK*

Date of submission 15 May 1992
Accepted for publication 29 June 1992

SMITH V.V., LAKE B.D., KAMM M.A. & NICHOLLS R.J.
(1992) *Histopathology* 21, 535–542

Intestinal pseudo-obstruction with deficient smooth muscle α-actin

We describe a 48-year-old woman with chronic constipation since early childhood who has an intestinal myopathy associated with a hitherto undescribed absence by immunostaining of smooth muscle α-actin confined to the intestinal circular muscle. There were no abnormalities in other contractile proteins (myosin, tropomyosin, filamin, caldesmon or desmin) and despite the abnormality of a contractile protein isoform in the circular muscle, no significant morphological changes were identified by light microscopy or ultrastructural examination. A possible developmental mechanism for the observed change is proposed. The use of specific antibodies to isoforms of contractile proteins may have potential value in the study of intestinal myopathies.

Keywords: actins, contractile filaments, immunohistochemistry, intestinal myopathy

Introduction

Chronic idiopathic intestinal pseudo-obstruction (CIIP) comprises a spectrum of diseases in which obstructive symptoms are associated with defective gut motility rather than an anatomical obstruction. It is generally classified as neuropathic or myopathic[1], depending on whether the primary defect is thought to lie within the complex enteric neuronal circuitry or in the smooth muscle of the bowel wall. Comparatively little is known about intestinal myopathies, but they may well be as numerous as their skeletal muscle counterparts. Smooth muscle abnormalities are often seen throughout the entire gastrointestinal tract and may involve other organs, particularly the urinary tract and the gall bladder[1,2]. In consequence, these disorders have been termed hollow visceral myopathies.

Routine histology is seldom adequate in identifying the subtle abnormalities present in many patients with CIIP. Examination of tissue using other techniques, such as electronmicroscopy, silver staining and histochemical methods, is usually required[3,4]. Myopathies have been described in adults[5–17] and in children[18–24] and may be associated with fibrosis and vacuolation of the smooth muscle cells. The fibrosis, when gross, is detected by light microscopy[5,6,10,12,13,16], but the identification of milder fibrosis and smooth muscle vacuolation requires ultrastructural examination[21,24,25]. The myopathic changes may be more pronounced in the longitudinal[5,6,10,12,14,21,26] or in the circular muscle coat[3,23,27]. In some examples abnormalities are seen equally in both layers of the muscularis propria[9,11,13,16,22] and occasionally may even involve the muscularis mucosae[9]. Abnormalities of nerves within the circular muscle, best detected by neurofilament immunostaining, may also be associated with intestinal myopathies[28]. In some patients no abnormality can be detected to account for the CIIP in spite of extensive investigations.

Accumulation of sarcoplasmic glycogen has also been described[29] and rare intramyocytic inclusions[15] have been found in one kindred with a myopathy of the internal anal sphincter, inherited as an autosomal dominant trait. The patterns of inheritance in intestinal myopathies are varied and may be autosomal dominant[4,5–7,10] or autosomal recessive[4,9,12–14]; in addition

Address for correspondence: Dr V.V.Smith, Department of Histopathology, Hospital for Sick Children, Great Ormond Street, London WC1N 3JH, UK.

the absence of demonstrable male-to-male transmission in some kindreds raises the possibility of X-linked dominant forms[7,10]. Sporadic cases are also reported[4,26].

In this report a patient is described with a newly identified intestinal myopathy involving only the circular muscle coat, in which an abnormality of smooth muscle α-actin, not seen in any of the controls, was demonstrable immunohistochemically using an antibody specific for this isoactin.

Clinical history

A 48-year-old woman had been constipated since early childhood. At the age of 35 years she developed severe abdominal pain and was thought to have intestinal obstruction. However, no mechanical cause was revealed at laparotomy. Later in the same year, because of persistent pain and vomiting, she underwent cholecystectomy and truncal vagotomy. The post-operative course was complicated by intra-abdominal sepsis and 6 days later a distal ileostomy was fashioned but fluid output from the stoma was high and resulted in recurrent dehydration. Because of this the ileostomy was taken down 1 year later and the bowel was re-anastomosed.

Since then she continued to experience chronic abdominal pain and constipation with superimposed episodes of acute abdominal pain and distension, vomiting and absolute absence of bowel motions. Plain abdominal radiographs during these acute episodes have shown multiple loops of distended bowel. A barium meal and follow-through between episodes of severe pain showed delayed gastric emptying with food residue in the stomach more than 12 h after the last meal. The oesophagus was of normal size but the distal duodenum and most of the rest of the small intestine were slightly dilated. No mechanical obstruction was demonstrated and barium passed into the colon after 90 min. Her treatment included erythromycin, cisapride and cyclical courses of antibiotics including tetracyclines, and narcotic analgesia during acute exacerbations.

At the age of 48 years, she experienced an acute episode of severe abdominal pain and distension persisting for more than 2 weeks. A laparotomy revealed mild dilatation of the whole of the small intestine but no anatomical obstruction. A full thickness biopsy specimen was taken from the jejunum for histological examination. The patient made a slow post-operative recovery over the next 6 weeks, during which she required intravenous nutrition. Her current clinical condition is unchanged and she continues to suffer chronic abdominal pain with occasional acute exacerbations of her symptoms.

Materials and methods

As well as the biopsy specimen from the patient, a number of surgical and autopsy full-thickness intestinal tissue samples were examined for comparative purposes as described below.

1 *Patient.* The resected full-thickness specimen of jejunum was divided. One portion was fixed in 4% phosphate buffered formalin for routine histology, a second piece in 2.5% glutaraldehyde for electron-microscopy and further blocks were snap frozen in liquid nitrogen for histochemical studies.

2 *Surgical specimens.* Nine jejunal, seven ileal and 38 large intestinal biopsies were studied as described above. The surgical samples were from patients diagnosed on clinical, electrophysiological and histopathological criteria to have intestinal myopathies ($n=19$), intestinal neuropathies (28), and other conditions (7) including jejunal atresia, diverticular disease, Crohn's disease and three unclassified pseudo-obstructions. The ages of these patients ranged from 15 days to 39 years, 11 were between 10–16 years and two were adults (27 & 39 years).

3 *Autopsy specimens.* Nine samples, six from colon and three from jejunum, were also examined. These tissues were obtained from patients who died from non-gastrointestinal causes. The age of these patients ranged between 8 weeks and 33 months. One specimen was from a baby born at 28 weeks gestation who died at the age of 11 weeks.

HISTOPATHOLOGY

Sections of paraffin wax-embedded tissue were stained with haematoxylin and eosin (H & E). In addition, on frozen tissue, the amount of connective tissue was assessed using the Gomori trichrome and picrosirius stains. Acid phosphatase activity was studied to detect any lysosomal activity, and accumulation of glycogen was estimated by examining sections stained by the periodic acid-Schiff (PAS) reaction, with and without salivary amylase digestion, after celloidin protection. Acetylcholinesterase activity was also demonstrated in the frozen samples. For details of these methods see Filipe & Lake[30].

IMMUNOHISTOCHEMISTRY

Immunohistochemical studies on cold (−20°C) acetone-fixed cryostat sections of snap-frozen tissue using the avidin-biotin-peroxidase complex (ABC) method[31] were based on a panel of antibodies comprising neural and muscle markers. Biotinylated antimouse and anti-

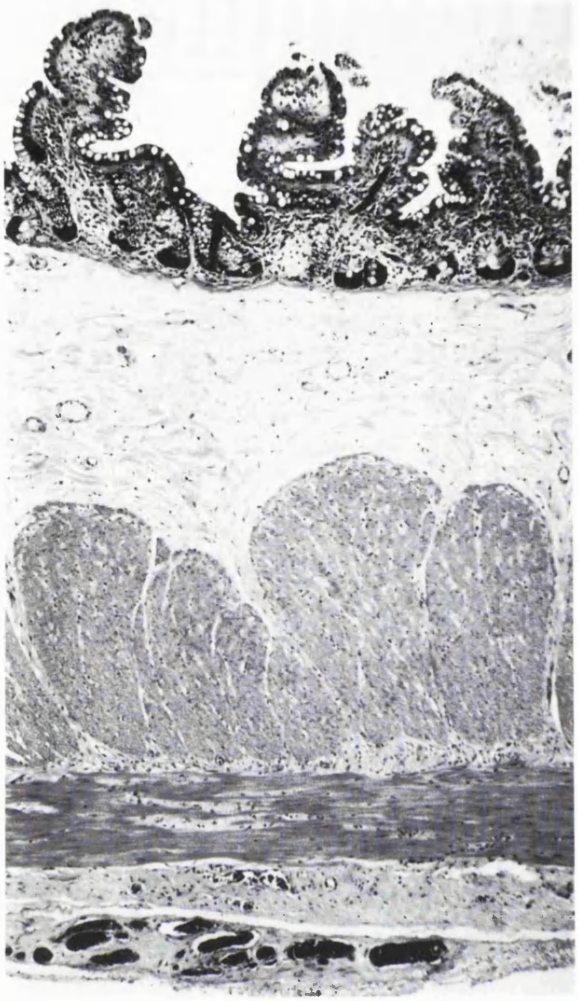


Figure 1. Routine H & E-stained paraffin section of the jejunal wall from the patient showing no histological abnormality. × 60.

rabbit antibodies and the ABC complex were obtained from Dako, High Wycombe, UK. The neural markers included monoclonal antibodies to phosphorylated and dephosphorylated neurofilaments (NN18, NE14, N52, NR4 from Sigma, Dorset, UK; BF10 from Boehringer Mannheim UK, Lewes, UK: SM132 from Sternberger Baltimore, Maryland, USA; RT97 from Professor Anderton, Institute of Psychiatry, London, UK). Other neural markers consisted of a monoclonal antibody to muscarinic receptors (M35 from Chemunex, Paris, France) and the neural cell adhesion molecule (Eric1 from Professor Walsh, Guy's Hospital, London, UK). The muscle markers included monoclonal antibodies to smooth muscle α-actin (1A4 from Sigma, Dorset, UK), smooth muscle α- and γ-actin (CGA7) and smooth, skeletal and cardiac muscle α- and γ-actin (HHF35) (both from Universal Biologicals Ltd, London, UK), to desmin clone DE-R-11 (Dako, High Wycombe, UK) and a polyclonal antibody to skeletal and smooth muscle myosin (Sigma, Dorset, UK). The muscle markers also included monoclonal antibodies to tropomyosin (TM311), filamin (FIL2) and caldesmon (CALD5), all from Sigma, Dorset, UK.

Controls: the neural markers acted as controls for muscle markers and vice versa. A negative control omitting the primary antibody was run for each specimen. Two 'irrelevant' antibodies were also applied, one a polyclonal antibody to α-feto-protein (Dako, High Wycombe, UK) and the other a monoclonal antibody to epithelial basement membrane protein clone GB3 (Seralab, Sussex, UK).

