Escherichia coli and Ulcerative colitis

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by

Björn Joakim Rembacken

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## CONTENTS

**STATEMENT OF INVOLVEMENT IN THE THESIS** ................................................................. 5

**INFLAMMATORY BOWEL DISEASE—CLINICAL CONSIDERATIONS** ....................... 6

- **HISTORICAL BACKGROUND** ...................................................................................... 6
- **CLINICAL FEATURES** .................................................................................................. 6
  - Complications and extra-intestinal manifestations ......................................................... 9
- **INVESTIGATIONS** ........................................................................................................ 13
- **HISTOLOGY** ................................................................................................................. 15
- **TREATMENT** ............................................................................................................... 16
- **PROGNOSIS** ............................................................................................................... 21

**AETIOLOGICAL CONSIDERATIONS** ............................................................................. 22

- **ETHNIC GROUPS** ....................................................................................................... 23
- **GENETIC FACTORS** .................................................................................................... 23
- **APPENDECTOMY AND COLITIS** .................................................................................. 28
- **THE ORAL CONTRACEPTIVE PILL** ............................................................................. 29
- **FOOD ALLERGY** ......................................................................................................... 29
- **SMOKING** .................................................................................................................. 29
- **THE MUCOSAL MUCUS LAYER** ................................................................................ 29
- **THE MUCOSAL MICROVASCULATURE** .................................................................. 30

**INFLAMMATORY PROCESSES** ..................................................................................... 32

- **THE NORMAL MUCOSAL IMMUNE SYSTEM** ............................................................ 33
- **MEDIATORS OF INFLAMMATION** .............................................................................. 34
  - Non-specific mediators of inflammation ................................................................. 34
  - Cytokines .................................................................................................................... 35
- **IMMUNOLOGICAL ABNORMALITIES IN INFLAMMATORY BOWEL DISEASE** ...... 40
  - Humoral immune system ......................................................................................... 40
  - Cell mediated immunity ......................................................................................... 41
  - Epithelium ................................................................................................................ 42
  - Future therapy to manipulate the mucosal immune response ............................... 48
- **ULCERATIVE COLITIS - AN AUTOIMMUNE DISEASE?** ............................................. 54

**THE COLONIC FLORA** .................................................................................................. 56

- **DIRECT DAMAGE BY AN INFECTIVE AGENT?** ....................................................... 57
  - *Diplostreptococcus* ................................................................................................ 57
  - *Bacteroides necrophorum* ...................................................................................... 57
  - *Clostridium difficile* ............................................................................................... 57
  - Cell wall deficient bacteria ..................................................................................... 58
  - *Saccharomyces cerevisiae* ..................................................................................... 58
  - *Escherichia coli* ....................................................................................................... 58
- **INDIRECT DAMAGE BY INTERFERENCE WITH COLONIC ORGANIC IONS?** ........... 59
- **INDIRECT DAMAGE BY BACTERIAL PRODUCTS?** .................................................... 60
- **A HARMFUL IMMUNE RESPONSE TRIGGERED BY MOLECULAR MIMICRY?** ... 61
- **ANTIBIOTICS TO TREAT UC LERATIVE COLITIS** .................................................... 62
E. COLI AND ULCERATIVE COLITIS ................................................................. 64

CLASSIFICATION OF PATHOGENIC E. coli ................................................ 64
Enteropathogenic E. coli ........................................................................ 65
Enterohaemorrhagic E. coli .................................................................... 66
Enterotoxigenic E. coli ........................................................................ 66
Enteroinvasive E. coli .......................................................................... 67
Enterotoxigenic E. coli ........................................................................ 67
QUANTITATIVE DIFFERENCES IN E. coli FROM PATIENTS WITH ULCERATIVE COLITIS ......................... 68
QUALITATIVE DIFFERENCES IN E. coli FROM PATIENTS WITH ULCERATIVE COLITIS ......................... 69
Adhesive properties of E. coli isolated from patients with ulcerative colitis 70

SUMMARY TO INTRODUCTION .................................................................. 71

METHODS AND LABORATORY TECHNIQUES ............................................. 73

GENERAL METHODS IN THE ISOLATION OF E. coli .................................. 73
Culture media used .............................................................................. 73
METHODS USED IN PCR TYPING OF E. coli ISOLATES ............................ 75
Background ......................................................................................... 75
Development of a typing system for E. coli ........................................... 75
Typing of known serotypes ................................................................ 76
Using REP-PCR to type “wild strains” of E. coli isolated from faeces 78
Using REP-PCR to type the non-pathogenic E. coli Nissle 1917 (Mutaflor) 79
REP-PCR protocol used in the study ..................................................... 80
METHOD FOR ISOLATION OF PLASMID DNA ......................................... 81
Method for curing plasmids by heat stress .......................................... 84

ASSESSMENT OF ADHESIVENESS .......................................................... 85
Background ......................................................................................... 85
Bacterial adhesins ............................................................................... 85
Mannose sensitive fimbrial adhesins .................................................... 86
Mannose resistant fimbrial adhesins .................................................... 86
In vitro measurement of bacterial adhesion ........................................ 87
Definition of adhesiveness ................................................................. 88
Validation of the buccal epithelial adhesion assay ................................ 89

IS A 98MDA PLASMID RESPONSIBLE FOR THE ADHESIVENESS OF E. coli ? ............................................. 91

BACKGROUND ....................................................................................... 91
METHODS ......................................................................................... 91
Plasmid curing by heat stress ............................................................... 92
Statistical analysis .............................................................................. 92
RESULTS ............................................................................................... 93
Plasmid carriage and adhesiveness ..................................................... 93
Plasmid curing experiments ............................................................... 94
DISCUSSION ......................................................................................... 95
STATEMENT OF INVOLVEMENT IN THE THESIS

The author undertook all the clinical assessments described including sigmoidoscopic assessments.

The author carried out the isolation and typing of *E. coli* and plasmid extraction with help from Anna Snelling and Laura Hibberts to allow sufficient numbers to be processed. Ann Buckingham carried out the development and validation of the rep-PCR assay in a preliminary study.

In order to preserve consistency in the buccal epithelial cell adhesion indices given in this thesis, this assay was undertaken by Jill Rothwell, under the direct supervision of the author.

Michael Dixon, at the Leeds University Department of Histopathology, examined the histological sections of rectal biopsies taken.
Chapter 1

INFLAMMATORY BOWEL DISEASE—CLINICAL CONSIDERATIONS

Although ulcerative colitis and Crohn's disease are different diseases, they share many clinical features including clinical symptoms, pattern of inflammation, radiological and histological features. Indeed in a proportion of patients a definite diagnosis cannot be made. Crohn's disease now affects about 1 in 1500 people and the annual incidence of ulcerative colitis is around 7 cases per 100,000 population in the United Kingdom. The sexes are affected equally with peaks in incidence in early and late adulthood. In Northern Europe, the United Kingdom and the United States, a female preponderance of approximately 30% has been reported. In other areas, no such difference has been detected.

