New techniques in the tissue diagnosis of gastrointestinal neuromuscular diseases

Charles H Knowles, Joanne E Martin

INTRODUCTION

The term gastrointestinal neuromuscular diseases (GINMD) describes a clinically heterogeneous group of disorders of children and adults in which symptoms are presumed or proven to arise as a result of neuromuscular (including interstitial cell of Cajal) dysfunction. Common to most of these diseases are symptoms of impaired motor activity which manifest as slowed or obstructed transit with or without evidence of transient or persistent radiological visceral dilatation. Such diagnoses include primary and secondary disorders of the oesophagus to the colon e.g. achalasia, gastroparesis, intestinal pseudo-obstruction and severe constipation. Pathologic abnormalities of the sensorimotor apparatus have been demonstrated in such disorders by a variety of methods since the 1960s; however, this remains an area of evolving interest especially with the increasing availability of newer techniques and more critical appraisal of those more established techniques.

This review outlines some of the more recent advances in this field, particularly in the area of small bowel disease manifesting as intestinal pseudo-obstruction. The area of Hirschsprung disease diagnosis, although numerically important (this being by far the most common GINMD) is not covered here since, although some contention exists, in general the techniques for this diagnosis are long and better established. The review covers the safe acquisition of tissue and advances in histopathological and allied techniques.

SAFE TISSUE ACQUISITION

Tissue may be taken with deliberate diagnostic intent or alternatively come as the by-product of emergency or planned surgical interventions. On this basis, tissues may take the form of mucosal, deep submucosal, seromuscular or full-thickness biopsies or resection.
specimens. Of particular note are recent advances in minimally invasive surgery that have permitted safe access and biopsy of a variety of intra-abdominal tissues including full-thickness bowel biopsy in adults with small bowel dysmotility, predominantly those with proven chronic idiopathic intestinal pseudo-obstruction (CIPO). A very recent study reported on the safety and diagnostic yield of a predominantly laparoscopically-assisted approach (Figure 1) to biopsy the small and large bowel in a cohort of 124 adults with suspected GINMD from 3 European centres. Median operating time was 50 min, conversion rate was 2% and length of stay was 1 d. There was an 8% readmission rate for obstructive symptoms; however, other morbidity was minimal and there were no mortalities. Overall the specific diagnostic yield was 81%, being high for jejunal biopsies (89%), but low for a small number of ileal and colonic biopsies. On this basis, an extracorporeal laparoscopically-assisted procedure appears safe and with acceptable yield if performed in the proximal small bowel for the indications in this study. Completely intracorporeal staple techniques may also be safe, but very little published data exists, at least for the jejunum. Laparoscopic gastric biopsies may also now be taken at the time of gastric pacing, and may be important in predicting outcome from this procedure on the basis of ICC pathology (personal communication: Gianrico Farrugia).

The potential to increase yield with multiple biopsies must be balanced against the risk of complications. Clearly, whilst there is some evidence from colectomy and post-mortem small bowel that sections should be taken at fixed intervals to avoid missing 'patchy' abnormalities of muscle or nerve, extending this finding to suggest multiple biopsies, even with a small risk for each is not currently advised. On this basis, as well as the potentially increased risks of leakage, laparoscopic full-thickness colonic biopsy is currently not advised, although seromuscular biopsies have been shown to be safe in a large series of paediatric patients and can also be used for determining the HSCR transition zone. The role of appendectomy as a diagnostic surrogate of GINMD has recently been suggested based on preliminary findings in diabetes, but needs further exploration. The evolving technique of NOTES (natural orifice transluminal endoscopic surgery) will in the future (in the author’s view) have an important role here, with proof of concept already demonstrated in the stomach. Regardless of technique, because of regional differences, whenever full-thickness biopsies are taken, the corresponding intestinal segment(s) should be precisely indicated to the pathologist.

**HISTOLOGICAL TECHNIQUES**

Although the histopathological diagnosis of GINMD (and exclusion of other disease) continues to be primarily based upon the analysis of H&E-stained sections with light microscopy, a number of other techniques can also be employed. A critical appraisal of the role of these techniques, particularly in comparison with the ‘yield’ of H&E, and guidelines for their use is currently being produced by an international working party: www.gastro2009.org/pdf/wp_project_desc07.pdf and is not covered here. Rather, descriptions of some newer diagnostic techniques are presented.