ELECTRONMICROSCOPY

Selected blocks from the glutaraldehyde-fixed tissue, were post-osmicated and processed into Araldite resin using standard methods. Ultrathin sections were cut to show the myenteric plexus and the circular and longitudinal muscle coats, and were contrasted with uranyl acetate and lead citrate before ultrastructural examination.

TISSUE COMPARISONS

Information is scanty about the immunostaining pattern in the normal human intestine with these antibodies. Immunoreactivity in the specimen from the patient was compared with immunostaining in normal intestine and in bowel from patients with intestinal disease (see above).

Results

PATIENT

Histological examination of the resected full-thickness intestine showed no abnormalities on routine H & E-stained sections of frozen or paraffin wax-embedded tissue (Figure 1). Neurons appeared morphologically normal in the submucosal and myenteric plexuses and were present in normal numbers. Acetylcholinesterase-positive nerve fibres were also unremarkable. In sections of the frozen tissue a slight increase in connective tissue was seen in the muscularis propria, appreciated only on the connective tissue stains. An excess of glycogen was noted, particularly in the circular muscle coat on PAS staining.

Ultrastructural studies

No ultrastructural abnormalities were detected in the ganglia or nerve tracts, and granular and agranular

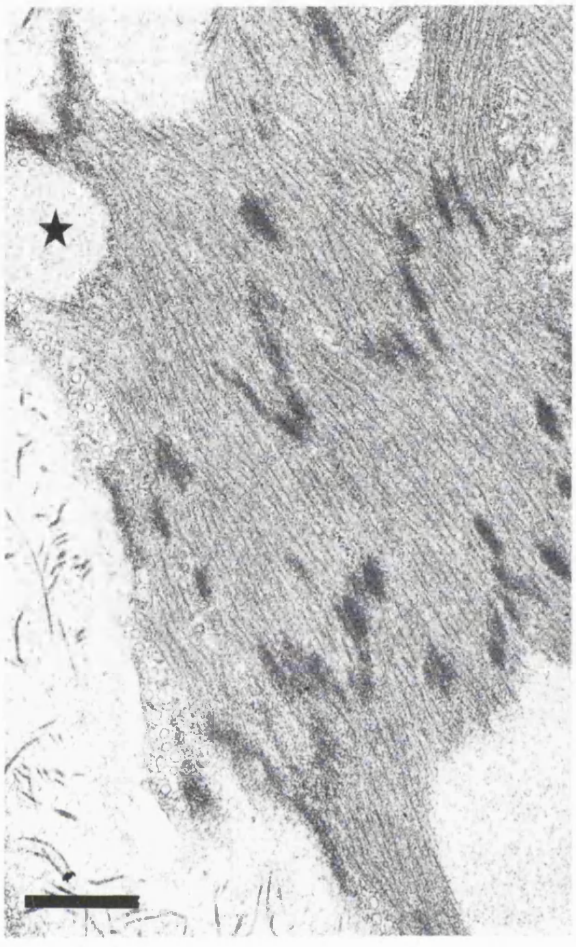


Figure 2. Electronmicrograph showing normal myofilaments, normal dense bodies and well-preserved caveoli in the patient's jejunum. Note the sub-sarcolemmal collections of glycogen (*). This appearance was seen in most fibres both in the circular and longitudinal muscles. Calibration bar 1 μm.

neurosecretory vesicles were normally distributed. The accumulation of glycogen in the muscularis propria was confirmed on electronmicroscopy. In general the myofibrillar appearance was unremarkable (Figure 2), apart from the occasional muscle fibres in both the circular and longitudinal muscles which showed disorganization of myofilaments. These included smooth muscle fibres with gross proliferation of intermediate filaments, a change generally thought non-specific and seen in many pathological conditions including conditions of chronic overload and smooth muscle hypertrophy[32] (personal communication, Gabella 1991). However, muscle hypertrophy was not a feature in our patient's specimen.

Immunohistochemical studies

Smooth muscle α-actin (1A4) immunostaining revealed a pattern quite different from the controls—it was absent within the smooth muscle cells of the circular muscle, with the exception of its innermost layer adjacent to the submucosa, where the smooth muscle cells stained strongly positive (Figure 3). In contrast, smooth muscle α-actin (1A4) immunostaining was normal in the smooth muscle cells throughout the longitudinal muscle, in the muscularis mucosae and in the smooth muscle of the blood vessel walls. Immunostaining, in the circular muscle, for tropomyosin (TM311), filamin (FIL2), caldesmon (CALD5), desmin (DE-R11) and myosin was of the same intensity as in the other intestinal muscle layers. Similarly, all muscle coats showed normal immunostaining with the antibody to pan-muscle α- and γ-actins (HHF35). However, the pattern of immunoreactivity with the antibody to smooth muscle α- and γ-actin (CGA7) was comparable to that seen with the antibody to smooth muscle α-actin (1A4). Although not absent, it was markedly reduced in the circular muscle and present in normal amounts in the longitudinal muscle, muscularis mucosae and blood vessel walls. Immunohistochemical studies using neural markers revealed no abnormalities.

TISSUE FROM INTESTINAL DISEASES AND NORMAL INTESTINE

The abnormality of smooth muscle α-actin detected in the patient was not seen in any of the control tissues outlined in the methods section. In all the other specimens, including the autopsy samples, strong immunostaining for smooth muscle α-actin was detected in the myocytes of the circular muscle. The immunoreactivity of all other antibodies used was the same in the controls as in the patient.

Discussion

Chronic idiopathic intestinal pseudo-obstruction is a heterogeneous group of disorders in which various morphological abnormalities of intestinal innervation and smooth muscle have been identified[4]. Standard histopathological techniques are often inadequate for their demonstration, although special techniques such as silver staining and electronmicroscopy help to identify structural abnormalities in some cases[1,3,4]. In a proportion of patients with CIIP no obvious morphological abnormality can be discovered using currently available techniques, even in the presence of a clear functional abnormality of gut motility (the patient described here falls into this category).

In this study, a number of monoclonal and polyclonal antibodies have been applied to a full-thickness specimen of morphologically normal jejunum from a patient with symptoms of CIIP since childhood. With all but one of

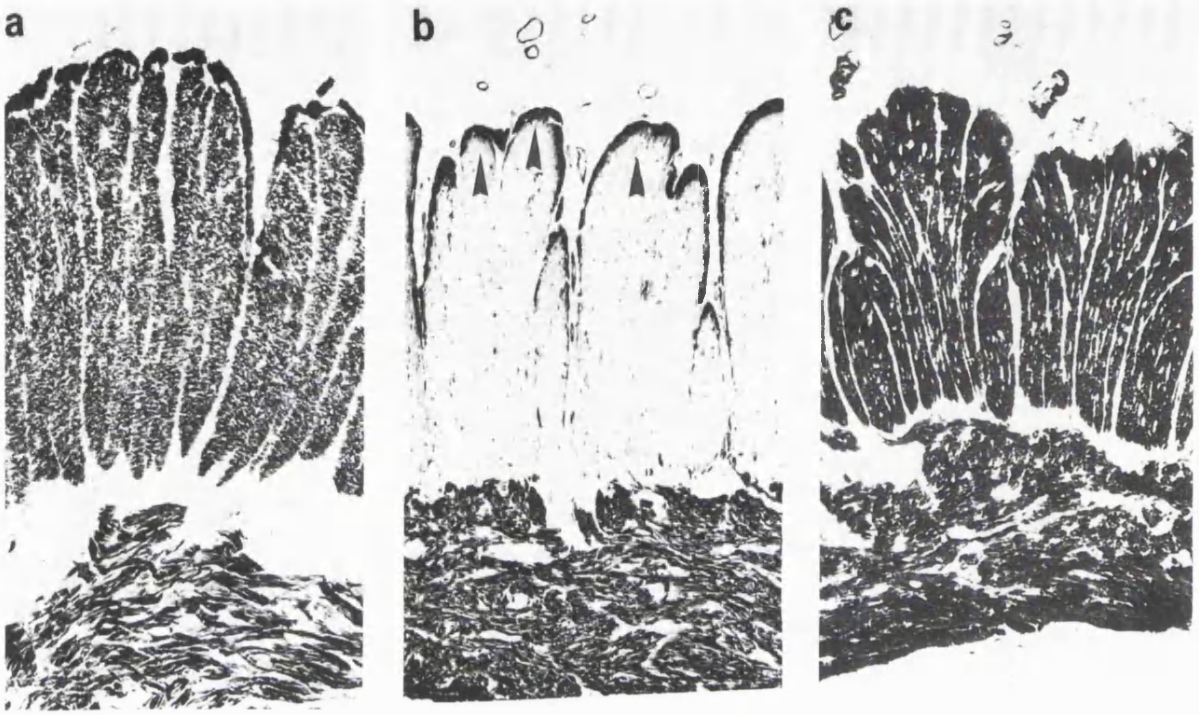


Figure 3. Cryostat sections of jejunum. **a** Immunostaining for smooth muscle α-actin (1A4) in the muscularis propria in a control case. × 100. **b** Absent immunostaining for α smooth muscle actin in the bulk of the circular muscle in the patient, with normal intensity in the longitudinal muscle and the 'topping' of the circular muscle (arrow). × 40. **c** Normal immunostaining for tropomyosin (TM311) in both the circular and longitudinal muscles in the patient. × 40.

these, immunostaining in the patient's jejunum was the same as in a number of other surgical full-thickness samples of intestine and autopsy bowel. The remarkable and unique feature of this case was a deficiency of smooth muscle α-actin immunostaining confined to the circular muscle. Despite the clear demonstration of this deficiency by immunohistochemistry, no significant ultrastructural differences were shown in the myofilament composition of the circular muscle and other smooth muscles of the gut wall. This is therefore the first description of a biochemical, as opposed to a structural, abnormality in CIIP. Biochemical confirmation of the absence of this protein or the presence of an altered protein was not possible due to insufficient tissue for this analysis. Moreover, immunohistochemistry has clear advantages over biochemical assays in that protein abnormalities confined to specific components of heterogeneous tissues such as the intestine can be accurately localized.

In smooth muscle contraction there is an interaction between thick and thin filaments. The thick filaments consist of myosin, no abnormality of which was detected in our patient, while the major component of thin filaments is actin. Actin exists in at least six different isoforms which are expressed in a tissue specific pattern. They differ in their amino acid sequence in the *N*-terminus and each is encoded by a separate gene[33,34]. The isoforms can be separated into three bands (α, β and γ) on isoelectric focusing and further subdivided into three α-isoforms localized to smooth muscle, skeletal muscle and cardiac muscle, and two γ- isoforms localized to smooth muscle and the cytoplasm of all cell types. The β-isoform is cytoplasmic, is present in all cells, and is not thought to be involved in muscle contraction[33]. The major isoactins in mature intestinal smooth muscle are smooth muscle γ- and α-actins, the γ isoform being the largest constituent and the smooth muscle α-isoactin being the smaller but nevertheless an important component[35]. As well as their major component of actin, the thin filaments also contain tropomyosin[31] and caldesmon[33] or filamin[33,36]. In the intestine from our patient these thin filament-associated proteins were present in all intestinal muscles with an intensity comparable with the controls.