Historical background

Sir Samuel Wilks, a physician at Guy's Hospital, distinguished the condition from ulceration caused by congestion, mercury poisoning and bacillary dysentery in 1859. He described "the case of Miss Bankes," in which the colon was dilated and with severe universal inflammation. Hawkins further described how the disease might be either intermittent or chronic and how the first attack tended to be the most severe. He also described cases presenting with bleeding and constipation rather than the more common diarrhoea. As the mucosal appearances were similar to dysentery and in view of reports of raised serum agglutination titres to Shigella in patients with ulcerative colitis most regarded the condition as infective. In 1942, Anderson suggested that more than half of patients with ulcerative colitis had an allergy to milk. In 1962, controlled trial of milk exclusion suggested that about 20% of patients respond to a milk free diet.

Clinical features

Ulcerative colitis and Crohn's disease commonly present with similar features and it may be useful to consider the clinical features of both types of inflammatory bowel disease. Indeed it may be several years before the clinical evolution allows a firm diagnosis to be made.

Virtually all patients with ulcerative colitis, and about half of patients with Crohn's disease present with rectal bleeding or bloody diarrhoea. Many patients complain of abdominal pain often related to defecation. The severity of ulcerative colitis may vary and the classification proposed by Truelove and Witts is simple and a valuable guide;
Mild disease is characterised by less than four stools a day, without systemic disturbance and a normal ESR. Moderate disease is defined as more than four stools a day but without significant systemic disturbance. Severe disease is characterised by six or more motions a day with blood and systemic disturbance as shown by fever, tachycardia (mean pulse rate more than 90/minute), anaemia and an ESR above 30.

Although Crohn's disease may affect any part of the gastrointestinal tract, the most common sites include the terminal ileum (65%), colon (20%) or perianal region (3-36%). In ulcerative colitis about 55% have proctitis, 30% left sided colitis, and 15% pan colitis\textsuperscript{10,11}. Patients with Crohn's disease at the terminal ileum usually present with abdominal or right iliac fossa pain. Diarrhoea is also common in these cases and may be aggravated by bacterial overgrowth, malabsorption of bile salts and colonic involvement.

Aphthous stomatitis is more common in Crohn's disease than in ulcerative colitis and a biopsy will usually show granulomas\textsuperscript{12}. Up to a third of patients with Crohn's disease develop perianal complications such as fistulae, fissures and abscesses, at some stage\textsuperscript{13}. 

Table I: Contrasts of the main contrasting features of ulcerative colitis and Crohn’s disease.

<table>
<thead>
<tr>
<th>Smoking</th>
<th>Ulcerative colitis</th>
<th>Crohn’s disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malnutrition or growth failure</td>
<td>non or ex-smokers</td>
<td>smokers</td>
</tr>
<tr>
<td>Autoantibodies</td>
<td>occasionally</td>
<td>common</td>
</tr>
<tr>
<td>Associated autoimmune disease</td>
<td>common</td>
<td>rare</td>
</tr>
<tr>
<td>Endoscopy</td>
<td>occasionally</td>
<td>rarely</td>
</tr>
<tr>
<td>Site of involvement</td>
<td>rectum and colon</td>
<td>70-85% small bowel</td>
</tr>
<tr>
<td>Thickened bowel wall</td>
<td>rare</td>
<td>characteristic</td>
</tr>
<tr>
<td>Narrowed bowel lumen</td>
<td>very rare</td>
<td>common</td>
</tr>
<tr>
<td>Skip lesions</td>
<td>very rare</td>
<td>common</td>
</tr>
<tr>
<td>Linear or apthous ulcers with</td>
<td>very rare</td>
<td>characteristic</td>
</tr>
<tr>
<td>normal surrounding mucosa</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Histology</th>
<th>Ulcerative colitis</th>
<th>Crohn’s disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transmural inflammation</td>
<td>very rare</td>
<td>characteristic</td>
</tr>
<tr>
<td>Submucosal fibrosis</td>
<td>very rare</td>
<td>characteristic</td>
</tr>
<tr>
<td>Granulomas</td>
<td>very rare</td>
<td>characteristic</td>
</tr>
<tr>
<td>Mucosal IgG subclass</td>
<td>IgG(_1) raised</td>
<td>IgG(_2) raised</td>
</tr>
<tr>
<td>Cytokines</td>
<td>IL-4, IL-5 normal</td>
<td>INF, IL-12 normal</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Management</th>
<th>Ulcerative colitis</th>
<th>Crohn’s disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corticosteroids</td>
<td>Benefit</td>
<td>benefit</td>
</tr>
<tr>
<td>5-aminosalicylates</td>
<td>benefit in maintaining remission</td>
<td>less benefit in maintaining remission</td>
</tr>
<tr>
<td>Response to antibiotics</td>
<td>possibly a benefit</td>
<td>definitely benefit</td>
</tr>
<tr>
<td>Nutritional therapy</td>
<td>no benefit</td>
<td>definite benefit</td>
</tr>
<tr>
<td>Azathioprine</td>
<td>possibly a benefit</td>
<td>no benefit</td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>benefit in acute stage</td>
<td>some benefit</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>little benefit</td>
<td></td>
</tr>
</tbody>
</table>
Complications and extra-intestinal manifestations

Local complication of Crohn's disease include; small bowel obstruction with postprandial abdominal pain and a reduction in the diarrhoea. As the transmural inflammation extends through the bowel wall, fistula may form. These sinus tracts may form abscesses or penetrate adjacent loops of bowel or nearby structures such as the bladder, stomach or even the skin surface to form entero-cutaneous fistulas. Table II:

Table II Extra-intestinal Manifestations of IBD

<table>
<thead>
<tr>
<th>Skin:</th>
<th>erythema nodosum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pyoderma gangrenosum</td>
</tr>
<tr>
<td></td>
<td>cutaneous vasculitis</td>
</tr>
<tr>
<td>Mouth:</td>
<td>stomatitis</td>
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<td></td>
<td>glossitis</td>
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<tr>
<td>Hepato-Biliary:</td>
<td>hepatitis</td>
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<tr>
<td></td>
<td>cholangitis</td>
</tr>
<tr>
<td></td>
<td>cirrhosis</td>
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<tr>
<td>Bone:</td>
<td>osteopenia</td>
</tr>
<tr>
<td></td>
<td>osteoparesis</td>
</tr>
<tr>
<td>Joints:</td>
<td>arthritis</td>
</tr>
<tr>
<td></td>
<td>arthralgia</td>
</tr>
<tr>
<td></td>
<td>ankylosing spondylitis</td>
</tr>
<tr>
<td></td>
<td>sacroiliitis</td>
</tr>
<tr>
<td>Vascular:</td>
<td>thrombophlebitis</td>
</tr>
<tr>
<td></td>
<td>vasculitis</td>
</tr>
<tr>
<td></td>
<td>polyarteritis nodosa</td>
</tr>
<tr>
<td></td>
<td>takayasu's arteritis</td>
</tr>
<tr>
<td></td>
<td>giant cell arteritis</td>
</tr>
<tr>
<td>Pancreas:</td>
<td>pancreatitis</td>
</tr>
<tr>
<td></td>
<td>pancreatic insufficiency</td>
</tr>
<tr>
<td>Pulmonary:</td>
<td>vasculitis</td>
</tr>
<tr>
<td></td>
<td>fibrosing alveolitis</td>
</tr>
<tr>
<td>Cardiac:</td>
<td>myocarditis</td>
</tr>
<tr>
<td></td>
<td>pericarditis</td>
</tr>
<tr>
<td>Musculoskeletal:</td>
<td>myositis</td>
</tr>
<tr>
<td></td>
<td>dermatomyositis</td>
</tr>
<tr>
<td>Neurological:</td>
<td>peripheral neuropathy</td>
</tr>
<tr>
<td></td>
<td>perineuritis</td>
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<tr>
<td></td>
<td>stroke</td>
</tr>
<tr>
<td></td>
<td>epilepsy</td>
</tr>
<tr>
<td>Haematological:</td>
<td>anaemia</td>
</tr>
<tr>
<td></td>
<td>neutropenia</td>
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<tr>
<td></td>
<td>thrombocytosis</td>
</tr>
<tr>
<td></td>
<td>coagulation disorders</td>
</tr>
<tr>
<td>Renal:</td>
<td>nephrolithiasis</td>
</tr>
<tr>
<td></td>
<td>hypertension</td>
</tr>
<tr>
<td></td>
<td>glomerulonephritis</td>
</tr>
<tr>
<td>Malignancy:</td>
<td>lymphoma</td>
</tr>
<tr>
<td></td>
<td>myelodysplasia</td>
</tr>
<tr>
<td></td>
<td>adenocarcinoma</td>
</tr>
</tbody>
</table>