**Tinctorial stains (Figure 2)**

Although there is vast variation in current practice, tinctorial stains can supplement H&E with particular use in the assessment of specific structures and

---

**Figure 1** Laparoscopically-assisted full thickness jejunal biopsy. The port sites are shown. After finding a suitable proximal jejunal loop, the bowel is exteriorised by extending slightly the umbilical port incision and biopsy and suture closure performed extracorporeally (Courtesy of B Nyborg, Huddinge, Stockholm).

**Figure 2** Tinctorial stains used in GI neuromuscular histopathology. A: Periodic acid Schiff staining showing polyglucosan bodies in a patient with intestinal pseudo-obstruction; B: Bifringence from amyloid visualised by Congo red staining (x 25-40).
cell types. With periodic acid Schiff (PAS) staining, inclusion bodies e.g. polyglucosan, lipofuscin granules (secondary autophagic lysosomes), and glycogen can be observed, and PAS combined with diastase treatment can differentiate between glycogen and other structures (glycogen disappears after diastase pretreatment), which may be of value where a glycogenosis or related metabolic disorder are suspected. Polyglucosan inclusion body myopathy has recently been described in GINMD\cite{16} and cannot easily be identified without use of PAS staining. Amyloid is a rare secondary cause of GINMD and can be detected with ease using Congo red staining. With Giemsa staining, mast cells and eosinophils can be seen easily, and the condition of the neuronal cytoplasm assessed (marginalization of the Nissl and chromatolysis). Various types of trichrome staining assist in the establishment of fibrosis and in differentiation from interstitial oedema (both cause increases in the distance between cells, and in early fibrosis this can be difficult to differentiate). Relevant to some rare cases of GINMD, Gomori trichrome staining is also used to diagnose mitochondrial neurogastro-intestinal encephalomyopathy (MNGIE) on the basis of finding ‘ragged red fibres’ on skeletal muscle biopsy\cite{17}.

**Immunohistochemistry (IHC) (Figure 3)**

The past thirty years has seen the use of IHC evolve in many areas of GI practice including that of GINMD diagnosis. With respect to mainly diagnostic rather than research applications, neuronal markers such as PGP9.5 and neuron specific enolase (NSE) may be employed to assist in the determination of neurons particularly if quantitation is considered important. This latter point is very contentious because heterogeneity of methods has meant that few normative data exist for any single method, especially when age and regional specificity are considered\cite{18}. Nevertheless, diagnoses reliant on decreases in numbers of neurons and ganglia\cite{19} have complemented findings made previously using the more laborious technique of silver staining\cite{20}. Alpha smooth muscle actin deficiency has been demonstrated by IHC in some children\cite{21} and adults\cite{9} with GINMD, and stresses the importance also of regional specificity-this being a normal variant in the ileum\cite{9}. Inflammatory neuropathies\cite{8,10,22} and much less commonly leiomyopathies\cite{23} may be best diagnosed by immunocyte IHC when large infiltrates (visible on H&E) are not apparent. This finding may prompt further autoimmune investigation (below) and dictate important changes in therapy\cite{22-24}. Finally, c-kit (CD117) IHC has now become established in detecting changes in ICC numbers that certainly accompany, and may be causative of some GINMD\cite{25}.

Research applications of IHC have predominantly addressed disease mechanisms and pathways. In GINMD, many studies have attested to alterations in neurochemically-stained subsets of neurons allied to their differing functions. Changes said to underlie abnormal neuronal development\cite{26}, retarded colonic transit e.g. reduced substance-P\cite{5}, failure of sphincter...
relaxation e.g. decreased nitric oxide, or visceral hyperalgesia e.g. increased transient receptor potential channels have been variously reported. Beyond mechanism and target identification, whether such changes may become clinical biomarkers of disease or guide treatment is the subject of much ongoing study, particularly if identifiable on endoscopic mucosal biopsy.

Electron microscopy (Figure 4)
Ultrastructural examination of neurons, muscle and interstitial cells of Cajal can be a useful adjunct to the above assessments in certain patients. These include some rare childhood myopathies where H&E findings are absent or equivocal (e.g. subtle fibrosis, atrophy of myocytes or myocyte vacuolation), the identification of rare inclusion bodies suggestive of mitochondrial disorders and some ultrastructural changes of ICC and myocytes, including a transformation to more secretory phenotypes.