The proportions of different isoforms of various contractile proteins change during smooth muscle development. The cytoplasmic isoforms predominate in undifferentiated smooth muscle cells in the early embryo, while

the smooth muscle specific isoforms (smooth muscle γ- and α-actins in the intestinal smooth muscle) predominate in mature fully differentiated cells[37–39]. For ordered peristalsis, the presence of intact, fully functional smooth muscle cells is vital. This is reflected by the presence of all contractile proteins (and their isoforms) in proportions appropriate for mature, fully differentiated smooth muscle cells. Thus, the absence or any change in the amounts of the contractile elements is likely to result in aberrant or abolished contractions. In the patient described here the abnormality of smooth muscle α-actin, one of the two major isoforms present in mature smooth muscle cells, probably explains her disturbance in intestinal motility.

Reliable immunohistochemical identification of actin isoforms depends on antibody specificity. Unless the antibody is made against the specific amino acid sequence in the *N*-terminus, cross reactivity with other isoforms is likely[40]. The antibody 1A4 is directed against the *N*-terminus of a (unique) synthetic decapeptide of smooth muscle α-actin, and is therefore specific for this isoform[41]. Unfortunately no other isoform-specific antibody is currently available. We used two antibodies (HHF35 and CGA7) which recognize α- and γ-actins which differed in their staining of the circular muscle in this patient. We believe this reflects a difference in the specificity of these two antibodies. The exact epitopes identified by antibodies such as HHF35 and CGA7 are unknown and the biochemical basis for their specificity is unclear[42,43]. Very weak immunostaining of the circular muscle was seen in our patient using the antibody CGA7, consistent with absent α-isoactin but implying the presence of γ-isoactin, whilst immunoreactivity was normal with HHF35. The normal immunoreactivity to HHF35 presumably reflects cross reactivity of this antibody with other contractile proteins. In the absence of specific antibodies to smooth muscle γ-isoactin it is not possible to exclude with certainty the possibility of an additional abnormality in this protein. A difference in immunoreactivity between various muscle layers in the same biopsy or between biopsies from different patients probably reflects significant abnormalities in the isoform content. In particular, the abnormality detected with the specific smooth muscle α-actin antibody (1A4) observed in this patient is highly significant.

The absent immunostaining for an actin isoform could reflect either an acquired phenomenon[44–48], or a specific developmental defect. In view of the life-long duration of symptoms in our patient, we believe the latter is more likely. The effect of vagotomy on isoactin composition is not known. However, the patient's symptoms began in early childhood, preceded her vagotomy by many years and have steadily deteriorated. It seems improbable, therefore, that the abnormality we have demonstrated in this patient is due to the surgical intervention.

The concept that the absence of a contractile protein limited to one muscle coat may be a developmental defect can be supported by consideration of the normal differentiation of intestinal smooth muscle. Cytodifferentiation of smooth muscle cells in the rat gut[49] proceeds in a specific order: cells expressing actin (α and γ) appear in the mesenchyme of the 15-day fetal rat in the circular, muscle-forming area and in the presumptive longitudinal muscle 48 h later. Such cells are noted in the muscularis mucosae shortly after birth and become fully established 2 weeks later. A distinct cell layer of actin-expressing cells arises in the perinatal period in the innermost part of the circular muscle. In humans the intestinal smooth muscle layers develop in the same order although the timing is different. The circular muscle develops at 9 weeks gestation, the longitudinal by 12 weeks and the muscularis mucosae is evident after the 21st gestational week[50]. It is probable that the development of the innermost circular muscle layer follows the same pattern as in the rat and appears later.

More intense immunoreactivity ('topping') can be seen in the innermost layer of the circular muscle compared with the bulk of the circular muscle using a variety of antibodies and the layer may even be recognized in routinely stained sections. The difference in staining may signify the separate origin of this layer from the rest of the circular muscle. In our patient, strong topping was seen with the antibody to smooth muscle α-actin, while the bulk of the circular muscle was unstained. One possible mechanism for this would be a transient developmental block at the crucial time of circular muscle development. The temporary nature of this developmental block would allow normal later development of the longitudinal muscle, muscularis mucosae and the innermost layer of the circular muscle.

In conclusion, we have demonstrated a previously undescribed abnormality in actin isoform content in the circular muscle in a patient with intestinal pseudo-obstruction. This is the first description of a protein abnormality of the smooth muscles in a patient with CIIP in whom there was no structural abnormality demonstrable by light- or electronmicroscopy.

Acknowledgements

We express our thanks to Professor G.Gabella for reviewing the electronmicrographs and thank Professors F.Walsh and B.Anderton for their gifts of antibodies.

This work was partly funded by the Joint Research Board of the Hospital for Sick Children and the Institute of Child Health, London, UK.

References

1. Christensen J, Dent J, Malagelada J-R, Wingate DL. Pseudo-obstruction. *Gastroenterology Int.* 1990; **3**; 107–119.
2. Malagelada JR. Clinical aspects of gastro-duodenal motor coordination. In: vanNueten JM, Schuurkes JHJ, Akkermans LMA eds. *Gastro-pyloro-duodenal Coordination*. Petersfield: Wrightson, 1990; 229–243.
3. Lake BD. Observations on the pathology of pseudo-obstruction. In Milla PJ ed. *Disorders of Gastrointestinal Motility in Childhood*. Chichester: John Wiley, 1988; 81–90.
4. Schuffler MD. Neuromuscular abnormalities of small and large intestine. In Whitehead R ed. *Gastrointestinal and Oesophageal Pathology*. Edinburgh: Churchill Livingstone, 1989; 329–353.
5. Schuffler MD, Lowe MC, Bill AH. Studies of idiopathic intestinal pseudo-obstruction. 1. Hereditary hollow visceral myopathy: clinical and pathological studies. *Gastroenterology* 1977; 73; 327–338.
6. Schuffler MD, Pope CE. Studies of idiopathic intestinal pseudo-obstruction. 2. Hereditary hollow visceral myopathy: Family studies. *Gastroenterology* 1977; **73**; 339–344.
7. Faulk DL, Anuras S, Gardner GD, Mitros FA, Summers RW, Christensen J. A familial visceral myopathy. *Ann. Intern. Med.* 1978; **89**; 600–606.
8. Colemont LJ, Camilleri M. Chronic intestinal pseudo-obstruction:- diagnosis and treatment. *Mayo Clin. Proc.* 1989; **64**; 60–70.
9. Alstead EM, Murphy MN, Flanagan AM, Bishop AE, Hodgson HJF. Familial autonomic visceral myopathy with degeneration of muscularis mucosae. *J. Clin. Pathol.* 1988; **41**; 424–429.
10. Rodrigues CA, Shepherd NA, Lennard-Jones JE, Hawley PR, Thompson HH. Familial visceral myopathy: a family with at least 6 involved members. *Gut* 1989; **30**; 1285–1292.
11. Smith JA, Hauser SC, Madara JI. Hollow visceral myopathy. A light- and electronmicroscopic study. *Am. J. Surg. Pathol.* 1982; **6**; 269–275.
12. Anurus S, Mitros FA, Nowak TV *et al.* Familial visceral myopathy with external opthalmoplegia and autosomal recessive transmission. *Gastroenterology* 1983; **84**; 346–353.
13. Anuras S, Mitros FA, Milano A, Kuminsky R, Decanio R, Green JB. A familial visceral myopathy with dilatation of the entire gastrointestinal tract. *Gastroenterology* 1986; **90**; 385–390.
14. Jacobs E, Ardichvili D, Perissino A, Gottignies P, Hanssens J-F. A case of familial visceral myopathy with atrophy and fibrosis of the longitudinal muscle layer of the entire small bowel. *Gastroenterology* 1979; **77**; 745–750.
15. Martin JE, Swash M, Kamm MA, Marher K, Cox EL, Gray A. Myopathy of internal anal sphincter with polyglucosal inclusions. *J. Pathol.* 1990; **161**; 221–226.
16. Smout AJPM, deWilde K, Kooyman CD, Ten Thije OJ. Chronic idiopathic intestinal pseudo-obstruction. Coexistance of smooth muscle and neuronal abnormalities. *Dig. Dis. Sci.* 1985; **30**; 282–287.
17. Lewis TD, Daniel EE, Sarna SK, Waterfall WE, Marzio L. Idiopathic intestinal pseudo-obstruction. Report of a case with intraluminal studies of mechanical and electrical activity, and response to drugs. *Gastroenterology* 1978; **74**; 107–111.
18. Bagwell CE, Filler RM, Cutz E *et al.* Neonatal intestinal pseudo-obstruction. *J. Pediatr. Surg.* 1984; **19**; 732–739.
19. Kascula ROC, Cywes S, Katz A, Louw JH. Degenerative leiomyopathy with massive megacolon. Myopathic form of chronic idiopathic intestinal pseudo-obstruction occurring in indigenous Africans. *Perspect. Pediatr. Pathol.* 1987; **11**; 193–213.
20. Vargas JH, Sachs P, Ament ME. Chronic intestinal pseudo-obstruction syndrome in pediatrics. Results of national survey by members of the North American Society of Pediatric Gastroenterology and Nutrition. *J. Pediatr. Gastroenterol. Nutr.* 1988; **7**; 323–332.
21. Puri P, Lake BD, Gorman F, O'Donnell B, Nixon HH. Megacystis-microcolon-hypoperistalsis syndrome: a visceral myopathy. *J. Pediatr. Surg.* 1983; **18**; 64–69.
22. Schuffler MD, Pagon RA, Schwartz R, Bill AH. Visceral myopathy of the gastrointestinal and genitourinary tracts in infants. *Gastroenterology* 1988; **94**; 892–898.
23. Nonaka M, Goulet O, Arahan P, Fekete C, Ricour C, Nezelof C. Primary intestinal myopathy, a cause of chronic idiopathic intestinal pseudo-obstruction syndrome (CIPS): clinicopathological studies of seven cases in children. *Pediatr. Pathol.* 1989; **9**; 409–424.
24. Milla PJ, Lake BD, Spitz L, Nixon HH, Harries JT, Fenton TR. Chronic idiopathic intestinal pseudo-obstruction in infancy: a smooth muscle disease. In Labo G, Bortolotti M eds. *Gastrointestinal Motility*. Verona: Cortinal International 1983; 125–131.
25. Krishnamurthy S, Schuffler MD. Pathology of neuromuscular disorders of the small intestine and colon. *Gastroenterology* 1987; **93**; 610–639.
26. Schuffler MD, Rohrmann CA, Chaffee RG *et al.* Chronic intestinal pseudo-obstruction. A report of 27 cases and review of the literature. *Medicine* 1981; **60**; 173–196.
27. Shaw A, Shaffer H, Teja K, Kelly T, Grogan E, Bruni C. A perspective for pediatric surgeons: chronic idiopathic intestinal pseudo-obstruction. *J. Pediatr. Surg.* 1979; **14**; 719–727.
28. Smith VV. Neurofilament antibodies will differentiate muscle and nerve disorders of chronic intestinal pseudo-obstruction. *Proc. R. Micr. Soc.* 1990; **25**; 52.
29. Dieler R, Schroder JM, Skopnik H, Steinau G. Infantile hypertrophic pyloric stenosis: myopathic type. *Acta Neuropathol. (Berl.)* 1990; **80**; 295–306.
30. Filipe IM, Lake BD. *Histochemistry in Pathology*. 2nd edn. Edinburgh: Churchill Livingstone, 1990.
31. Hsu SM, Raine L, Fanger H. Use of avidin biotin peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabelled antibody (PAP) procedures. *J. Histochem. Cytochem.* 1981; **29**; 577–580.
32. Gabella G. Hypertrophy of visceral smooth muscle. *Anat. Embryol.* 1990; **182**; 409–424.
33. Hartshorne DJ. Biochemistry of the contractile process in smooth muscle. In Johnson LR ed. *Physiology of the Gastrointestinal Tract*. Volume 1. 2nd edn. New York: Raven, 1987; 423–482.
34. Sawtell NM, Lessard JL. Cellular distribution of smooth muscle actins during mammalian embryogenesis: expression of the alpha vascular but not the gamma enteric isoform in differentiating striated myocytes. *J. Cell. Biol.* 1989; **109**; 2929–2937.
35. Fatigati V, Murphy RA. Actin and tropomyosin variants in the smooth muscles. *J. Biol. Chem.* 1984; **259**; 14383–14388.
36. Lehman W, Sheldon A, Madonia W. Diversity in smooth muscle thin filament composition. *Biochim. Biophys. Acta* 1987; **914**; 35–39.
37. Perry SV, Grand RJA. Mechanisms of contraction and the specialised protein components of smooth muscle. *Br. Med. Bull.* 1979; **35**; 219–226.
38. Owens GK, Thompson MM. Developmental changes in isoactin expression in the rat aortic smooth muscle cells *in vivo*. *J. Biol. Chem.* 1986; **261**; 13373–13380.
39. Glukhova MA, Frid MG, Koteliansky VE. Developmental changes in expression of contractile and cytoskeletal proteins in human aortic smooth muscle. *J. Biol. Chem.* 1990; **265**; 13042–13046.