There is also an increased risk of colorectal cancer in both ulcerative colitis and colonic Crohn’s disease. Disease for more than eight years extending proximal to the sigmoid colon is the two major determinants of increased risk of colorectal cancer in ulcerative colitis\textsuperscript{14}.

Extra-intestinal manifestations are common in both ulcerative colitis and Crohn’s disease\textsuperscript{15,16}. The different extra-intestinal features of inflammatory bowel disease is outlined in Table II.
Skin

Non-specific rashes are usually related to the therapy. Erythema nodosum, which is more common in Crohn's disease, may also be a reaction to sulphasalazine. The aetiology is unknown, but immune complex deposition causing vasculitis or panniculitis has been suggested.

Pyoderma gangrenosum affects between 1-2% of patients. It is more common in ulcerative colitis than Crohn's disease. Most patients have active pan-colitis. The skin disease may continue after colectomy suggesting that if continuing intestinal inflammation or exposure to an antigen derived from the intestinal lumen is involved in initiating the skin lesion, they are not required for its persistence.

Photograph 1

This particular patient presented to hospital with the typical necrotic rash of pyoderma gangrenosum (behind her ear). She had noticed an increased bowel frequency in the preceding six months. A colonoscopy confirmed ulcerative colitis and she improved with azathioprine and prednisolone.

Mouth

Up to 10% of patients with active disease are troubled by aphthous ulceration. Angular stomatitis is associated with iron deficiency anaemia.

Liver

Primary sclerosing cholangitis is the most common form of chronic liver disease in ulcerative colitis and may be present in between 2-7% of patients. Conversely about 70% of patients with PSC have ulcerative colitis. Primary sclerosing cholangitis affects men twice as common as women and in most cases (90%) patients have a colitis affecting the entire colon.
Symptoms of colitis usually predate the diagnosis of PSC, but PSC may precede the onset of colitis by years and indeed may occur after total colectomy. The outcome is unrelated to the activity, severity or course of the colitis.

Patients with ulcerative colitis and the HLA B8 DR3 haplotype have ten times the background risk of developing PSC. A large number of other immunological abnormalities have been described, but no trigger or infective agent has been identified. Das demonstrated that a monoclonal antibody to the colonic epithelium 40kD protein also reacts with bile duct epithelium as well as skin. The overall frequency of the HLA B8 DR3 is 70% which is greater than controls but no more common than in patients in with uncomplicated colitis.

The typical radiological appearances of PSC includes narrowing and dilatation of the intra- and extra-hepatic biliary tree (photograph 2 below).

### Photograph 2
Cholangiogram from an adult patient with severe primary sclerosing cholangitis characterised by areas of stricturing and dilatation of the intrahepatic bile ducts.

**Joints**
An acute sero negative mono-arthritis may affect between 10-15% of patients. Less commonly the patients suffer a more chronic arthritis affecting the small joints of the hand and wrist. The aetiology is unknown although absorption of a luminal antigen through diseased intestine has been suggested. Bacterial antigens have been detected in the synovial fluid of patients with reactive arthritis following intestinal infection with Yersinia or Salmonella species, and there is evidence of molecular mimicry between the HLA B27 antigen and Yersinia, Salmonella, Shigella and Klebsiella species.
**Eye**

The true incidence of eye complications of the inflammatory bowel disease is not known but the reported incidence varies between 3.5% and 11.8%\textsuperscript{29,30}. Uveitis, episcleritis and scleritis are by far the commonest ophthalmic complications\textsuperscript{31}. Nearly half of the patients would have more than one ocular complication and up to two-thirds also have another extraintestinal manifestation, most commonly arthritis or ankylosing spondylitis\textsuperscript{32,33}. Most patients already have diagnosed inflammatory bowel disease prior to development of ophthalmic complications, but in a minority ocular disorders precede the diagnosis of the inflammatory bowel disease\textsuperscript{34}. Cataracts have been described in patients on long-term steroids. Secondary glaucoma\textsuperscript{33} can be a result of uveitis or scleritis, or can be steroid induced.

**Lung**

A number of pulmonary associations have been described, including abnormalities of lung function tests, bronchiectasis and bronchial inflammatory changes\textsuperscript{35} and which correlate poorly with clinical disease activity. Butland\textsuperscript{36} suggested an autoimmune basis for the bronchiectasis, which he described in seven patients. Antinuclear and anti-smooth muscle antibodies were detected in six and five of seven patients respectively.

**Rare associations**

Pericarditis has been described in association with ulcerative colitis\textsuperscript{37,38}. There has been no evidence of a viral aetiology on the basis of serology, and no evidence of bacterial infection or immune complex mediated disease. However treatment with corticosteroids is said to be effective.

Coomb's test positive haemolysis is a rare complication\textsuperscript{39,40} more common in women. The severity of the haemolysis is unrelated to the activity of the bowel disease and it may respond to corticosteroids or immunosuppressants. No cross-reactivity between anti-colon antibodies and red cell antibodies has been found\textsuperscript{41}.
Investigations

Patients are initially evaluated clinically and with blood tests. After infection has been excluded the nature and extent of inflammation should be established by either colonoscopy or double contrast barium enema examination. In Crohn’s disease, the small intestine may be visualised by contrast studies to define the distribution and severity of the disease and detect any associated fistulae.

Photograph 3

Detail of a small bowel meal showing a diseased terminal ileum giving rise to a “cobblestoned” appearance in Crohn’s disease.

Photograph 4

Detail of a barium enema showing confluent ulceration from the rectum to mid-descending colon in a patient with ulcerative colitis.
Colonoscopy is superior to radiology for colonic and terminal ileal disease as it allows detection of superficial disease and biopsies without a radiation load to the patient. Furthermore, strictures can be dilated with balloons introduced through the instrument channel.

Photograph 5
Narrowed and ulcerated terminal ileum in a patient with Crohn’s disease.

Photograph 6
Oedematous, granular and erythematous rectal mucosa with superficial ulceration in a patient with active ulcerative colitis.