ADJUNCTIVE INVESTIGATIONS

Proteomic investigations
In cases characterized by clinical (adult onset, personal or family history of autoimmunity) and histopathological findings (especially inflammatory neuro or myopathies) an autoimmune pathogenesis may be suggested. A variety of antibodies directed to nuclear proteins and, to a lesser extent, membrane-bound receptors of the enteric neuromuscular compartment have been found in patients with secondary GINMP, especially of paraneoplastic origin. The presence of some of these autoantibodies in patients with idiopathic disorders affecting gut motility has prompted their attempted identification in several recent studies. In nearly all cases, proof of pathogenicity remains weak in comparison with established autoimmune diseases of the neuromuscular junction. For a very recent full review, see Kashyap & Farrugia, 2008. If clinically suspected, it is, however, reasonable to take a sample of serum for antibody testing. This should be sent to an established neuroimmunology unit where a variety of methods such as radioimmunoprecipitation assays may be employed (Figure 5). Established antibody tests include those for anti-Hu (anti voltage-gated calcium channels (particularly in paraneoplasia), anti smooth muscle (particularly in myopathies and scleroderma), anti-ganglionic acetylcholine receptor (particularly if associated with dysautonomia) and anti voltage-gated potassium channel antibodies. One other blood-based investigation occasionally indicated in the investigation of pseudo-obstruction is the thymidine phosphorylase leucocyte activity assay in patients suspected on the basis of clinical findings to have MNGIE.

Genomic investigations
Recent history has witnessed a colossal expansion in data regarding the human genome in health and disease. In keeping with this, several studies have demonstrated molecular genetic changes that accompany, and in some instances, contribute to various forms of intestinal dysmotility. Whilst offering interesting research insights, few presently have great value in clinical practice, and these are in the most part limited...
to quite characteristic clinical syndromes. Most utilised are candidate single gene approaches, and these have been applied to screening for RET mutations in patients with Hirschsprung disease\(^{43}\) or suspected multiple endocrine neoplasia (MEN) 2 syndromes\(^{44}\), and thymidine phosphorylase mutation analysis in patients with MNGIE\(^{45}\). A variety of tests may also be appropriate in patients in which GI dysmotility may accompany other systemic diseases such as muscular dystrophy, cystic fibrosis and neurofibromatosis. In most cases, the information delivered is used to guide genetic counselling, and prognosis rather than influence diagnosis (except prenatally) or treatment (except in MEN where prophylactic surgery may be required to prevent subsequent neoplasia\(^{46}\)).

ACKNOWLEDGEMENTS

We thank Dr. B Nyborg, Huddinge Hospital, Stockholm for providing Figure 1.

