Non-invasive investigation of inflammatory bowel disease

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Received 2001-03-20 Accepted 2001-04-15

Abstract
The assessment of inflammatory activity in intestinal disease in man can be done using a variety of different techniques. These range from the use of non-invasive acute phase inflammatory markers measured in plasma such as C reactive protein (CRP) and the erythrocyte sedimentation rate (ESR) (both of which give an indirect assessment of disease activity) to the direct assessment of disease activity by intestinal biopsy performed during endoscopy in association with endoscopic scoring systems. Both radiology and endoscopy are conventional for the diagnosis of inflammatory bowel disease (IBD). However these techniques have severe limitations when it comes to assessing functional components of the disease such as activity and prognosis. Here we briefly review the value of two emerging intestinal function tests. Intestinal permeability, although ideally suited for diagnostic screening for small bowel Crohn’s disease, appears to give reliable predictive data for imminent relapse of small bowel Crohn’s disease and it can be used to assess responses to treatment. More significantly it is now clear that single stool assay of neutrophil specific proteins (calprotectin, lactoferrin) give the same quantitative data on intestinal inflammation as the 4-day faecal excretion of 111InIndium labelled white cells. Faecal calprotectin is shown to be increased in over 95% of patients with IBD and correlates with clinical disease activity. It reliably differentiates between patients with IBD and irritable bowel syndrome. More importantly, at a given faecal calprotectin concentration in patients with quiescent IBD, the test has a specificity and sensitivity in excess of 85% in predicting clinical relapse of disease. This suggests that relapse of IBD is closely related to the degree of intestinal inflammation and suggests that targeted treatment at an asymptomatic stage of the disease may be indicated.

Subject headings inflammatory bowel diseases; permeability; NCAM; membrane glycoproteins


INTRODUCTION
Distinguishing irritable bowel syndrome from inflammatory bowel disease
Gastroenterologists are often faced with the diagnostic difficulty of differentiating patients with the irritable bowel syndrome (IBS) from those with organic intestinal pathology, in particular inflammatory bowel disease (IBD). Many symptoms are common to both conditions including abdominal pain, bloating, excessive flatus and altered bowel habit while other clinical features such as a predominance of diarrhoea and rectal bleeding will increase the likelihood of organic disease. Although symptoms are a surprisingly good guide to a diagnosis, most clinicians proceed to and rely on laboratory tests to aid in the differential diagnosis. Certainly, fulfilling the ROME criteria[1,2] and having a normal full blood count, routine biochemical screening, ESR and CRP are reassuring indicators pointing to IBS. As a result a number of investigators[3,4] have recommended a straightforward approach to evaluation and treatment of patients with IBS based on the use of the Rome criteria as a means of cost effective management. Despite this the use of the Rome criteria has not been universal and is largely confined to use as entry criteria into research studies of patients with IBS. The concern for gastroenterologists is that some patients with organic intestinal disease will be incorrectly diagnosed if excess reliance is placed upon these criteria. They may therefore feel compelled to exclude all organic disease using invasive diagnostic investigations as objective evidence for there being no other significant pathology. This has significant implications for health care costs as well as exposing patients to the inherent risks associated with invasive procedures.

Managing inflammatory bowel disease
Once IBD is diagnosed the treatment involves induction and subsequently maintenance of remission based largely on clinical disease activity indices[3,6] and the physicians global assessment of well-being. The problem with the use of clinical disease activity scores is that they are a composite of quantitative subjective symptoms that are affected by non inflammatory processes such as fibrous strictures, fistulas and previous surgical intervention. As a guide to clinical decision making, many clinicians therefore use nonspecific laboratory tests to document relapse of disease and radiology and radio isotopic techniques to distinguish between actively inflamed disease and fibrotic strictures. In addition a number of blood tests (erythrocyte sedimentation rate (ESR), orosomucoid, C -reactive protein (CRP), platelet, and white cell counts, IL-6, TNF-α, IL-1β)[7-11] which reflect the systemic consequences of inflammation, have been proposed as predictors and/or markers of clinical relapse of IBD with varying degrees of success. However, the overall predictive values of these different variables in identifying patients at risk of relapse have in general been disappointing. This is possibly due to the fact that these measures are non-specific, affected by a variety of non-intestinal diseases[12] and most importantly do not measure the intestinal inflammation directly. Patients with clinically active IBD can have normal serological inflammatory indices while clinically quiescent disease may be associated with abnormal blood tests. In particular, there is a major discrepancy between severity of
symptoms and macroscopic evaluation of disease activity in patients with Crohn’s disease limited to the colon.