40. Kuroda M. Change of actin isomers during differentiation of smooth muscle. *Biochim. Biophys. Acta* 1985; **843**; 208–213.
41. Otey CA, Kalnoski MH, Bulinski JC. Identification and quantification of actin isoforms in vertebrate cells and tissues. *J. Cell. Biochem.* 1987; **34**; 113–124.
42. Skalli O, Ropraz P, Trzeciak A, Benzonana G, Gillessen D, Gabbiani G. A monoclonal antibody against alpha smooth muscle actin. A new probe for smooth muscle differentiation. *J. Cell Biol.* 1986; **103**; 2787–2796.
43. Tsukada T, Tippens D, Gordon D, Ross R, Gown AM. HHF35, a muscle actin specific monoclonal antibody. 1. Immunocytochemical and biochemical characterization. *Am. J. Pathol.* 1987; **126**; 51–60.
44. Gown AM, Vogel AM, Gordon D, Lu PL. A smooth muscle specific monoclonal antibody recognizes smooth muscle actin isoenzymes. *J. Cell Biol.* 1985; **100**; 807–813.
45. Li YF, Bowers RL, Haley-Russell D, Moody FG, Weisbrodt NW. Actin and myosin isoforms in gallbladder smooth muscle following cholesterol feeding in prairie dogs. *Gastroenterology* 1990; **99**; 1460–1466.
46. Coflenski JT, Adler KB, Woodcock-Mitchell J, Mitchell J, Evans JN. Proliferative changes in the pulmonary arterial wall during short term hyperoxic injury to the lung. *Am. J. Pathol.* 1988; **132**; 563–573.
47. Gottlieb AI, Langille BL, Wong MKK, Kim DW. Biology of disease. Structure and function of endothelial cytoskeleton. *Lab. Invest.* 1991; **65**; 123–137.
48. Lewes W, Gonzalez B. Actin isoform mRNA alterations induced by Doxorubicin in cultured heart cells. *Lab. Invest.* 1990; **62**; 69–76.
49. Kedinger M, Simon-Assmann P, Bouziges F, Arnold C, Alexandre E, Haffen K. Smooth muscle actin expression during rat gut development and induction in fetal skin fibroblastic cells associated with intestinal embryonic epithelium. *Differentiation* 1990; **43**; 87–97.
50. Desa DJ. Alimentary tract. In Wigglesworth JS, Singer DB eds. *Textbook of Fetal and Perinatal Pathology*. Volume 2. Boston: Blackwell Scientific Publications, 1991; 903–979.

Gut 1992; 33: 1477–1481

Gastric antral dysrhythmias in children with chronic idiopathic intestinal pseudoobstruction

S P Devane, A M Ravelli, W M Bisset, V V Smith, B D Lake, P J Milla

Abstract

Chronic idiopathic intestinal pseudoobstruction is a serious disorder of intestinal neuromuscular function resulting in recurrent episodes of intestinal obstruction, and is caused by primary disease of the enteric nerves or enteric smooth muscle. Gastric electrical control activity detected by the non-invasive technique of surface electrogastrography was investigated in 11 children (0·1–16 years) with proven chronic idiopathic intestinal pseudoobstruction (four with known disease of the enteric nerves, three with disease of smooth muscle cells, and four without defined pathology), to determine whether abnormalities were present and whether these were useful in detecting the underlying pathology. Abnormalities were present in eight of 11 patients. Persistent tachygastria (electrical control activity frequency >5 cycles/minute) was found in three patients, all with a proven neuropathy. A continuously irregular frequency was found in five patients, three with a proven myopathy and two with undefined pathology. A normal electrical control activity frequency was present in three patients, one with a proven neuropathy and two with undefined pathology. It is suggested that this non-invasive technique may provide a useful screening test of the pathophysiological basis of the functional obstruction in children with chronic idiopathic intestinal pseudoobstruction.

(*Gut* 1992; 33: 1477–1481)

Chronic idiopathic intestinal pseudoobstruction is an uncommon but serious disorder of intestinal neuromuscular function which results in recurrent episodes of intestinal obstruction without a mechanical cause, in vomiting, and in an inability to tolerate oral feeds. Chronic idiopathic intestinal pseudoobstruction is usually caused by disease of the enteric nerves or smooth muscle. This is most often a primary disorder in children but in adults is more commonly secondary to diseases such as diabetes mellitus or scleroderma. Contractile activity of the muscle coats of the gut is regulated by myogenic, neural, and humoral factors. Myogenic control is related to the intrinsic excitability of gastrointestinal smooth muscle cells, and particularly to the constant rhythmic polarisation and depolarisation of the plasmalemmal membranes which gives rise to the electrical slow wave or electrical control activity. Neural and humoral factors modulate these processes to produce patterns of activity appropriate to the circumstances of the gastrointestinal tract at a given time. Abnormalities of electrical control activity have been associated with dysmotility of the gastric antrum, paroxysmally with nausea and vomiting[1] and persistently with chronic intractable vomiting.[2]

At the present time the diagnosis of chronic idiopathic intestinal pseudoobstruction is based upon invasive investigations, including the demonstration of characteristic patterns of small intestinal motor activity by intraluminal manometry,[3 4] and the histological examination of full thickness intestinal biopsies. As children with diffuse chronic idiopathic intestinal pseudoobstruction frequently vomit, we have investigated gastric electrical control activity as detected by the non-invasive technique of surface electrogastrography in the fasting state in such children to determine if persistently abnormal electrical control activity was present, and if it was related to the underlying pathology of enteric nerves and muscle coats.

Methods

PATIENTS

Eleven children with a presumptive diagnosis of chronic idiopathic intestinal pseudoobstruction were investigated. Clinical details of the patients are given in Table I. All had severe symptoms of intestinal pseudoobstruction, including episodic vomiting, abdominal distension, and intolerance of food. None of the patients had evidence of a central nervous system or an autonomic nervous system disorder. The diagnosis was confirmed in all 11 patients by contrast radiography and by antroduodenal manometry performed in a manner previously described.[5] All had markedly abnormal manometric recordings. In eight of the 11 patients, full thickness intestinal biopsy specimens were available for histological examination. Fifteen control children (age 0·2–17 years, median 11 years) without symptoms of gastrointestinal motor dysfunction formed a control group for surface electrogastrography recordings.

Fasting gastric electrical control activity was recorded and analysed using a modification of

TABLE I *Clinical details of the 11 patients with chronic idiopathic intestinal pseudoobstruction*

Patient number	*Age*	*Symptoms*
1	3 years	Persistent severe vomiting
2	9 months	Persistent vomiting and distension
3	9 months	Persistent vomiting and distension
4	12 years	Intermittent vomiting
5	5 years	Intermittent vomiting and distension
6	16 years	Intermittent vomiting and distension
7	13 months	Persistent vomiting and distension
8	5 weeks	Persistent vomiting
9	6 years	Intermittent vomiting and distension
10	4 weeks	Persistent vomiting
11	19 months	Persistent vomiting

Institute of Child Health, University of London, Guildford Street, London
S P Devane
A M Ravelli
W M Bisset
V V Smith
B D Lake
P J Milla

Correspondence to:
Dr S P Devane, Department of Child Health, 4th Floor, NWB, King's College Hospital, Denmark Hill, London SE5 9RS.