REFERENCES

1. De Giorgio R, Sarnelli G, Corinaldesi R, Stanghellini V. Advances in our understanding of the pathology of chronic intestinal pseudo-obstruction. Gut 2004; 53: 1549-1552
2. Knowles CH. New horizons in the pathogenesis of gastrointestinal neuromuscular disease. J Pediatr Gastroenterol Nutr 2007; 45 Suppl 2: S97-S102
3. Wingate D, Hongo M, Kellow J, Lindberg G, Smout A. Disorders of gastrointestinal motility: towards a new classification. J Gastroenterol Hepatol 2002; 17 Suppl: S1-S14
4. Greig JD, Miles WF, Nixon SJ. Laparoscopic technique for small bowel biopsy. Br J Surg 1995; 82: 363
5. Hutson JM, Chow CW, Borg J. Intractable constipation with a decrease in substance P-immunoreactive fibres: is it a variant of intestinal neuronal dysplasia? J Pediatr Surg 1996; 31: 580-583
6. King SK, Sutcliffe JR, Hutson JM. Laparoscopic seromuscular colonic biopsies: a surgeon’s experience. J Pediatr Surg 2005; 40: 381-384
7. Familoni BO, Abell TL, Voeller G. Measurement of gastric and small bowel electrical activity at laparoscopy. J Laparoendosc Surg 1994; 4: 325-332
8. Tornblom H, Lindberg G, Nyberg B, Veress B. Full-thickness biopsy of the jejunum reveals inflammation and enteric neuropathy in irritable bowel syndrome. Gastrointestinal Endoscopy 2002; 123: 1972-1979
9. Knowles CH, Silk DB, Darzi A, Veress B, Feakins R, Raimundo AH, Crompton T, Browning EC, Lindberg G, Martin JE, Darzi A, Martin JE, Nyberg B, Lindberg G, Safety and diagnostic yield of laparoscopically assisted full-thickness bowel biopsy. Neurogastroenterol Motil 2008; 20: 774-779
10. McCallum RW, Chen JD, Lin Z, Schirmer BD, Williams RD, Ross RA. Gastric pacing improves emptying and symptoms in patients with gastroparesis. Gastroenterology 1998; 114: 456-461
11. Fitzgibbons PL, Chandrasoma PT. Familial visceral myopathy. Evidence of diffuse involvement of intestinal smooth muscle. Am J Surg Pathol 1987; 11: 846-854
12. Miller SM, Narasimhan RA, Schmalz PF, Sofer EE, Walsh RM, Krishnamurthi V, Pasricha PJ, Szurszewski JH, Farrugia G. Distribution of interstitial cells of Cajal and nitrergic neurons in normal and diabetic human appendix. Neurogastroenterol Motil 2008; 20: 349-357
13. Knowles CH, De Giorgio R. Observations on a vestigial organ: a potential surrogate for enteric neuroendmesenchymal disease. Neurogastroenterol Motil 2004; 16: 317-326
14. Rajan E, Gostout CJ, Lukren MS, Talley NJ, Locke GR, Szarka LA, Sumiyama K, Bakken TA, Stoltz GJ, Knipschild MA, Farrugia G. Endoscopic ‘no hole’ full-thickness biopsy of the stomach to detect myenteric ganglia. Gastrointest Endosc 2008; 68: 301-307
15. Knowles CH, Nickols CD, Feakins R, Martin JE. A systematic analysis of polyglycozan bodies in the human gastrointestinal tract in health and disease. Acta Neuropathol 2003; 105: 410-413
16. Mueller LA, Camillieri M, Emslie-Smith AM. Mitochondrial neurogastrointestinal encephalomyopathy: manometric and diagnostic features. Gastroenterology 1999; 116: 959-963
17. Csendes A, Smok G, Braghetto I, Gonzalez P, Henriquez A, Csendes P, Pizurno D. Histological studies of Auerbach’s plexuses of the oesophagus, stomach, jejunum, and colon in patients with achalasia of the oesophagus: correlation with gastric acid secretion, presence of parietal cells and gastric emptying of solids. Gut 1992; 33: 150-154
18. Wedel T, Roblick UJ, Ott V, Eggers R, Schiedek TH, Krammer HJ, Bruch HP. Oligoneuronal hypoganglionosis in patients with idiopathic slow-transit constipation. Dis Colon Rectum 2002; 45: 54-62
19. Krishnamurthy S, Schuffner MD, Rohrman CA, Pope CE. 2nd. Severe idiopathic constipation is associated with a distinctive abnormality of the colonic myenteric plexus. Gastroenterology 1985; 88: 26-34
20. Smith VV, Lake BD, Kamm MA, Nicholls RJ. Intestinal pseudo-obstruction with deficient smooth muscle alpha-actin. Histopathology 1992; 21: 533-542
21. Smith VV, Gregson N, Foggensteiner L, Neale G, Milla PJ. Acquired intestinal aganglionosis and circulating autoantibodies without neoplasia or other neural involvement. Gastroenterology 1997; 112: 1366-1371
22. Ruuska TH, Karkkoski R, Smith VV, Milla PJ. Acquired myopathic intestinal pseudo-obstruction may be due to autoimmune enteric leiomyositis. Gastroenterology 2002; 122: 1133-1139