Baron\textsuperscript{44}, has suggested a grading of the mucosal appearances in ulcerative colitis. The first mucosal sign is loss of the vascular pattern followed by erythema, friability and granularity. Severe colitis is associated with spontaneous bleeding and ulceration.

Imaging with radiolabelled leucocytes may define the distribution of disease and detect any intra-abdominal abscesses non-invasively. Scintigraphic scanning with monoclonal antibodies to upregulated cellular adhesion molecules such as E selectin is also possible\textsuperscript{45}.

Transabdominal ultrasound is a non-invasive radiation-free method to investigate patients presenting with pain and swellings in the right iliac fossa or perianal disease. Changes in mucosal and superior mesenteric arterial blood flow may be detectable by colour Doppler ultrasound\textsuperscript{46}. Cross-sectional imaging such as computed tomography (CT) or magnetic resonance imaging (MRI) may be superior to contrast studies in detecting fistulae or the assessment of extramural disease such as abscesses. MRI has been shown to be superior to CT for the assessment of pelvic and perianal disease\textsuperscript{47}.
Histology

Crohn’s disease is characterised by a dense accumulation of activated T cells and macrophages, which in some cases are organized into typical granulomas. The earliest microscopic lesion in Crohn disease consists of a focal accumulation of lymphocytes and macrophages near an intestinal crypt. These may enlarge and form granulomas in any layer of the bowel wall from the mucosa to the serosa.

In contrast, in ulcerative colitis, the cellular infiltrate is more variegated and acute inflammatory events, such as neutrophils forming crypt abscesses, are prominent. Lymphocytes and macrophages are present, but granulomas are not.

In ulcerative colitis, the inflammation is limited to the upper and mid-lamina propria whilst the inflammation is usually transmural in Crohn’s disease and may be accompanied by fissured ulcers penetrating deep into the wall. Microscopic focality is a feature of Crohn’s disease with areas of inflamed mucosa being separated by normal epithelium. Crypt abscesses may be formed in both active disease ulcerative colitis and Crohn’s disease.

It may be difficult to distinguish ulcerative colitis from infective colitis. Acute Chlamydial proctitis may also resemble active ulcerative colitis. Chronic infective colitis such as chronic shigellosis and amoebiasis is particularly difficult to distinguish from ulcerative colitis. A predominant acute rather than chronic inflammatory infiltrate makes infective colitis more likely. Furthermore, disruption of the crypt architecture, crypt atrophy and basal lymphoid aggregates are not features of infective colitis. Examples of the different histological appearances of Crohn’s disease and ulcerative colitis are given in photograph 7 and 8 below.

Photograph 7
Continuous inflammatory infiltrate confined to the mucosa with crypt distortion, crypt abscesses and ulceration of the epithelium.

Photograph 8
Mucosal and submucosal inflammation with granulomas and deep ulceration in Crohn’s disease.
Treatment

Unless surgery is contemplated the management of colonic Crohn's disease is broadly similar to that of ulcerative colitis.

Undernourished patients need nutritional supplements including iron, calcium, magnesium, zinc, and fat-soluble vitamins. Patients are at particular risk of osteoporosis and biphosphonates, calcium, vitamin D, and hormone replacement therapy should be considered.

Codeine phosphate and loperamide are often used to reduce bowel frequencies but should be used with caution as they may precipitate acute colonic dilatation in active colitis. There have been reports of relapses precipitated by non-steroidal anti-inflammatory drugs and these should, if possible also be avoided.

Patients with terminal ileal Crohn's disease or previous resections and diarrhoea may benefit from cholestyramine (4 g 1-3 times daily) to bind bile salts. Sick inpatients may require intravenous fluids, blood transfusions and subcutaneous heparin to reduce the risk of systemic venous thromboembolism.

Active disease

In inflammatory bowel disease, prednisolone (40-60 mg/day) brings about a remission of active disease within 4 weeks in 70-80% of patients. Once the patient has begun to improve the dose is usually tapered by 5 mg every 7-10 days or according to the clinical response. Budesonide (9 mg/day) is an alternative and is associated with less adrenal suppression and has a therapeutic efficacy approaching that of 40 mg prednisolone. However it is comparatively expensive and is therefore only used in patients in whom minimisation of steroid induced side effects is particularly important.

Liquid formula diets are often used instead of corticosteroids in children with Crohn's disease and patients with both ileitis and colitis may respond. Elemental (amino acid based), oligomeric (containing peptides), and polymeric (containing whole protein) feeds all have similar efficacy to corticosteroids if taken for 4-6 weeks as the sole nutritional source. However, cost, high relapse rates and the difficulty many patients have in adhering to the diet limits the usefulness of this therapy. There is no evidence that elemental diets or other dietary intervention have any specific therapeutic effect in ulcerative colitis.
Dietary intervention in patients with ulcerative colitis is aimed at reducing symptoms and providing adequate nutrition to compensate for reduced intake and increased colonic losses\textsuperscript{63,64}.

High dose oral mesalazine (Pentasa 2 g bd or Asacol 1.2 g tds) may induce a remission in up to 40% of patients with active inflammatory bowel disease when given for up to 4 months\textsuperscript{65,66}.

Cyclosporine does not appear to confer any benefit in patients with acute ileo-caecal Crohn's disease but is often successful in inducing remission in ulcerative colitis\textsuperscript{67,68}. The remission is then maintained with an immunosuppressive drug such as azathioprine\textsuperscript{69,70}. Over half of patients with a severe attack of ulcerative colitis may avoid colectomy on this regimen according to Stack et al\textsuperscript{71}.

**Maintenance of remission**

Since attacks recur, maintenance treatment is important. Unfortunately, meta-analysis has shown that, unlike in ulcerative colitis, long term aminosalicylates does not prevent relapse\textsuperscript{72} in Crohn's disease. As prednisolone has long term side effects it has no routine prophylactic role in inflammatory bowel disease. Furthermore, budesonide has been shown not to increase the remission rate at 1 year follow up\textsuperscript{73}.

Sulphasalazine and 5-aminosalicylic acid preparations are equally effective in ulcerative colitis\textsuperscript{74} but the-aminosalicylates are better tolerated. Nevertheless, about 10% of patients are intolerant of the newer preparations\textsuperscript{75}. The use of sulphasalazine, the oldest (and least expensive) of these, has become less popular because of side effects including nausea, skin rashes, and reversible oligospermia. The newer oral 5-aminosalicylates are better tolerated but are not free from side effects. Mesalazine may cause rash, headache, nausea, diarrhoea, pancreatitis, or blood dyscrasias in up to 5% of patients; interstitial nephritis occurs in around 1 in 500\textsuperscript{76} and so regular monitoring of renal function is mandatory\textsuperscript{77}.

Azathioprine, 6-mercaptopurine or methotrexate\textsuperscript{78} are used to prevent relapse or to treat patients with Crohn's disease refractory to corticosteroids. A clinical response can be expected in up to 40% of patients but may take up to 4 months to occur, even when using intravenous azathioprine\textsuperscript{79}. Once the condition has responded, the dose of steroid is tapered down and if possible stopped altogether\textsuperscript{80}. Though the evidence supporting the use of azathioprine in ulcerative colitis is weaker than that in Crohn's disease, a recent survey confirmed its widespread use by British gastroenterologists\textsuperscript{81}.
Side effects of azathioprine are uncommon but potentially serious and include bone marrow depression, acute pancreatitis and chronic hepatitis. The side effects of methotrexate include bone marrow depression, hepatic fibrosis, pneumonitis and opportunistic infections. The manufacturer advices weekly monitoring for the first 8 weeks. As there is little evidence to support this recommendation, the British National Formulary recommends weekly monitoring for the first 4 weeks and then at least every three months.