**Intestinal function tests**

Although imperfect the above approach to diagnosis and management of patients with IBD remains the norm and in general it works well for the vast majority of patients. However, few would argue with the notion that there is scope for improvement. Where is such improvement to come from?

Recently, investigators have turned to direct tests of intestinal function. Such tests provide new, direct and different information. They have the potential to be used as a diagnostic screen for intestinal disorders as well as providing prognostic information for the behaviour of the disease. At present there are three kinds of intestinal function tests that could fulfill the above promise, two of which (intestinal permeability and white cell scans) have a 20 year history. The third, namely direct assay of faeces for inflammatory markers, we suspect has the greatest potential. There follows a brief outline of how these tests can provide information that is not obtainable by other methods and their possible use in the day-to-day management of patients with IBD.

**INTESTINAL PERMEABILITY**

Permeability refers to that property of a membrane that enables passage of a solute by unmediated diffusion. The diffusion of a solute across a simple membrane is determined by the structure of the membrane (in terms of its composition, charge, thickness, etc.), the physicochemical properties of the solute (like molecular size, shape, charge and solubility) and its interaction with the media or solvent. Intestinal permeability is assessed non-invasively in vivo by measuring urinary excretion of orally administered substances.

The ideal permeability probe is water-soluble, non-toxic, non-degradable and not metabolised before, during or after permeating the intestine[13]. The probes should preferably not be naturally present in urine, be completely excreted in the urine following intravenous administration and be easily and accurately measurable. Fordtran et al[14] were instrumental in the development of ideas for assessing intestinal permeability in man but it was Menzies who introduced oligosaccharides as test substances for the non-invasive assessment of intestinal permeability[15] in 1974, and later formulated the principle of differential urinary excretion of orally administered test substances[16]. The importance of the differential urinary excretion principle is that it overcomes most if not all the problems associated with the use of a single test substance, where urinary excretion is dependent on a number of pre- and post-mucosal factors as well as intestinal permeability. The differential principle advocates that a nonhydrolyzed disaccharide (i.e. lactulose) and a monosaccharide (L-rhamnose or mannitol) are ingested together. As the pre- and post-mucosal determinants of urinary excretion affect the two test substances equally and the differential 5 hour urinary excretion ratio (ratio of lactulose/L-rhamnose) is not affected by these variables the urinary excretion ratio becomes a specific measure of intestinal permeability.

Tests of intestinal permeability were initially designed to allow reliable non-invasive detection of patients with untreated coeliac disease[16]. The tests have since come to be viewed as synonymous with assessing intestinal barrier function. In clinically active small bowel Crohn’s disease the vast majority of patients (>95%) have an increase in the differential urinary excretion of ingested di-/mono-saccharides (lactulose/L-rhamnose or mannitol) and half of those with Crohn’s colitis are abnormal[17]. These figures are marginally improved with the use of 51CrEDTA, which requires a 24-hour, as opposed to a 5-hour urinary collection. The vast majority of patients with ulcerative colitis have normal small intestinal permeability when assessed by these methods. However, tests of intestinal permeability have not found widespread application as screening tests to discriminate between patients with Crohn’s disease and IBS. The reason for this is probably that the urinary sugar analysis is time consuming and demanding, and there may be some concern that the tests lack specificity being abnormal in a variety of small intestinal diseases (Table 1). At first sight the test appears to identify a number of “clinically irrelevant” diseases, which usually translates into disease for which no treatment is available, but in practice the tests seem often to identify small intestinal pathology where none was previously expected, thus expanding the number of identifiable small bowel pathologies.