Accepted for publication 30 March 1992

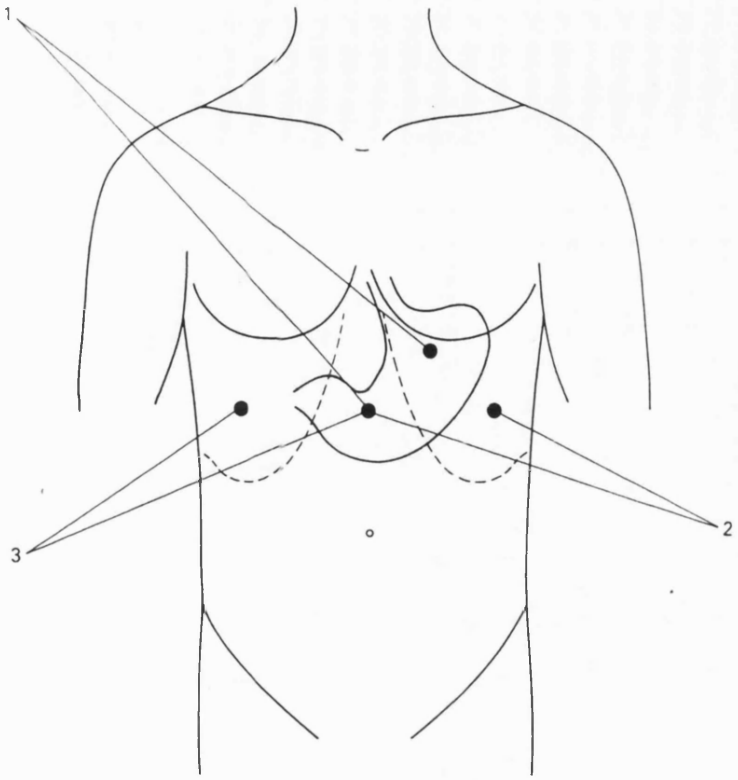


Figure 1: Electrogastrographic electrode placements; the potential difference between the electrodes of pairs 1, 2, and 3 was measured.

the method described by Van der Schee *et al.*[6] The potential differences across three pairs of Ag/AgCl electrodes matched for impedance and placed as shown in Figure 1 were measured for a one hour period after an overnight fast (or a fast of at least four hours in the smallest infants). Skin impedance was reduced to less than 5 kOhms by light skin abrasion. After amplification and passage through an analogue low pass filter (Gould Electronics, time constant 3·2 seconds, cut off slope 6 dB/octave) to remove high frequencies originating from cardiac electrical activity, the signal was digitalised at a frequency of 1 Hz using an analogue to digital convertor (Data Translation 2801A) and recorded on the hard disk of a desktop personal computer (Zenith Data Systems Z248) for later off line analysis. A running spectral analysis of the real time signal captured on disk was performed. Modular computerised algorithms for data capture, digital conditioning of the captured signal, division into subsections, and frequency analysis (PC-Dats, Prosig Computer Consultants) were used. These modules were ordered and invoked by a Fortran code routine written by the authors (RM-Fortran, Ryan McFarland). Each one hour recording was subdivided into 53 overlapping segments of 256 seconds (75% overlap), each segment was digitally filtered (band-pass Butterworth filter, 0·015–0·25 Hz, slope 24 dB/octave) and the frequencies present were determined by Fast Fourier Transformation. The peak frequency present in each segment was extracted and the dominant frequency of the one hour recording (that present in the greatest number of segments) was obtained.

Full thickness intestinal biopsy specimens were obtained in eight of the children. In one patient (patient 4) only routine sections were available for analysis. Biopsy specimens from the remaining seven subjects were divided for routine microscopy (fixation in buffered formalin), for silver staining (fixation in formol saline) and for histochemistry (snap frozen in hexane at −80°C); a portion was taken for electron microscopy (fixation in 2·5% glutaraldehyde in 0·1 M cacodylate buffer containing 2·5 mM $CaCl_2$ at pH 7·4, postfixation in 1% osmium tetroxide and processed into araldite by conventional methods). General morphology was assessed in routine sections. Connective tissue was demonstrated in routine and cryostat sections stained with trichrome and picrosirius methods. Acid phosphatase activity was used to detect lysosomal degradation in smooth muscle, and acetylcholinesterase staining was used to identify nerve fibre distribution in cryostat sections.[7] The ultrastructure of the intestine was examined in seven patients.

The analysis of the electrogastrogram and the histology was conducted by independent observers in a blind manner.

Results

ELECTROGASTROGRAPHIC FINDINGS

The dominant frequencies and the histological abnormalities found in the 11 patients are shown in Table II. A persistently and abnormally fast electrical control activity frequency (>5 cycles/minute), a tachygastria, was found in three of the subjects with chronic idiopathic intestinal pseudoobstruction and in none of the control subjects. A clear dominant electrical control frequency close to 3 cycles per minute was found in three of the subjects with chronic idiopathic intestinal pseudoobstruction and in all of the control subjects. In five of the subjects with chronic idiopathic intestinal pseudoobstruction, a continuously irregular frequency was present such that no clear dominant frequency could be found in the recording (Fig 2).

HISTOPATHOLOGICAL FINDINGS

In the one subject (patient 4) from whom routine sections only of large bowel and ileum were available, histological examination showed an inflammatory process predominantly affecting the myenteric plexus particularly in the

TABLE II *Dominant electrical control activity frequency and histological category of the 11 patients with chronic idiopathic intestinal pseudoobstruction*

Patient number	*Dominant frequency (cycles/minute)*	*Histology*
1	6·4	Neuropathy
2	8·3	Neuropathy
3	9·4	Neuropathy
4	2·8	Neuropathy
5	None	Myopathy
6	None	Myopathy
7	None	Myopathy
8	None	Inconclusive examination
9	None	Insufficient material for full evaluation
10	2·2	No histology available
11	3·7	No histology available

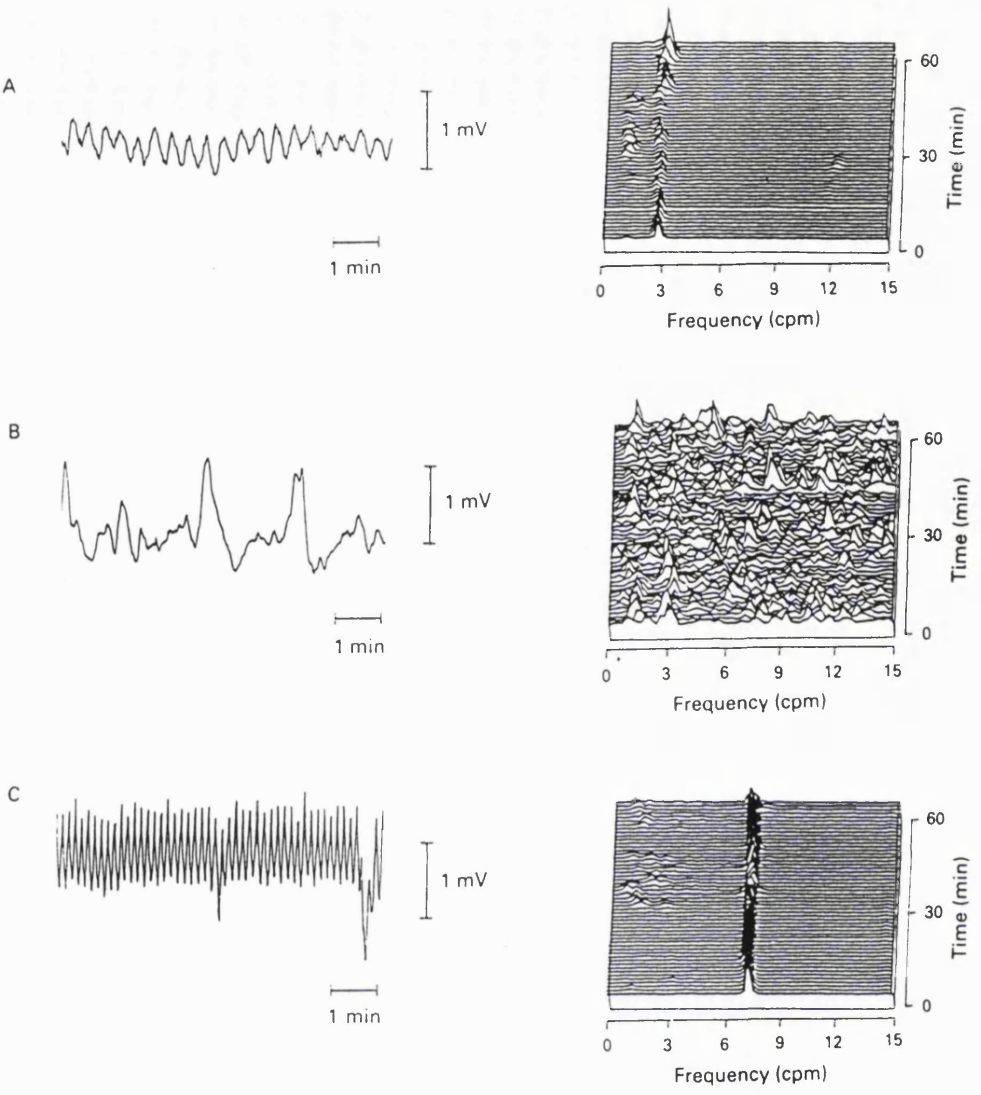


Figure 2: Representations of the recorded electrical control activity from (a) a subject with a normal frequency of electrical control activity; (b) a subject with a continuously irregular electrical control activity frequency; (c) a subject with a tachygastria. In each case, a short extract from the original recording, and a pseudo-3-dimensional representation of the frequency distribution in each of the 256 second segments obtained for analysis from a one hour recording is shown.

appendix but extending to the small intestine. Neurones in the myenteric plexus were not identified in the colon and were sparse and appeared degenerate in the appendix and ileum.

Pathological changes either in the neural plexus or in the smooth muscle coats were not evident in routine or cryostat sections, or on histochemical staining, in the seven subjects from whom full thickness bowel samples were available for further analysis. In three of these patients (patients 5, 6, and 7), ultrastructural examination, however, showed abnormalities of smooth muscle fibres whilst normal argyrophilic neurones were seen in the silver preparations. In one patient (patient 8) there was no conclusive ultrastructural evidence of a myopathic change, and the neurones appeared ultrastructurally normal. No material was available for silver staining from this patient. In the remaining three subjects (patients 1, 2, and 3) silver staining revealed a neuropathic abnormality with absent or severely depleted and ill defined argyrophilic neurones in the myenteric plexus. Electron microscopy of the bowel from one of these patients (patient 1) also showed atrophic and shrunken neurones in the myenteric plexus. Intestinal smooth muscle from these patients was ultrastructurally unremarkable.

RELATIONSHIP BETWEEN ELECTROGASTROGRAPHIC, HISTOPATHOLOGICAL, AND MANOMETRIC FINDINGS

The four patients with proven neuropathy had severely disturbed propagation and organisation of antroduodenal motor activity. All three patients with proven myopathy had abnormal conformation of phase III of the migrating motor complex with a low amplitude of contraction. Two of the four patients without a proven histological diagnosis had low amplitude poorly formed phase III complexes. Of the remaining two patients, one had both low amplitude of contractions and severe disorganisation of activity, and one (patient 8) has no motor activity detected.

All three subjects with a tachygastria (patients 1, 2, and 3) had a proven neuropathy of the myenteric plexus whereas three of the five patients with a continuously irregular frequency of the electrical control activity had clear evidence of a myopathy of the muscle coats on ultrastructural studies. In the remaining two patients the ultrastructural changes in the muscle coats were inconclusive in one case (patient 8) and the histological material was inadequate for diagnosis in the other case (patient 9).