23. De Giorgio R, Barbara G, Stanghellini V, De Ponti F, Salvio B, Tonini M, Velo P, Bassotti G, Corinaldesi R. Clinical and morphofunctional features of idiopathic myenteric ganglionitis underlying severe intestinal motor dysfunction: a study of three cases. Am J Gastroenterol 2002; 97: 2454-2459
24. Lyford GL, He CL, Soffer E, Hull TL, Strong SA, Senagore AJ, Burgart LJ, Young-Fadok T, Szurszewski JH, Farrugia G. Pan-colonic decrease in interstitial cells of Cajal in patients with slow transit constipation. Gut 2002; 51: 496-501
25. Facer P, Knowles CH, Thomas PK, Tam PK, Williams NS, Anand P. Decreased tyrosine kinase C expression may reflect developmental abnormalities in Hirschspring’s disease and idiopathic slow-transit constipation. Br J Surg 2001; 88: 545-552
26. Bruley des Varannes S, Chevalier J, Pimont S, Le Neel JC, Klotz M, Schafer KH, Galmiche JP, Neunlist M. Serum actin. Gut 2006; 55: 319-326
27. Bhat YM, Bielefeldt K. Capsaicin receptor (TRPV1) and non-erosive reflux disease. Eur J Gastroenterol Hepatol 2006; 18: 263-270
28. Smith VV, Milla PJ. Histological phenotypes of enteric smooth muscle disease causing functional intestinal obstruction in childhood. Histopathology 1997; 31: 112-122
29. Barnett HL, McDonnell WM, Appelqvist JD, McCreery WO. Familial visceral neuropathy with neuronal intranuclear inclusions: diagnosis by rectal biopsy. Gastroenterology 1992;
32 Ohlsson B, Veress B, Lindgren S, Sundkvist G. Enteric ganglioneuritis and abnormal interstitial cells of Cajal: features of inflammatory bowel disease. Inflamm Bowel Dis 2007; 13: 721-726
33 Lennon VA, Sas DF, Busk MF, Scheithauer B, Malagelada JR, Camilleri M, Miller LJ. Enteric neuronal autoantibodies in pseudoobstruction with small-cell lung carcinoma. Gastroenterology 1991; 100: 137-142
34 De Giorgio R, Bovara M, Barbara G, Canossa M, Sarnelli G, De Ponti F, Stanghellini V, Tonini M, Cappello S, Pagnotta E, Nobile-Orazio E, Corinaldesi R. Anti-HuD-induced neuronal apoptosis underlying paraneoplastic gut dysmotility. Gastroenterology 2003; 125: 70-79
35 Vernino S, Low PA, Fealey RD, Stewart JD, Farrugia G, Lennon VA. Autoantibodies to ganglionic acetylcholine receptors in autoimmune autonomic neuropathies. N Engl J Med 2000; 343: 847-855
36 Pardi DS, Miller SM, Miller DL, Burgart LJ, Szurszewski JH, Lennon VA, Farrugia G. Paraneoplastic dysmotility: loss of interstitial cells of Cajal. Am J Gastroenterol 2002; 97: 1828-1833
37 Knowles CH, Lang B, Clover L, Scott SM, Gotti C, Vincent A, Martin JE. A role for autoantibodies in some cases of acquired non-paraneoplastic gut dysmotility. Scand J Gastroenterol 2002; 37: 166-170
38 Tornblom H, Lang B, Clover L, Knowles CH, Vincent A, Lindberg G. Autoantibodies in patients with gut motility disorders and enteric neuropathy. Scand J Gastroenterol 2007; 42: 1289-1293
39 Vincent A. Measuring and evaluating the significance of autoantibodies in neurological disorders. Clin Appl Immunol Review 2002; 3: 127-151
40 Kashyap P, Farrugia G. Enteric autoantibodies and gut motility disorders. Gastroenterol Clin North Am 2008; 37: 397-410, vi-vii
41 Lennon VA, Kryzer TJ, Griesmann GE, O'Suilleabhain PE, Windebank AJ, Woppmann A, Miljanich GP, Lambeth EH. Calcium-channel antibodies in the Lambert-Eaton syndrome and other paraneoplastic syndromes. N Engl J Med 1995; 332: 1467-1474
42 Nishino I, Spinazzola A, Hirano M. Thymidine phosphorylase gene mutations in MNGIE, a human mitochondrial disorder. Science 1999; 283: 689-692
43 Angrist M, Bolk S, Thiel B, Puffenberger EG, Hofstra RM, Buys CH, Cass DT, Chakravarti A. Mutation analysis of the RET receptor tyrosine kinase in Hirschsprung disease. Hum Mol Genet 1995; 4: 821-830
44 Mulligan LM, Kwok JB, Healey CS, Elsdon MJ, Eng C, Gardner E, Love DR, Mole SE, Moore JK, Papi L. Germ-line mutations of the RET proto-oncogene in multiple endocrine neoplasia type 2A. Nature 1993; 363: 458-460
45 Decker RA. Long-term follow-up of a large North American kindred with multiple endocrine neoplasia type 2A. Surgery 1992; 112: 1066-1072; discussion 1072-1073

www.wjgnet.com