There have been attempts to use intestinal permeability as an index of disease activity in Crohn’s disease. In general these have been disappointing because the degree of increase in the differential urinary excretion of lactulose/L-rhamnose or the excretion of 35CrEDTA is dependent on localisation and extent of disease within the small bowel as well as activity of the inflammation[16]. Abnormalities in intestinal permeability may, however, be used as a predictor of imminent relapse of quiescent Crohn’s disease. Three studies have now shown that, in patients with Crohn’s disease in clinical remission, an increased intestinal permeability can predict those at significant risk of relapse of disease in the next few months[17-19]. The strength of this association is difficult to assess from the published studies. Nevertheless, less than 20% of those with normal intestinal permeability appear to relapse over the ensuing 6 months. Interestingly, elevated levels of IL-6 in serum, which can be viewed as a surrogate marker of intestinal inflammation, also has a predictive value for relapse of Crohn’s disease[20], but receiver operating curve (ROC) analysis shows relatively low sensitivity and specificity (70 and 50%, respectively). The permeability ratio differs from such indices in that it is not based on concentrations of plasma proteins but rather represents functional changes in the intestinal mucosa, a direct consequence of intestinal inflammation.

The clinical implications of these findings are discussed later.

**WHITE CELL SCANS AND FAECAL EXCRETION**

Intense neutrophil recruitment to the intestinal mucosa is a feature common to inflammatory bowel diseases[20]. When a patient’s own radiolabelled neutrophils are re-injected they migrate to sites of acute inflammation as well as to the liver, spleen and bone marrow[21]. Segal, Saverymuttu and Chadwick were instrumental in the introduction, validation and implication of the 111Indium white cell technique for use in gastroenterology[20,22]. The technique visualises inflamed segments of bowel and quantitates the degree of inflammatory activity[20,23-26].

A number of studies have established that abdominal scans are abnormal in virtually all patients with active IBD: their accuracy in localisation of disease and distinguishing between actively inflamed and fibrous stricturing disease has implications for treatment. It was suggested that the technique could be used to discriminate, with an accuracy approaching 100%, between patients with IBD and IBS at the first outpatient visit. In practice this suggestion was not followed up with relevant research.

When combined with measurement of the 4 day faecal
excretion of labelled white cells for quantitation of the inflammatory activity the technique becomes a formidable tool for research and investigation. The faecal excretion of the labelled white cells quantitate inflammation accurately and can be used to document therapeutic efficacy of various treatments in IBD. It has also been used to define a number of enteropathies (NSAIDs, alcohol, chronic renal failure, hypogammaglobulinaemia, HIV-AIDS, etc.) where none were suspected or impossible to demonstrate by techniques other than perhaps the intestinal permeability tests (Table 1). The method is not disease specific, resembling that of the permeability tests, but it is specific for intestinal inflammation. This is not a drawback as it is a simple matter to distinguish between the inflammatory activity in patients with IBD and the above enteropathies, colonic cancer, diverticulitis, etc., since patients with active IBD have excretion values often an order of magnitude higher than the others.

<table>
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<tr>
<th>Nonsteroidal anti-inflammatory drugs</th>
<th>Inflammatory bowel disease</th>
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<td>Alcohol</td>
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<td>Renal failure</td>
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<td>Abdominal radiation</td>
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<td>Cytotoxic drug treatment</td>
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Why has the white cell technique not been universally adapted for use as a diagnostic screen in IBD, and to assess disease activity? It requires expensive labeling facilities including labelling cabinets. The labeling procedure is time consuming, taking over 2 hours. The cost of isotope and material is in excess of $200 (US $300) and the radiation dose is not trivial if abdominal scans are carried out, being equivalent to that of a barium enema. A complete 4-day faecal collection is also demanding and unpleasant for patients, occasionally requiring hospital admission.

Other methods have attempted to build on this success. One such is 99mTc labeling of white cells. This is purported to give superior quality abdominal scintigraphy (which is not clinically important), but does not allow late (>4 hours) scanning, because the label comes off and is excreted into the bowel independent of white cell excretion. Furthermore a faecal collection provides no quantitative information on intestinal inflammation (as the Technetium comes off the white cells and is excreted in faeces) and the labeling requires the same facilities as the white cells.