It is not clear how long patients should be maintained on these drugs. In one study by Bouhnik et al, the risk of relapse after 4 years was similar whether azathioprine or 6-mercaptopurine was continued or stopped. An unblinded controlled trial by Neurath et al. suggested that mycophenolate mofetil, which inhibits purine synthesis in lymphocytes, produces a quicker response than azathioprine and with fewer adverse effects.

**Antibiotics**

Antibiotics such as metronidazole, ciprofloxacin, clarithromycin, rifabutin, and clofazimine, have been reported to help inducing a remission in patients with active Crohn’s disease with about 50% of patients with moderately active Crohn’s colitis or perianal disease responding to oral metronidazole.

Trials of antibiotic therapy have usually treated patients for up to 3 months. However, side effects may become troublesome including nausea, diarrhoea, an unpleasant taste, alcohol intolerance, and a peripheral neuropathy, which can be irreversible.

**Anti-tumour necrosis factor antibody**

Infliximab, a mouse-human chimeric antibody (cA2) to tumour necrosis factor may be used for patients with refractory Crohn’s disease. A single infusion produced improvement in 64% of patients and remission in 33% compared with 17% and 4% respectively after placebo. Up to 62% of perianal fistulae heal with three intravenous infusions over 6 weeks of antitumour necrosis factor antibody compared with 26% given placebo. However, reopening of the fistulae was common in the 3 months after treatment was stopped.

The application of this new therapy is limited by its high cost (£1000 per infusion) and high incidence of infusion reactions (20%).
Common minor side effects include headache, nausea, and upper respiratory tract infections. Serious, infections including salmonella enterocolitis, pneumonia, and cellulitis have been reported. Rapid healing and fibrosis of intestinal strictures may precipitate obstruction\textsuperscript{89}. Treatment with anti-tumour necrosis factor has been disappointing in ulcerative colitis and a benefit has only been reported in one of three published studies in ulcerative colitis\textsuperscript{90,91,92}.

Another limitation of this treatment is that patients tend to relapse in the ensuing months and repeated infusions at 4-8 weeks may be required\textsuperscript{93}. There have been reports of delayed hypersensitivity reactions in patients given repeated infusions. In addition, anti-double stranded DNA and cardiolipin antibodies may cause a lupus syndrome. There have also been case reports of lymphomas developing although it is not yet clear if these are a complication of the disease or due to the drug\textsuperscript{94}.

**Probiotic therapy**

It has been proposed that a defective epithelial barrier may cause a loss of tolerance to normal resident enteric bacteria. Once these bacterial products gain access to the submucosa, they may drive a variety of proinflammatory signalling pathways perpetuating the inflammation\textsuperscript{95}.

Data from experimental models imply that certain luminal bacteria are more pro-inflammatory than others. *Bacteroides* species have been found to be particularly pathogenic in many experimental models\textsuperscript{96}, whereas *Lactobacillus* species seem to have an anti-inflammatory effect by the suppression of bacterial adherence of other more pathogenic bacterial species\textsuperscript{97} and reduction in the production of pro-inflammatory cytokines\textsuperscript{98}.

How then might altering the enteric bacterial flora affect the gut? Probiotics have been defined as living organisms which, upon ingestion, improve the health of the host beyond their inherent basic nutrition\textsuperscript{99}. The following desirable properties of an ideal probiotic strain has been proposed.

- Resistance to acid and bile
- Attachment to human epithelial cells
- Colonization of the human intestine
- Production of an antimicrobial substance
- Good growth characteristics
- Beneficial effects on human health
Probiotics have been used in the treatment of infective diarrhoea, and antibiotic-induced diarrhoea\textsuperscript{100,101,102,103}. Lactobacilli strains appear to have protective immunomodulating properties by the induction of a systemic Th2 response\textsuperscript{104,105,106}, inhibiting the adhesion of pathogenic bacteria to the intestinal wall\textsuperscript{107}, restore permeability defects induced by cow's milk in weanling rats\textsuperscript{108}, induce growth factors\textsuperscript{109}, and enhance the synthesis of antibodies to microbial pathogens\textsuperscript{110}.

A probiotic mixture, VSL #3 (Yovis; Sigma-Tau, Pomezia, Italy), containing 300 billion/g of viable lyophilized bacteria of 4 strains of lactobacilli, 3 strains of bifidobacteria, and 1 strain of \textit{Streptococcus salivarius} subsp. \textit{thermophilus} has been used to treat inflammatory bowel disease.

In a pilot study, Venturi et al\textsuperscript{113} showed a significant increase in faecal concentrations of lactobacilli, bifidobacteria, and \textit{S. salivarius} subsp. \textit{thermophilus} when used for maintenance of remission in ulcerative colitis patients. In this study, 75\% of the patients maintained remission over the year on therapy. In the second trial, 40 patients with chronic pouchitis who initially achieved remission after combination antibiotic treatment were randomized to placebo or VSL #3 for 9 months. All 20 patients randomized to placebo relapsed; in contrast, 17 of 20 patients treated with VSL #3 were still in remission at 9 months\textsuperscript{114}. This probiotic combination has also shown efficacy in the maintenance treatment of ulcerative colitis\textsuperscript{115}, and in preventing postoperative recurrence of Crohn's disease\textsuperscript{116}.

**Surgery in ulcerative colitis**

In ulcerative colitis, restorative proctocolectomy with ileal reservoir is usually carried out after failure of medical treatment either through a lack of efficacy or unacceptable side effects. Occasionally, a colectomy may be required because of severe epithelial dysplasia or colorectal carcinoma, in a patient with long standing colitis. Until recently, surgical treatment implied permanent ileostomy but the creation of an ileal reservoir or pouch, with ileoanal anastomosis is now the standard operation\textsuperscript{117,118}.

Pouchitis, a non-specific inflammation of the ileal reservoir, is the most frequent long term complication and may occur in up to one third of patients. Two thirds of patients with pouchitis encounter recurrent attacks\textsuperscript{119,120,121}. Metronidazole is the first line treatment for pouchitis.
**Surgery in Crohn’s disease**

Patients with resistant ileocaecal disease not responding to drug or dietary therapy are often considered for local resection. Unfortunately, there is a 40-50% risk of symptomatic recurrence at 4 years after resection\(^1\). Patients with small bowel obstruction are usually given a 48-72 hour therapeutic trial of intravenous corticosteroids before surgery is organised. Short strictures may be treated by enteroscopic or colonoscopic balloon dilatation\(^2\) with or without intralesional injection of triamcinolone\(^3\).

Abscesses without an enteric connection may be treated by ultrasound or CT guided drainage\(^4\) rather than by surgical resection. An attempt is often made to manage fistulae without distal obstruction conservatively with enteral or parenteral nutrition, azathioprine, intravenous cyclosporine or anti-tumour necrosis factor antibody\(^5,\)\(^6\). Although some fistulae heal, many patients still require surgery. Suppurating perianal Crohn's disease requires surgical drainage\(^7\).