Discussion

Chronic idiopathic intestinal pseudoobstruction is a disorder of motor function of the gastrointestinal tract as a result of disease of enteric nerves or smooth muscle cells. We and others[8-10] have described the histological characteristics of full thickness intestinal biopsies in patients with visceral myopathy and neuropathy. A variety of abnormalities including inflammatory cell infiltration and Schwann cell proliferation, hypoganglionosis, and hyperganglionosis have been described in those with disease of the enteric nervous system. In muscle disease degeneration of muscle fibres and replacement with fibrous tissue have been seen. In our patients, four had evidence of a neuropathy (one with associated inflammatory changes) and three had evidence of a myopathy.

Manometric and electrophysiological studies of intestinal myoelectrical and motor activity in patients with pseudoobstruction have shown clearly different patterns of activity in those with disorders of the enteric nervous system compared with those with muscle disease.[3 4 8 11] In the former, disturbed conformation and propagation of phase III of the migrating motor complex together with bizarre waveforms of normal or increased amplitude are usually seen, whereas in the latter very low amplitude or absent contractions are the rule. In our patients, those with proven neural and muscular pathology conformed to these patterns of motor activity. In addition, two others without a histologically proven diagnosis had a pattern suggestive of a myopathic process.

Smooth muscle cells of the gastric body and antrum exhibit inherent rhythmic polarisation and depolarisation of the electrical potential across the cell membrane. The frequency of this rhythmic activity is determined by a group of cells on the greater curvature which have been called the gastric pacemaker. This slow wave or electrical control activity is entrained throughout the stomach because of electrical coupling between adjacent muscle cells and a differential in excitability of the muscle cells between the gastric corpus and the antrum.[12] In the human antrum, the frequency of the electrical control activity is close of 0·05 Hz (3 cycles/minute). This electrical control activity can be detected by electrodes placed surgically in the bowel wall[13] or by electrodes placed against the mucosal surface by intubation techniques. The former has provided useful information in cases requiring laparotomy.[14] The latter suffers from unstable mucosal contact, although the use of improved fixation techniques such as magnetic stabilisation has reduced this problem.[15] The invasive nature of intubation techniques, however, restricts its usefulness in pediatric practice. Recordings of gastric electrical control activity made from surface electrodes have been shown to correlate well with mucosal recordings,[16] and this together with improvements in detection methods and their wider availability has led to increased use of surface electrogastrography in the investigation of gastrointestinal symptoms. The advent of powerful desktop computers and the introduction of the technique of running spectal analysis by Van der Schee[6] has allowed objective analysis of the recorded signals, superceding the use of visual analysis techniques.[17]

Abnormality of the electrical control activity has been shown to occur in association with motion sickness,[18] in patients with idiopathic gastroparesis,[19] unexplained nausea and vomiting,[1] and anorexia nervosa,[17] and in response to vagotomy[20] and to administration of glucagon.[15] One case report has been published describing a gastric electrical control activity close to 0·05 Hz (3 cycles/minute) with normal histology in a child with an idiopathic motility disorder.[14] Tachygastria has been reported in a child with gastric atony,[21] who later developed the full clinical picture of CIIP.[22] To our knowledge, this is the first report of persistent antral dysrhythmias in a series of patients with chronic idiopathic intestinal pseudoobstruction and known disease of enteric nerves or smooth muscle.

Three of the four patients with a known neuropathy but none of the control group and none of the patients with a known myopathy had a persistent tachygastria in the fasting state. Thus detection of a persistent tachygastria in the fasting state is highly suggestive of a neuropathic basis for the pseudoobstruction. The mechanism whereby a neuropathy may produce a tachygastria is uncertain. The neural abnormalities that could induce a tachygastria include loss of intrinsic inhibitory innervation or lack of extrinsic autonomic inhibition. We have no evidence in any of our patients, after investigation of the central and autonomic nervous systems, that extrinsic neural control was disordered. It is clear, however, that a variety of conditions affecting the extrinsic nerves and the central nervous system may result in tachygastria, ranging from children with cerebral palsy,[23] to motion sickness,[18] anorexia nervosa,[17] and vagotomy.[24] In these conditions, with the exception of cerebral palsy, the tachygastria is usually paroxysmal, and the increased frequency is somewhat variable. We were impressed that the tachygastria in our patients with neuropathic pseudoobstruction was both persistent and raised consistently in the fasting state. We were unable to explore systematically the electrical control activity in the postprandial state because of the patients' intolerance to food. We would suggest that in neuropathic pseudoobstruction it is more likely that the tachygastria is induced by loss of intrinsic inhibitory innervation which may normally modify the frequency of polarisation of the plasmalemmal membrane of the gastric smooth muscle cells.

The presence of a continuously irregular frequency such that no dominant frequency could be shown was found in the three patients with a proven myopathy and in two patients with a manometric pattern suggestive of muscle disease. Electrical control activity is caused by the entrainment of the fluctuations of the transmembrane potentials of individual smooth muscle cells. The apparent continuously irregular frequency recorded, which contains high and low frequency components, may have several explanations. It may be the result of inability to maintain a constant frequency of electrical control activity and this may be due to

the effects of muscle disease on groups of muscle cells producing populations with altered frequencies. Alternatively, it is possible that the abnormal recordings are the result of poor summation of the electrical signal in the abnormal muscle syncytium and poor projection onto the body surface. A reduced amplitude of the electrical control activity may result in a marked diminution of the signal to noise ratio making it difficult to extract the true dominant frequency. In addition, it may be considered that the frequency pattern results from a technically unsatisfactory recording. We think the last explanation is unlikely as unsatisfactory recordings produced on our equipment result in a signal with an overwhelming predominance of low frequencies of <0·025 Hz (unpublished observations). At the present time, it is not possible to distinguish between the other alternative explanations, though it may be that both factors operate to modulate the pattern of frequencies recorded at the body surface. In the future, computerised spatial mapping of the distribution of frequencies in the manner now being used for electroencephalographic mapping of epileptic foci may provide a non-invasive alternative to multiple implanted electrodes to help distinguish between the above explanations.

In conclusion we have found a persistent tachygastria in the fasting state in three of four patients with chronic idiopathic intestinal pseudoobstruction with a known neuropathic basis for their condition, and in none of 15 control patients, none of three patients with chronic idiopathic intestinal pseudoobstruction as a result of muscle disease, and none of four with chronic idiopathic intestinal pseudoobstruction with an unknown basis. In the three patients with muscle disease there was absence of a dominant frequency. We suggest that surface electrogastrography may offer a non-invasive means of screening patients with suspected neuromuscular disease of the gut causing persistent vomiting and episodes of functional obstruction.

SD would like to acknowledge generous support from the Janssen Research Foundation. This work was published in part in abstract form following presentation at the International Symposium on Gastrointestinal Motility, Vienna, September 1989.

1 Geldof H, Van der Schee EJ, van Blankenstein M, Grashuis JL. Electrogastrographic study of gastric myoelectric activity in patients with unexplained nausea and vomiting. *Gut* 1986; **27**: 799–808.
2 Cucchiara S, Janssens J, Vantrappen G, Geboes K, Ceccatelli P. Gastric electrical dysrhythmias (tachygastria and tachyarrhythmia) in a girl with chronic intractable vomiting. *J Pediatr* 1986; **108**: 264–7.
3 Stanghellini V, Camilleri M, Malagelada J-R. Chronic idiopathic intestinal pseudo-obstruction: clinical and intestinal manometric findings. *Gut* 1987; **28**: 5–12.
4 Hyman PE, McDiarmid SU, Napolitano J, Abrams CE, Tomomasa T. Antroduodenal manometry in children with chronic intestinal pseudo-obstruction. *J Pediatr* 1988; **112**: 899–905.
5 Fenton T, Harries JT, Milla PJ. Disordered small intestinal motility: a rational basis for toddler's diarrhoea. *Gut* 1983; **24**: 897–903.
6 Van der Schee EJ, Grashius JL. Running spectrum analysis as an aid in the representation and interpretation of electrogastrographic signals. *Med Biol Eng Comput* 1987; **25**: 57–62.
7 Filipe IM, Lake BD, eds. *Histochemistry in pathology*. 2nd ed. Edinburgh: Churchill Livingstone, 1990: 444–63.
8 Milla PJ, Lake BD, Spitz L, Nixon HH, Harries JT, Fenton TR. Chronic idiopathic intestinal pseudo-obstruction in infancy: a smooth muscle disease. In: Labó G, Bortolotti M, eds. *Gastrointestinal motility*. Verona: Cortina International, 1983: 125–31.
9 Krishnamurthy S, Schuffler MD. Pathology of neuromuscular disorders of the small intestine and colon. *Gastroenterology* 1987; **93**: 610–39.
10 Navarro J, Sonsino E, Boige N, Nabarra B, Ferkadji L, Mashako LMN, *et al*. Visceral neuropathies responsible for chronic intestinal pseudo-obstruction syndrome in pediatric practice: analysis of 26 cases. *J Pediatr Gastroenterol Nutr* 1990; **11**: 179–95.
11 Wozniak E, Fenton TR, Milla PJ. Fasting small intestinal motor activity in chronic idiopathic intestinal pseudo-obstruction. *Pediatr Res* 1984; **18**: 1060.
12 Kelly KA. Differential responses of the canine gastric corpus and antrum to electrical stimulation. *Am J Physiol* 1974; **226**: 230–4.
13 Alvarez WC, Mahoney LJ. Action current in stomach and intestine. *Am J Physiol* 1922; **58**: 476–93.
14 Bland EL, Karaus M, Glicklis M, Sarna SK, Werlin SL. Gastrointestinal myoelectric activity in an infant with congenital idiopathic motility disorder. *Dig Dis Sci* 1989; **34**: 1124–31.
15 Abell TL, Malagelada J-R. Glucagon-evoked gastric dysrhythmias in humans shown by an improved electrogastrographic technique. *Gastroenterology* 1985; **88**: 1932–40.
16 Hamilton JW, Bellahsene BE, Reichelderfer M, Webster JG, Bass P. Human electrogastrograms, comparison of surface and mucosal recordings. *Dig Dis Sci* 1986; **31**: 33–9.
17 Abell TL, Malagelada J-R, Lucas AR, Brown ML, Camilleri M, Go VLW, *et al*. Gastric electromechanical and neurohormonal function in anorexia nervosa. *Gastroenterology* 1987; **93**: 958–65.
18 Koch KL, Stern RM, Vasey MJ, Seaton JF, Demer LM, Harrison TH. Gastric myoelectric and endogenous neuroendocrine responses to illusory self-motion in man. *Gastroenterology* 1988; **95**: 875.
19 Bortolotti M, Pinotti R, Barbara L. Gastric myoelectric activity in patients with chronic idiopathic gastroparesis. *Gastroenterology* 1989; **95**: 857.
20 Nelsen TS, Kohatsu S. Clinical electrogastrography and its relationship to gastric surgery. *Am J Surg* 1968; **116**: 215–22.
21 Telander RL, Morgan KG, Kreulen DL, Schmalz PF, Kelly KA, Szurszewski JH. Human gastric atony with tachygastria and gastric retention. *Gastroenterology* 1978; **75**: 497–501.
22 Malagelada JR. *Manometric diagnosis of gastrointestinal motility disorders*. New York: Thieme Medical, 1986: 109.
23 Devane SP, Bisset WM, Milla PJ. Persistent tachygastria in severe nausea and vomiting. *Pediatr Res* 1989; **26**: 275.
24 Geldof H, Van der Schee EJ, Van Blankenstein M, Smout AJPM, Akkermans LMA. Effects of highly selective vagotomy on gastric myoelectric activity, an electrogastrographic study. *Dig Dis Sci* 1990; **35**: 969–75.