Newer techniques include E-selectin scanning. This method is derived from the more conventional labelled white cell scintigraphy, but uses a labelled antibody to E-selectin, which is over-expressed in endothelial cells at sites of inflammation. It has the advantage of studying a more fixed entity that (unlike white cells) will not be shed at a variable rate into the bowel lumen and is applicable to the occasional patient with intestinal inflammation who is neutropenic.

In our opinion, the greatest impact that the white cell technique has had is that it emphasised that if a sensitive method is to be established for assessing intestinal function there are no shortcuts. Neurologists assess spinal fluid, respiratory physicians assess sputum, urologists urine and the gastroenterologist needs to come terms with the fact that faecal analysis is essential to obtain maximal information about the state of the intestine. emphasised that there is life beyond morphological assessment of the gut (x-ray and colonoscopic studies). raised the possibility of dramatically changing our views on the treatment of IBD. Many patients with IBD in full clinical remission are shown to have significant intestinal inflammation. At present treatment is non-specifically directed at maintaining remission (5-ASA, azathioprine, etc.). It seems highly probable that those patients with substantial inflammatory activity should be targeted for more aggressive therapy, in particular if they can be shown to be at significant risk of clinical relapse of disease. The analogy with the treatment of rheumatoid arthritis springs to mind. Here, first line treatment is directed to wards reducing the acute inflammatory component of the disease followed by a number of second line agents that can alter the natural history of the disease, reduce the frequency, duration and severity of relapses as well as reducing the joint damage.

### FAECAL MARKERS

Faecal analysis is unpleasant but has been with us for a long time. Measure of electrolytes and osmolality helped in the differential diagnosis of diarrhoea in children. Faecal fats were a widespread screening test for steatorrhoea for a while and faecal occult bloods have become the yardstick for colorectal screening with which other methods need to be compared. An improvement on these techniques was the introduction of radioisotopically labelled compounds (labelled red blood cells, proteins, white cells) which provided quantitative and functional data and which was event specific (blood loss, inflammation, protein losing enteropathy, etc.) but non-specific for disease.

The inflamed hyperpermeable mucosa of patients with inflammatory bowel disease is associated with increased protein loss into the bowel lumen. Studies using radiolabelled proteins have demonstrated that there is faecal protein loss in patients with active Crohn’s disease and it may therefore be a useful marker of disease activity. Other studies have shown faecal α1 antitrypsin clearance to be a useful indicator of protein losing enteropathy and that in patients with inflammatory bowel disease, 72 hour faecal clearance of α1 antitrypsin correlates with that of 51Cr-albumin, and moderate rectal bleeding does not affect the α1 antitrypsin determination. Random faecal α1 antitrypsin levels have been shown to be as useful as more prolonged collection in measuring Crohn’s disease activity and correlated with several other laboratory measures that have been proposed as indicators of Crohn’s disease activity.

Concerns about costs, radiation, and the need for prolonged faecal collections all worked against these techniques for routine use, although many remain very important for research studies. The idea then emerged that it might be possible to assay for cell proteins or substances that are specifically associated with a certain cell type and which would then provide information on a specific component of the inflammatory cascade. Ferguson’s Edinburgh group was instrumental in expanding this idea. Concerned about bacterial degradation of markers they used a whole gut lavage method involving ingestion of polyethylene-based purgatives.
(Kleenprep or GoLitely) for obtaining clear liquid faecal samples for analysis. The analysis took to various markers, such as immunoglobulins, neutrophils-specific elastase, and haemoglobin. Separate studies showed that Crohn’s disease could be identified with ease, and that the method had a greater sensitivity for colorectal cancer than the conventional faecal occult blood technique. Ideally suited for research, the method has as yet not found wide application for routine screening purposes, possibly because of the drawback of patients needing to ingest large volumes of liquid.