To reduce the risk of relapse after surgical resection azathioprine is usually used. Alternative medications include high dose aminosalicylates (3-4 g/day mesalazine)\(^8\), budesonide (6 mg/day)\(^9\) and metronidazole\(^10\).

**Prognosis:**

Most patients with ulcerative colitis have intermittent exacerbations of their disease. About 10% will have a severe first attack requiring surgery with another 10% pursuing a chronic continuous course. Patients with extensive or total colitis are much more likely to undergo colectomy within one year of diagnosis. After the first year, the course of disease appears to be similar for all patients\(^1\)\(^2\),\(^1\)\(^3\). In Crohn’s disease, about 70% of patients will require surgery during their lifetime\(^1\)\(^4\). Unfortunately, the majority (70%) of patients will have endoscopic evidence of recurrent Crohn’s disease within one year of surgery\(^1\)\(^5\).

Patients with Crohn’s colitis and ulcerative colitis both have an increased risk of colorectal cancer. The risk appears to be highest in those with extensive disease for more than 10 years.

In a large collaborate study between Oxford, Stockholm and Birmingham, the cumulative risk of developing cancer in patients with ulcerative colitis was 7.2% at 20 years and 16.5% after 30 years\(^1\)\(^6\),\(^1\)\(^7\).
To reduce the risk of patients with Crohn's colitis or ulcerative colitis developing colonic cancer, many units carry out regular colonoscopic surveillance to detect dysplasia. However, such surveillance regimens are not cost-effective. A review of the published literature reporting on a total of 3807 colonoscopies, carried out in patients with extensive ulcerative colitis for more than 8-10 years, only yielded 8 early cancers\textsuperscript{135}.

**AETIOLOGICAL CONSIDERATIONS**

There are a number of etiological theories of inflammatory bowel disease offering a useful framework the potential mechanisms by which the intestinal microflora may have an effect\textsuperscript{136}. These theories must take several features of the condition into consideration.

<table>
<thead>
<tr>
<th>Case</th>
<th>Cases/10^5 population/year</th>
</tr>
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<tbody>
<tr>
<td>Czechoslovakia</td>
<td>1.3</td>
</tr>
<tr>
<td>Italy</td>
<td>1.9</td>
</tr>
<tr>
<td>France</td>
<td>3.0</td>
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<tr>
<td>Sweden</td>
<td>4.7</td>
</tr>
<tr>
<td>UK (London)</td>
<td>6.2</td>
</tr>
<tr>
<td>UK (Cardiff)</td>
<td>7.2</td>
</tr>
<tr>
<td>UK (Scotland)</td>
<td>11.3</td>
</tr>
<tr>
<td>Denmark</td>
<td>9.5</td>
</tr>
<tr>
<td>Iceland</td>
<td>7.4</td>
</tr>
<tr>
<td>Norway</td>
<td>14.8</td>
</tr>
<tr>
<td>Faroe Islands</td>
<td>20.3</td>
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</tbody>
</table>

As can be seen in table III, the incidence of inflammatory bowel disease is higher in the United Kingdom (6-11 per 100 000), Scandinavia (4-9 per 100 000) and North America (4-7 per 100 000) than in Japan or in Southern and most of Eastern Europe (0.5–2 per 100 000)\textsuperscript{137}. In Israel, the incidence of inflammatory bowel disease (3.6 per 100 000) is less than in the white populations of the United States and North Europe.

Even within countries there are differences in incidence between rural and urban, locations. In 1963, Acheson\textsuperscript{138} reviewed over 500 American veterans with ulcerative colitis and found that few came from farming communities. Similar findings have been reported from Italy\textsuperscript{139} and the UK\textsuperscript{140}. These findings lend some support for the view that a rural life style may lessen the risk of ulcerative colitis.
Ethnic groups

In Baltimore, the incidence of ulcerative colitis in the black population is one-third that of the white population (1.4 vs. 4.6 per 100 000)\(^1\).

Ulcerative colitis has been reported to be 3 - 5 times more common in Jews living in Western communities with reported rates of up to 145 per 100 000\(^2,3\). However, Israeli-born or non-Ashkenazi Jews are less prone to develop either ulcerative colitis\(^4\) or Crohn's disease\(^5\) than those born in Europe or America.

Mayberry et al described a two-fold increase in incidence amongst South Asians in Leicestershire compared to whites. The highest prevalence was found amongst Sikhs and the lowest in the Bangladeshi communities\(^6\) (table below).

<table>
<thead>
<tr>
<th>Cases/10^5 population/year</th>
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<tbody>
<tr>
<td>Gujurati Hindus</td>
</tr>
<tr>
<td>Punjabi Sikhs</td>
</tr>
<tr>
<td>Bangladeshi Muslims</td>
</tr>
<tr>
<td>Europeans – Leicester</td>
</tr>
<tr>
<td>Europeans – London</td>
</tr>
</tbody>
</table>

Table IV

Different prevalence of inflammatory bowel disease amongst the Asian communities versus that of the European population in Leicester and London.

Paradoxically, although the anatomical extent of disease was reported to be similar to Europeans, South Asians appeared to have fewer operations\(^7\). This may suggest a more ready acceptance of diarrhoea by South Asians or a less ready acceptance of a stoma. The reason for these ethnic differences is not known. It has been suggested that the betel nut derivative, paan, may be protective against ulcerative colitis\(^8\). A reduced fat consumption has also been proposed as protecting against ulcerative colitis\(^9\). The smoking habits also vary within South Asian populations with Muslims being more likely to smoke tobacco than Hindus\(^0\).

Genetic factors

If ulcerative colitis was solely caused by an environmental determinant triggering the disease in adulthood, one would expect spouses to have a higher than average risk of contracting the disease. Instead, most studies have found a low incidence in the spouses of patients with ulcerative colitis. There has only been one published series of 19 couples reported in which both husband and wife were affected by the disease\(^1\).
Between 10 and 20% of patients with ulcerative colitis will have other affected relatives\textsuperscript{152}. The association is strongest with first degree relatives who may have either ulcerative colitis or Crohn's disease. An epidemiological study of twins found that of the 16 monozygotic twin pairs, in whom one member had ulcerative colitis, only one pair was concordant for the disease, whereas all 20 dizygotic twins were discordant\textsuperscript{153}. This gives a concordance rate of 6.3% for ulcerative colitis whereas this value was 58.3% for Crohn's disease, suggesting a much stronger genetic influence for Crohn's disease than for ulcerative colitis. In this study, there was no case of ulcerative colitis in one member and Crohn's disease in the other.

Another analysis of the inheritance pattern of inflammatory bowel disease have suggested the presence of a dominant gene in 9-13 % of colitics and a recessive gene in 7% cases of Crohn's disease\textsuperscript{154}. This analysis indicated that penetrance was low at 0.2 - 0.26, suggesting the need for a second agent to trigger disease. Perhaps a combination of genetic and environmental factors determine susceptibility and severity of the disease.