Archives of Disease in Childhood 1992; 67: 218–221

Persistent gastrointestinal symptoms after correction of malrotation

S P Devane, R Coombes, V V Smith, W M Bisset, I W Booth, B D Lake, P J Milla

Abstract

Persistent vomiting, diarrhoea, or intolerance of feeding, are well recognised problems in children after surgical correction of intestinal malrotation. Conversely, intestinal malrotation is a common accompaniment of chronic idiopathic intestinal pseudo-obstruction. We investigated motor activity of the small intestine during fasting in eight children who had persistent vomiting, intolerance of full enteral feeding, or severe diarrhoea after surgical correction of intestinal malrotation. Abnormality of motor function similar to that found in neuropathic pseudo-obstruction was found in seven of the eight patients. Persistence of symptoms after surgical correction of a malrotation is associated with a motility disturbance which seems to be due to a defect of intrinsic enteric innervation. Such a defect may be important in the aetiology of the malrotation.

Malrotation of the midgut results from a disturbance of the process by which the herniated midgut rotates and returns to the enlarging fetal abdominal cavity in the twelfth week of gestation. After birth, small intestinal malrotation frequently presents with acute intestinal obstruction and requires surgical intervention.[1] Symptoms persist after surgery and disordered feeding consistent with abnormal motility is well recognised. As malrotation is common in neuropathic pseudo-obstructive disorders of the midgut, we have studied the motor function of the small intestine in eight children with persistent feeding problems, vomiting, or diarrhoea after surgery in order to investigate whether abnormalities of motility were present which might underlie such problems.

Patients and methods

PATIENTS

Between January 1987 and April 1988, 81 children (aged 1 day to 13 years) with a history of malrotation and intestinal obstruction were seen in the Hospital for Sick Children, London, or the Children's Hospital, Birmingham. Eight (table 1) had persistent severe gastrointestinal symptoms and feeding problems after Ladd's procedure for correction of malrotation, carried out at a median age of 16 months (range 1 month to 13 years). Seven of the eight patients had persistent vomiting. In five of these (patients 1, 3, 6, 7, and 8) vomiting occurred as the enteral intake was increased after surgery, and persisted until the time of referral for investigation. The vomiting was associated with the persistent aspiration of significant amounts of fluid from the stomach, and full enteral feeding could not be established. Three of these five patients were being fed parenterally, one was on continuous gastrostomy feeding, and one was on overnight continuous nasogastric feeding.

In one patient (patient 2), vomiting occurred intermittently and was associated with episodes of abdominal and intestinal distension. One patient (patient 5) had bouts of vomiting associated with severe abdominal pain relieved by cisapride and was intermittently dependent on parenteral nutrition. One patient (patient 4), who had had an ileal resection because of a volvulus, had persistent severe diarrhoea after ingestion of food, not relieved by an exclusion diet, and associated with failure to thrive. One child with vomiting (patient 3) had accompanying pyloric stenosis and a congenitally short small intestine. One patient (patient 8) subsequently developed neurofibromatosis. The patients were referred for investigation at a median time of seven months after surgery (range 0·5 months to five years).

The results of the manometric studies were compared with seven control subjects (aged 2·9–13 years, median 6·9 years), undergoing investigation for suspected gut disease, who were found after investigation not to have a gastrointestinal disease. This group consisted of two patients with chest disease undergoing pancreatic function tests, two patients with short stature undergoing jejunal biopsy, one

Institute of Child Health, University of London
S P Devane
V V Smith
W M Bisset
B D Lake
P J Milla

Institute of Child Health, University of Birmingham
R Coombs
I W Booth

Correspondence to:
Dr S P Devane, Department of Child Health, 4th Floor, New Ward Block, King's College Hospital, London SE5 9RS.

Accepted 10 October 1991

Table 1 Clinical features of 8 patients with persistent gastrointestinal symptoms after correction of malrotation

Patient No	*Symptoms*	*Route of feeding*	*Age at operation*	*Age at study*
1	Vomiting on feeding	Parenteral and nasogastric	<1 week	6 weeks
2	Intermittent distension and vomiting	Oral	<1 week	9 months
3	Vomiting on feeding	Via gastrostomy	<1 week	5 months
4	Profuse diarrhoea on feeding, failure to thrive	Oral	<1 week	28 months
5	Vomiting on feeding	Parenteral and oral	8 years	13 years
6	Vomiting on feeding, constipation	Parenteral	22 weeks	7 months
7	Vomiting on feeding	Parenteral	3 weeks	5 weeks
8	Vomiting after bolus feeding	Overnight nasogastric	11 weeks	23 months

patient with episodic abdominal pain undergoing a pancreatic function test, and two patients with a history of episodes of bowel disturbance due to emotional stress in one case and to transient dissaccharide intolerance in the other case.

MANOMETRY

Motor function of the small intestine after fasting, in particular phase III activity, was used as a probe of the integrity of the enteric nervous system and intestinal smooth muscle. Motor activity was assessed manometrically after a 12 hour fast as previously described.[2] Activity was recorded for a minimum of three cycles of the migrating motor complex where this was present, or for a minimum period of four hours if not present. Mean migrating motor complex duration (cycle length), mean phase III duration, mean phase III pressure wave frequency, a mean phase III motility index for sum of wave amplitudes/duration), and mean propagation velocity of the phase III activity front were measured at the duodenojejunal flexure.

HISTOLOGY

In those patients with severe and persistent symptoms where serious neuromuscular disease was suspected, biopsy specimens of the gastrointestinal tract were taken. Rectal suction biopsy specimens were obtained in four subjects (patients 3, 4, 7, and 8) and examined in our laboratory. Full thickness small intestinal biopsy specimens were obtained from four subjects (patients 1, 2, 3, and 5), and were divided into three for fixation in buffered formalin, glutaraldehyde, and for snap freezing. Morphology was assessed on haematoxylin and eosin stained paraffin and cryostat sections, and the amount of connective tissue was estimated using picrosirius and trichrome stains. Acetylcholinesterase activity was detected in snap frozen tissue. Gluteraldehyde fixed tissue was processed for examination by transmission electron microscopy using standard methods.

Results

MANOMETRY

The indices measured on the manometric recordings are shown in table 2, and representative segments of the recordings from three of the patients are shown in the figure. Patient 1 had no detectable motor activity at the time of the study. Patient 2 had very low amplitude pressure waves as shown by a low motility index, with both failure of propagation and retrograde propagation of some phase III activity fronts. Patients 3 and 4 had a slow frequency of contractions in phase III of the migrating motor complex, suggesting abnormal smooth muscle electrical control activity. In addition, patient 3 showed retrograde propagation of the phase III activity front. Patients 5 and 6 showed failure of propagation of some phase III activity fronts. Patient 7 had abnormally slow propagation of the activity front of phase III activity. Patient 8 had normal fasting activity of the small intestine.

HISTOLOGY

Histological abnormalities in the small intestine were found in three of the four patients in whom full thickness intestinal biopsy specimens were obtained. These abnormalities consisted of distended neuronal axons with ill defined membranes (patient 1), hypoganglionosis of the ileum (patient 2), and vacuolated nerve tracts on electron microscopy (patient 3). Abnormally large nerve trunks in the gastric antrum were found in the fourth of these patients (patient 5). Abnormally large and prominent nerves in the submucosa and myenteric plexus were found in one rectal biopsy (patient 8).

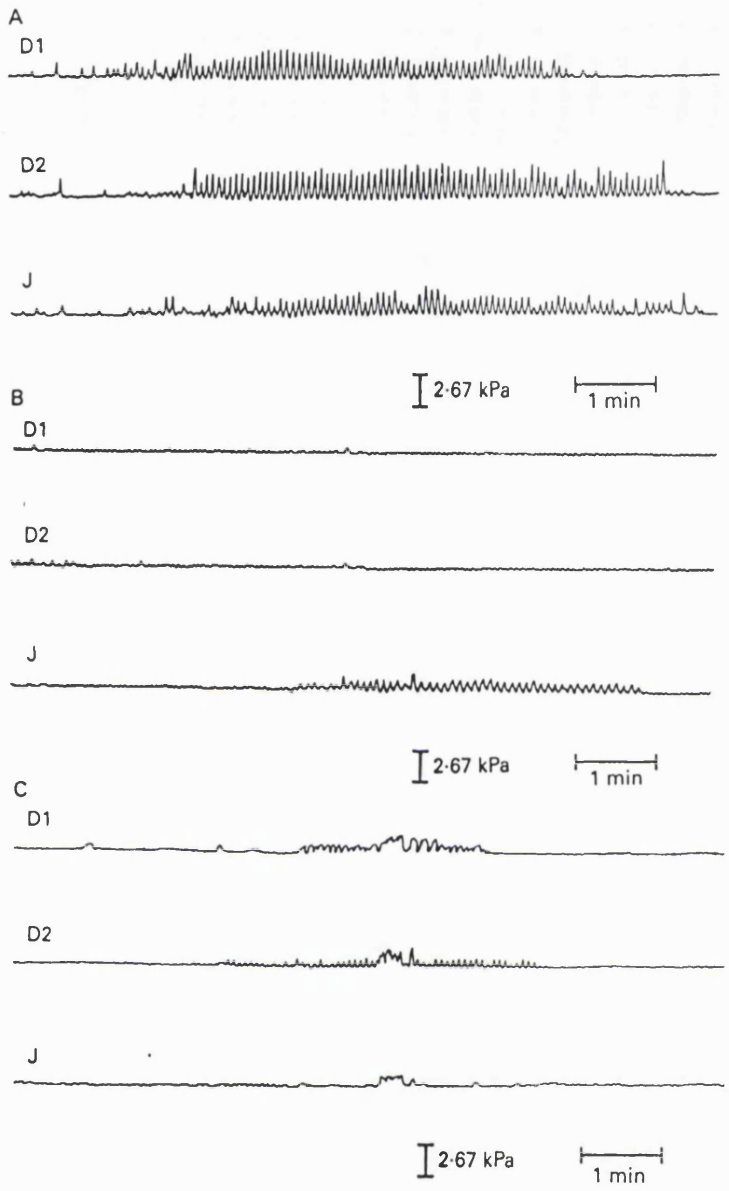
Discussion

The factors which direct the intestine to rotate when the herniated fetal midgut returns to the abdominal cavity at 12 weeks' gestation are not known. By the time the intestine returns into the fetal abdominal cavity, the smooth muscle cells and the neurones which form the enteric nervous system have migrated into place. Although little is known of functional activity of either the enteric nervous system or intestinal smooth muscle cells at this stage of gestation, there is some knowledge of the development of individual neurotransmitters[3] and it is known from animal studies that complex maturational processes influenced by the local microenvironment take place before enteric neurones achieve their final mature form.[4] Malrotation is common in children with pseudo-obstruction, being

Table 2 Indices of motor function of the small intestine obtained from manometric recordings in eight patients with persistent gastrointestinal symptoms after correction of malrotation

Patient No	*Cycle length (min)*	*Phase III duration (min)*	*Phase III frequency (contractions/min)*	*Phase III motility index (kPa/min)*	*Phase III velocity* (cm/min)*	*Non-propagation (fraction)*
1	—	—	—	—	—	0/0
2	30·3	3·1	13·0	6·93	−6·0	2/3
3	32·1	2·4	9·3	39·85	−1·7	0/3
4	43·6	5·0	10·9	11·60	6·6	0/6
5	115·2	7·1	11·0	72·50	3·2	1/13
6	22·7	8·0	11·4	35·50	2·7	6/22
7	35·1	2·5	12·9	22·25	2·0	0/4
8	52·7	3·7	12·8	22·90	19·7	0/5
Normal subjects (n=7)						
Median	79·7	6·2	12·0	24·95	20·7	None
Range: Lower	35·3	3·2	11·5	10·10	2·2	
Upper	157·0	7·3	13·0	36·30	48·5	

*Negative numbers denote net orad propagation, positive numbers normal aborad propagation.