Direct analysis of markers in faeces would be a major advance on this method. Here the problem is initially the bacterial degradation of the marker necessitating swift sample handling. One such marker, TNF, has been successfully used in children and in HIV infection in adults. However, it is now clear that it is not necessary for the marker to be completely non-degraded, provided that the antibody (most of these assays are ELISA’s or radio immunoassay) is directed at an epitope of the molecule which resists degradation. One such assay is that for lactoferrin. Lactoferrin is a relatively specific marker for neutrophils, in which it is present in cytoplasmic granules.

**Faecal calprotectin**

The greatest experience with analysis of faecal proteins is with calprotectin. It accounts for up to 50% of the neutrophilic cytosolic protein while being resistant to colonic bacterial degradation. It is easily measured in faeces by a commercially available ELISA.

Calprotectin was first isolated from granulocytes by Fagerhol et al. and named L1 protein, but was later named calprotectin upon identification of its calcium binding and antimicrobial properties. The protein is a heterocomplex protein consisting of two heavy (L1H) chains and one light (L1L) chain, which are non-covalently linked. Calprotectin appears to play a regulatory role in the inflammatory process and functions in both an antimicrobial and antiproliferative capacity. It has both bactericidal and fungicidal properties with minimal inhibitory concentrations comparable to those of many antibiotics. It is released from the cells during cell activation or cell death. The C-terminal sequence of the L1H chain has been shown to be identical to the N-terminus of peptides known as neutrophil immobilising factors (NIF).

It has been suggested that NIF activity of the L1H chain depends upon its phosphorylation and that such an activity of calprotectin could be important for the accumulation of granulocytes, while calprotectin released from dead neutrophils, macrophages and epithelial cells might exert antimicrobial activity, possibly by depriving microorganisms of zinc. Calprotectin may inhibit metalloproteinases which may also involve the deprivation of zinc suggesting that it may limit their participation as enzymatic cofactors for invading organisms. Interest in calprotectin as a marker for inflammation in the gut followed the realisation that 111 Indium labelled granulocyte scans could be used to both visualise and quantitate the acute inflammation in the gut of patients with inflammatory bowel disease. These findings led to the idea that an increased influx of granulocytes into the intestinal mucosa in conditions of inflammation might give increased levels of proteins from such cells in faeces.

Others have demonstrated that eosinophilic granulocytes are the main cellular source of calprotectin in the normal gut mucosa. However, relatively high levels of calprotectin are found in the stools of normal individuals-about six times the plasma levels (which are about 0.5mg/L). This is compatible with data suggesting that in normal individuals most circulating neutrophils migrate through the mucosal membrane of the gut wall and thereby terminate their circulating life. Subsequent lysis within the gut lumen and release of cytosolic calprotectin thereby accounts for the median faecal levels of 2.0mg/L seen in healthy controls. The diagnostic use of faecal calprotectin in a broad spectrum of intestinal diseases has been studied by a number of groups with remarkable agreement between the results to date.

**Inflammatory bowel disease**

It is almost possible to extrapolate all the findings obtained with the white cell faecal excretion technique to the calprotectin method. Both techniques correlate with histopathological assessment of disease activity in ulcerative colitis and there is a very good correlation between the 4-day faecal excretion of white cells and faecal calprotectin concentrations. A correlation which is maintained when single stool calprotectin concentrations are used as opposed to 1 or 4 day collections. The faecal calprotectin concentration has a narrow normal range with an upper limit of 10mg/L. As with the white cells, faecal calprotectin has potential as a screening procedure to differentiate between patients with IBD and IBS and it may be useful for documenting a fall in intestinal inflammation in response to successful treatment of disease. Calprotectin concentration is rarely within the normal range in patients with IBD despite full clinical remission and is therefore a highly sensitive method for detecting such patients irrespective of disease activity. In over 100 patients with Crohn’s disease of varying severity and activity only 4 had normal calprotectin concentrations.