In 1996, Hugot et al\textsuperscript{155} reported a genetic link with a locus on chromosome 16 and Crohn's disease. This link was subsequently confirmed in other studies\textsuperscript{156} and by an international IBD Genetics consortium\textsuperscript{157}. Ohmen et al\textsuperscript{158} reported that the chromosome is primarily involved in non-Jewish Caucasians in the United States. Later, a link with the same locus and ulcerative colitis was suggested\textsuperscript{159}. The report of a putative susceptibility gene on chromosome 16\textsuperscript{160} raised considerable interest as this region also contains several candidate genes such as a CD11 integrin cluster including complement receptor type 3, B lymphocyte marker CD19, adhesion molecule sialophorin and the interleukin 4 receptor, all which may have a contributory or modifying role in the pathogenesis of Crohn's disease\textsuperscript{161}.

A study by Satsangi et al\textsuperscript{162} could not confirm a link with chromosome 16 but reported a link with a locus on chromosome 12 and also implicated further loci on chromosome 3 and 7. Brant et al\textsuperscript{163} could not confirm the link with chromosome 3 and 7 whilst Cho et al\textsuperscript{164} confirmed the locus on chromosome 16 in Crohn's disease and reported suggestive linkage evidence for loci on chromosomes 1,3 and 4. Satsangi et al, obtained information from 433 adult patients with inflammatory bowel disease. Compared with the prevalence in the general population, the relative risks in siblings of patients with Crohn's disease calculated from these data were respectively 36.5 for Crohn's disease and 16.6 for ulcerative colitis\textsuperscript{165}.
A high degree of concordance for disease type, extent, extra-intestinal manifestations was noted. However, the median age of onset in the parents was significantly higher than in offspring.

There are several possible explanations for the differences in results between these studies. Differences in the clinical definition of the patients studied could account for negative results. Individual loci may have various degrees of importance in different ethnic groups. Finally, and perhaps most importantly, because of the strong likelihood of interaction with a variety of environmental factors, different loci may differ in importance not only between ethnic groups but also between geographic regions. Finally, linkage studies only investigate patients with multiple affected family members. As a positive family history is only found in 10-20% of patients, this group may represent a subgroup of patients with a specific genetic susceptibility.

Reports of associations between inflammatory bowel disease and chromosome 6 are intriguing. Cytokines play a central role in the initiation and regulation of the immune response. The genes that encode proteins which are involved in the regulation of the immune response (the human leucocyte antigen class II genes) are located in the major histocompatibility complex (MHC) on the short arm of chromosome 6. The class II molecules consist of an alpha and a beta chain that form a groove in which the antigenic peptide, after partial digestion of antigen by antigen presenting cells is conferred to the T cell receptor166,167.

The three different HLA class II molecules are HLA-DP, -DQ and -DR. Subunits of HLA-DP and -DQ are each encoded by polymorphic alpha and beta chain genes. In the case of HLA-DR there is a non-polymorphic alpha chain gene and up to three distinct highly polymorphic beta chain genes. Generally, patients and sex, age and ethnically matched controls are typed for the serological antigens DR1 – 10 and the split antigens for DR2 (DR15,16), DR3 (DR17, 18), DR5 (DR11, 12) and DR6 (DR13,14).

The HLA-DR beta chain gene B1, is always present in all individuals and is by far the most polymorphic and has therefore been used to study the relationship between HLA class II genes and inflammatory bowel disease. An association between DRB1 and severe or extensive ulcerative colitis has been reported in Dutch168,169 British170,171 and Japanese172 populations. However, the studies of HLA associations in colitis have yielded inconclusive results. A meta-analysis of HLA associations in inflammatory bowel disease reviewed 29 studies and only found a positive association between ulcerative colitis and HLA-DR2 and HLA-DR9173.
Ethnic differences were important and the association between ulcerative colitis and DR9 was not detected when only white populations were included in the meta-analysis.

The link with HLA-DR2 is intriguing as several authors have shown an association between a reduced production of tumour necrosis factor (alpha) and the presence of HLA-DR2\textsuperscript{174,175}. Other groups have looked at the TNF-alpha secretion by isolated peripheral blood monocytes (PBMC) after stimulation with lipopolysaccharide\textsuperscript{176,177} and found reduced production in ulcerative colitis compared with PBMC isolated from patients with Crohn's disease or healthy controls.

There is clinical evidence that TNF alpha production is less important in ulcerative colitis than Crohn's disease. Treatment with TNF monoclonal antibodies in Crohn's disease has all shown a benefit\textsuperscript{178,179,180}. In contrast, this treatment has only been of benefit in one of three published studies in ulcerative colitis\textsuperscript{181,182,183}.

Yang et al reported an association between HLA-DR2 and p-ANCA compared with ANCA negative controls\textsuperscript{184}. However, the results were not conclusive and other workers have not been able to substantiate this\textsuperscript{168}. In above meta-analysis\textsuperscript{173}, the link with DR2 was only detected in the homogeneous Japanese, Finnish and Sicilian populations. In more heterogeneous white populations some studies confirmed an increased frequency\textsuperscript{185,186,187,188,189,190} others equal frequencies\textsuperscript{191,192} and one study even reported reduced frequency of DR2\textsuperscript{193} in patients with ulcerative colitis. The possible reasons for these conflicting findings include inadequate ethnic matching of controls, small sample sizes and different HLA typing techniques (serological versus molecular). One of the most important explanation may be that most studies do not take disease heterogeneity into account. There are marked differences in clinical behaviour, response to treatment and prognosis between colitics. These different clinical subgroups may be influenced by different genetic backgrounds and it is perhaps not surprising that various studies have yielded conflicting results.

Hugot \textit{et al.} continued to employ classic positional-cloning methods and in 2001 both Hugot \textit{et al} and Ogura \textit{et al} independently identified the Crohn's disease susceptibility gene "NOD2" at chromosome 16\textsuperscript{194,195}. A disease-related truncating mutation of the carboxy terminal of NOD2 was described in 8\% of patients with familial Crohn's disease, as compared with 4\% of patients with ulcerative colitis or healthy controls.
However, only patients with Crohn's disease were homozygous for this mutation, and homozygosity increased the risk of Crohn's disease by a factor of 20 to 40.

Overall 29% of Crohn's disease patients carried at least one NOD2 variant allele confirming that additional genes must be involved to confer susceptibility. However, there is evidence for a “gene dosage effect” as the relative risk of developing Crohn's disease was 3 among heterozygotes, and 38 among homozygotes and 44 among compound heterozygotes. NOD2 is similar to the “R factor genes” of plants that confer resistance to infection and has been shown to bind endotoxins intracellularly and activate nuclear factor-kappa B (NF-κB). NF-κB is a final intracellular signal conduction pathway which activate the production of a number of inflammatory signals including TNF, IL-1, IL-6 and IL-12. The truncated gene found in patients with Crohn's disease appears to result in impaired binding to endotoxin and thus decreases the activation of NF-κB. This is paradoxical as Crohn's disease is characterised by raised NF-κB and increased TNFa production.