Segments of recorded motor activity of the small intestine while fasting showing phase III of the migrating motor complex, from (A) patient 8, showing normal amplitude and frequency of contractions, and normal aborad propagation; (B) patient 4 showing a low frequency of contractions; and (C) patient 2 showing a low amplitude of contractions and failure of propagation of activity to the most distal channel. The ports for channels D1 and D2 are positioned sequentially in the duodenum and the port for channel J is at the duodenojejunal junction; all ports are 5 cm apart.

associated with approximately 12% of all types of pseudo-obstructive disorders in which there is disease of the enteric nervous system either from an early age or expressed congenitally.[5] Thus motor activity may play a part in directing the gut to its correct position within the abdominal cavity. Alternatively, abnormality of the microenvironment in the malrotated intestine may interfere with normal maturation of the neurohumoral control of motor function.

Intestinal motor activity results from the control of inherently contractile smooth muscle cells by the enteric nervous system and polypeptide hormones. The latter are particularly important in the postprandial state whereas fasting activity reflects the activity and integrity of the enteric nervous system. In normal subjects, fasting activity of the small intestine fluctuates in a cyclical manner over a period of approximately 60 to 90 minutes. This cycle is called the migrating motor complex and it consists of three phases, phases I, II, and III. Phase III is a period of rhythmic contractile activity at a frequency of 11–12 cycles per minute (figure). It is followed by a period of quiescence, phase I, and preceeded by irregular contractions, phase II. This motor complex migrates aborally along the small intestine.

The manometric abnormalities present in patients with pseudo-obstruction have been described in both adults[6] and children.[7–9] In the former study, the most common abnormality found was abnormal propagation and/or conformation of the migrating motor complex (found in 25 of the 42 subjects) together with bizarre waveforms. In the latter studies, in children, the fasting abnormalities included in addition absent migrating motor complexes and very low amplitude or absent contractions. In those manometric investigations where full thickness intestinal biopsies have also been done and adequately studied, very low amplitude contractions with poorly differentiated phase III periods have been associated with smooth muscle disease.[8] In contrast, contractile activity of normal or increased amplitude where there is disorganisation, especially loss of cyclic activity, lack of propagation, abnormal propagation or presence of bizarre clusters of contractions, is usually found in patients with neuropathic disorders.[9]

Five of the eight patients in our series had very abnormal motor activity. Four had abnormal propagation and/or conformation of phase III of the migrating motor complex (with or without a low amplitude of contraction), similar to the abnormalities characterised by Stanghellini *et al*[6] and by Wozniak *et al*.[9] One had no motor activity, which was similar to one of the patterns described by Hyman *et al*.[7] Of the other three patients, one had abnormally slow propagation of the phase III complex, and one had an abnormally slow frequency of contraction in phase III, when compared with the values obtained in subjects without gastrointestinal disease. These findings suggest that the majority of our patients who continued to have gastrointestinal problems after correction of malrotation have disturbances of motor function similar to those found in patients with idiopathic intestinal pseudo-obstruction, particularly those with neuropathic disease involving the enteric nervous system.

The histopathology of neuromuscular disease of the intestine has been the subject of a number of reports[10 11] and both muscle and nerve disorders have been described. A recent report of visceral neuropathies, excluding aganglionosis of the colon and Hirschsprung's disease, summarised the most common intestinal conditions.[12] Such conditions included hypoganglionosis, hyperganglionosis or neuronal intestinal dysplasia, glial cell hyperplasia, and absent or diminished argyrophil neurones associated with pyloric stenosis, a short small intestine, and malrotation.[13]

In the present study, one of our patients had hypoganglionosis of the small intestine whose

proximal extent was not defined, and had total colonic aganglionosis. Two of our patients had abnormal enteric nervous on electron microscopy. Therefore three of the four patients who had full thickness biopsies had abnormalities in the small intestine previously associated with pseudo-obstruction. The significance of the gastric antral abnormality in the fourth patient (patient 5) is uncertain.

Intestinal neuronal dysplasia can be diagnosed from suction biopsies of the rectum by assessment of acetylcholinesterase-positive nerve cells in the lamina propria, hyperplasia of submucosal neurones and the presence of displaced neurones within the lamina propria.[14] In our series of patients, rectal biopsies were helpful in excluding intestinal neuronal dysplasia and in demonstrating the abnormality in patient 8. It may be that the large nerve trunks seen in this patient, who later developed neurofibromatosis, may indicate early evidence of the spectrum of disorders which include glial cell hyperplasia[12] and intestinal neuronal dysplasia.

One child (patient 4) had a short small intestine due to surgical resection and presented with diarrhoea. There are no published manometric studies of motor function of the small intestine in children with short gut syndrome. Therefore it is not possible to know whether the abnormality found in this case (a slow frequency of contraction in phase III) is secondary to the resection.

Abnormalities of motor function may occur in the period immediately after intestinal surgery. Prolonged ileus is not uncommon in the period immediately after surgery for malrotation. In six of our eight cases the manometric study was performed more than 10 weeks after surgery. The long term effect of surgery on small intestinal activity has not been systematically investigated in children, but in adults who had a proctocolectomy and ileoanal anastomosis, jejunoileal fasting activity was not greatly altered 4–24 months after the operation.[15] We suggest that, in view of the nature of Ladd's procedure and of these adult studies, it is unlikely that the motor abnormalities were caused by surgery in these patients.

Two of the subjects (patients 6 and 7) have now died having failed to take enteral feeding without resultant vomiting, two subjects (patients 1 and 2) are well on parenteral feeding, one (patient 5) is well on enteral feeding and cisapride, and the three remaining subjects suffer intermittent gastrointestinal symptoms on enteral feeding.

Our findings support the notion that in those children where symptoms of gastrointestinal disturbance continue despite surgical correction there is disordered intestinal motor activity. The pattern of disordered activity suggests that it results from either disorder or disease of the enteric nervous system. We therefore postulate that in some children with malrotation aberrant early development of either the enteric nervous system or its microenvironment results both in malrotation of the intestine and its later disordered function.

This project was supported, in part, by the Janssen Research Foundation, and this paper was presented in part to the European Society for Paediatric Gastroenterology and Nutrition, Copenhagen, May 1988.

1 Rees JR, Redo SF. Anomalies of intestinal rotation and fixation. *Am J Surg* 1968;116:834–41.
2 Fenton T, Harries JT, Milla PJ. Disordered small intestinal motility: a rational basis for toddler's diarrhoea. *Gut* 1983;24:897–903.
3 Chavyvialli JA, Paulin C, Descos F, Dubois PM. Ontogeny of vasoactive intestinal peptide in the human fetal digestive tract. *Regul Pept* 1983;5:245–56.
4 Le Douarin NM. The ontogeny of the neural crest in avian embryo chimeras. *Nature* 1980;286:663–9.
5 Vargas JH, Sachs P, Ament ME. Chronic ideopathic pseudo-obstruction in pediatrics. *J Pediatr Gastroenterol Nutr* 1988;7:323–32.
6 Stanghellini V, Camilleri M, Malagelada J-R. Chronic idiopathic intestinal pseudo-obstruction: clinical and intestinal manometric findings. *Gut* 1987;28:5–12.
7 Hyman PE, McDiarmid SU, Napolitano J, Abrams CE, Tomomasa T. Antroduodenal manometry in children with chronic intestinal pseudo-obstruction. *J Pediatr* 1988;112: 899–905.
8 Milla PJ, Lake BD, Spitz L, Nixon HH, Harries JT, Fenton TR. Chronic idiopathic intestinal pseudo-obstruction in infancy: a smooth muscle disease. In: Labb G, Bortolotti M, eds. *Gastrointestinal motility*. Verona: Cortina International, 1983:125–31.
9 Wozniak E, Fenton TR, Milla PJ. Fasting small intestinal motor activity in chronic idiopathic intestinal pseudo-obstruction. *Pediatr Res* 1984;18:1060.
10 Rohrmann CA, Ricci MT, Krishnamurthy S, Schuffler MD. Radiologic and histologic differentiation of neuromuscular disorders of the gastrointestinal tract: visceral myopathies, visceral neuropathies, and progressive systemic sclerosis. *AJR* 1984;143:933–41.
11 Krishnamurthy S, Schuffler MD. Pathology of neuromuscular disorders of the small intestine and colon. *Gastroenterology* 1987;93:601–39.
12 Navarro J, Sonsino E, Boige N, *et al*. Visceral neuropathies responsible for chronic intestinal pseudo-obstruction syndrome in pediatric practice: analysis of 26 cases. *J Pediatr Gastroenterol Nutr* 1990;11:179–95.
13 Tanner MS, Smith B, Lloyd JK. Functional intestinal obstruction due to deficiency of argyrophil neurons in the myenteric plexus: familial syndrome presenting with short small bowel, malrotation, and pyloric hypertrophy. *Arch Dis Child* 1976;51:837–41.
14 Puri P, Lake BD, Nixon HH, Mishalany H, Claireaux AE. Neuronal colonic dysplasia: an unusual association of Hirschsprung's disease. *J Pediatr Surg* 1977;12:681–5.
15 Stryker SJ, Borody TJ, Phillips SF, Kelly KA, Dozois RR, Beart RW Jr. Motility of the small intestine after proctocolectomy and ileal pouch anastomosis. *Ann Surg* 1985;201: 351–6.