Since the method is so much simpler than the white cell technique, requiring only a single stool sample, extraction and an ELISA, it has potential as a screening test to distinguish between patients with IBD and IBS in an outpatient setting. One study in over 225 patients showed that a cut off of 30mg/L had a 100% sensitivity and 94% specificity for this purpose. Another showed that this was also the case when over 600 unselected consecutive patients were studied. Indeed a patient presenting with positive ROME criteria and a normal faecal calprotectin has virtually no chance of having IBD. As a result of these studies it is now our practice not to investigate such patients by radiology or colonoscopy with considerable cost saving implications. The white radiolabelled cell technique demonstrated reduced intestinal inflammation in response to 5-ASA treatment and elemental diets. We have shown (unpublished) that improvement in calprotectin parallels the improvement in the excretion of labelled white cells in response to treatment with elemental diets. These techniques prove to be much more reliable and reproducible than the changes in clinical disease indices. It seems likely that the assay of faecal calprotectin will become an integral part of the assessment of therapeutic efficacy of the acute inflammation in future treatment trials in patients with IBD.

Apart from screening and assessing response to treatment, the faecal calprotectin has a further major advantage over the white cell labeling technique in predicting relapse of IBD. It has been shown that, in patients with clinically quiescent IBD (ulcerative colitis and Crohn’s disease), faecal calprotectin values above 50mg/L may be used to predict clinical relapse of disease within a few months with over 80% sensitivity. Symptoms of inflammatory bowel disease often appear to be the direct consequence of the inflammatory process itself and often vary dependent upon...
the location of the inflammation. Most patients with quiescent IBD have low-grade inflammation and it is possible that symptomatic relapse occurs only when the inflammatory process reaches a critical intensity. Furthermore, as inflammation is a continuous process it may be that the direct assessment of the level of inflammatory activity may provide a quantitative pre-symptomatic measure of imminent clinical relapse of the disease.

The clinical implications of this, if substantiated, are considerable as it might offer targeted treatment at an earlier stage, with less side effects, to avert the relapse, as well as assessment of new therapeutic strategies to maintain symptomatic remission. At present this is done with some degree of success with the rather indiscriminate use of sulphasalazine, 5-ASA and azathioprine, all of which are associated with side effects. However the calprotectin method offers guidance as to whom to treat at this stage and with what kind of vigour. Theoretically such treatment should lead to a dramatic reduction in the frequency and severity of clinical relapses with an improvement in the patient’s quality of life.

In addition, the identification of patients at high risk of relapse will improve the design of clinical trials to assess the efficacy of therapeutic regimes designed to maintain patients in remission. In most such trials, patients studied tend to be a heterogeneous mix of those with high and low risk of relapse. This introduces possible bias when assessing the response to a particular treatment regime due to the imbalance of high risk patients in each treatment arm. Stratification by risk group using faecal calprotectin would reduce the possibility of such a bias. It is also possible that a lack of power in detecting a response to treatment may be due to the study of a large number of patients at low/intermediate risk of relapse, in whom all treatments may show the same efficacy, and therefore clinical trials studying a homogenous high risk group may be more powerful in detecting a difference in treatment efficacy. Much work remains to be done, some is already on its way, but what is clear is that gastroenterologists need to move with the times and start thinking along the lines that rheumatologists do, that is, to implement treatments that alter the natural history of the disease. We are now in possession of tests that have the potential to revolutionise our approach to treatment of patients with IBD. There are some hurdles to overcome. The most frequent criticism of the “faecal” tests is that they are unacceptable to patients and unpleasant to work with.

The faecal calprotectin and lactoferrin methods are the first wave of techniques that allow non-invasive assessment of specific and selective cellular components of the intestinal inflammatory cascade. At present these are useful for a variety of purposes, outlined above, but it is likely that it will be possible to estimate the participation of other cells. Many other cells of the inflammatory cascade are numerically increased in biopsy specimens from patients with a variety of gastroenterological conditions. Some, such as mast cells and eosinophils, are thought to play a central role in mediating the pathogenesis of chronic intestinal inflammation. It may therefore be possible, as for neutrophils and calprotectin, to identify mast cell granule proteins, such as tryptase and chymase, in faecal samples and use them as markers of a specific component of the intestinal inflammatory response.

The long-term objective might be to fully automate a faecal sample assay method that provides specific information on the activity of acute inflammation (neutrophils), chronic inflammation (T-cells) and allergy (mast cells).

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Edited by Rampton DS and Ma JY