There are two hypotheses on how the gene confers susceptibility to Crohn's disease. One is that NOD2 has a role in apoptosis. The other theory is that the NOD2 protein is involved in the recognition of microbes. The NOD2 gene has a region of 10 leucine-rich repeats towards its carboxy terminal. Such regions of leucine-rich repeats are a feature of proteins that identify molecular patterns of microbial products, called “pattern-recognition receptors”. An example is the family of toll-like receptors (TLR) and CD14. Each toll-like receptor recognises a different microbial product. For example, TLR-4 is the receptor for bacterial endotoxins, TLR-5 is the receptor for bacterial flagellin, and TLR-9 binds to bacterial CpG (cytidine phosphate guanosine) nucleotides.

The enteric bacterial flora is the common driving force in all animal models of Crohn's disease and the discovery of NOD2 mutations clearly links genetic susceptibility and enteric bacteria. It is tempting to speculate that NOD2 participates as pattern-recognition receptors in the dialogue between the normal enteric flora, the epithelium, and the immune system.
Appendectomy and colitis

It has been noted that a history of appendicitis is rare in patients with ulcerative colitis\textsuperscript{200}. This suggests a protective effect of appendicectomy. However, the decrease in appendicectomy rate in Britain during the past 50 years has not been associated with an increase in incidence of ulcerative colitis\textsuperscript{201}. Another explanation is that appendicitis and ulcerative colitis are alternative inflammatory responses which are genetically or environmentally determined.

Mormons living in Britain and Ireland have been reported to have a lower incidence of appendectomy and a higher incidence of ulcerative colitis than the general population\textsuperscript{202}. A recent study from Sweden found that patients who underwent appendectomy for appendicitis or mesenteric adenitis had a reduced risk (hazard ratio 0.58) whilst those who underwent appendicectomy for non-specific abdominal pain had no reduction in risk\textsuperscript{203}. Interestingly, this inverse relationship was only found for those who underwent surgery before the age of 20 years (figures I and II below).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figures.png}
\caption{Figure I and II}
\end{figure}

It therefore appears to be the inflammatory process itself, which protects against ulcerative colitis rather than the appendectomy. Studies have reported an increased risk of appendectomy among the children of parents who smoke\textsuperscript{204,205}. However, the inverse relationship between appendectomy and ulcerative colitis remain after adjusting for smoking status\textsuperscript{206,207,208}.

A weak positive relationship has been reported between Crohn’s disease and appendectomy\textsuperscript{209,210,211}. Crohn’s disease is mediated by type 1 helper T-cells whereas ulcerative colitis is mediated by type 2 helper T-cells. It is possible that appendicitis is mediated by type 1 helper T-cells explaining the inverse relation to ulcerative colitis.
The oral contraceptive pill
A higher incidence of ulcerative colitis in women taking the oral contraceptive pill has been reported. However, this association is weak and disappear when the data is corrected for social class and smoking habits\textsuperscript{21-2}.

Food allergy
Although antibodies to milk protein has been described in patients with ulcerative colitis\textsuperscript{21-3}, no relationship between the height of the titre and disease seventy has been found. Food allergy is unlikely to be involved in the aetiology as treating patients with active disease with parenteral nutrition does not improve outcome\textsuperscript{21-4}. Furthermore diverting the faecal stream by an ileostomy does not reduce disease activity.

Smoking
Another recognised environmental factor is cigarette smoke. Patients with ulcerative colitis do not generally smoke\textsuperscript{21-5}. A number of authors have demonstrated a relative risk of developing ulcerative colitis in current smokers, compared to those who have never smoked, of 0.2 to 0.6\textsuperscript{21-6,21-7,21-8}. Ex-smokers appear to have an increased risk compared to those who have never smoked.

In contrast, current smoking carries an increased risk of Crohn's disease, suggesting that the relationship between inflammatory bowel disease and smoking is not a result of the disease affecting smoking habits. Smoking can reduce the colonic blood flow\textsuperscript{21-9}, decrease the mucosal permeability\textsuperscript{21-20,21-21} and affect mucus production\textsuperscript{21-22,22-3}. However, the precise mechanism behind the protective effect of smoking is not known.

The effect of nicotine patches, added to the treatment of colitics receiving sulphasalazine, mesalazine or low dose steroids has been demonstrated in a double blind trial\textsuperscript{22-4}. The authors reported complete resolution of symptoms in 17 of the 35 patients randomised to receive nicotine compared with 9 of the 37 patients given placebo.

The mucosal mucus layer
Mucus is a complex visco-elastic gel, which acts as a lubricant and an interface with the colonic bacterial flora. Mucus is the first line of defence between the mucosa and luminal pathogens.
A large proportion of mucus is made up of highly glycosylated, high molecular weight glycoproteins, varying in size between $0.5 \times 10^6$ and $6 \times 10^6$ Daltons. There are well described changes in intestinal mucin in inflammatory bowel disease. Goblet cell mucus is severely depleted in active ulcerative colitis and there is an increase in the production of sialomucin and reduction in sulphomucin as assessed by staining with alcian blue and high diamine$^{225}$.

Podolsky and Isselbacher described a deficiency in one subtype of mucin (type IV mucus) in ulcerative colitis$^{226}$. The same mucin abnormality has been described in the monozygotic twin of a patient with ulcerative colitis$^{227}$. In contrast, the type IV mucus is normal in Crohn's disease, radiation colitis, ischaemic and infective colitis$^{228}$. It has been suggested that an abnormality in the mucus layer may render the host more susceptible to another agent such as bacteria which are only pathogenic when this alteration is present. It has also been argued that the division of mucin in to subclasses is artificial and possibly a consequence of the method used.

Bacteroides and Enterobacter species have been found to be able to invade the mucus layer of patients with Crohn's disease in immunohistochemical studies. This is probably not simply an opportunistic colonisation of ulcers as the same number of bacteria can be found in ulcerated and non-ulcerated areas. Normally, IgA is secreted into the mucus layer to prevent such colonisation. It has been found that patients with inflammatory bowel disease have reduced levels of secretory IgA$^{229}$. The administration of Lactobacilli and Bifidobacteria has been shown to induce remission in some patients with pouchitis perhaps by stimulation of the mucosal IgA secretion.

**The mucosal microvasculature**

There is evidence for widespread inflammatory vasculitis in inflammatory bowel disease$^{230}$.

There is activation of the clotting cascade with a reduction of the partial thromboplastin time, thrombocytosis, increased level of factor VII and fibrinogen$^{231}$ activated platelets and disorders of factor V, VIII decreased level of antithrombin III$^{232}$.

Hudson and colleagues$^{233}$ have reported increased plasma concentration of VII:C (factor VII coagulant activity), fibrinogen and lipoprotein (a) in patients with inflammatory bowel disease. Increased concentration of VII:C complicates microvascular damage and inflammation in the interstitial wall by augmenting focal fibrin deposition on the luminal surface of inflamed vessels.
There is increased activity of Factor VII - a potent procoagulant in the presence of tissue factor\textsuperscript{234}. Lipoprotein (a) competes and binds to plasminogen receptor binding sites on the endothelial cells. This in turn may impair fibrinolysis and increase platelet accumulation. Fibrinogen, an acute phase protein, increases plasma viscosity and activates platelets; both are known to compromise microcirculation.

Thus, the changes in vessel wall by vasculitis and changes in blood constituents may be responsible for the local and systemic vaso-occlusive phenomena seen in inflammatory bowel disease.
INFLAMMATORY PROCESSES

The intestinal epithelium serves as a dynamic barrier regulating the uptake of nutrients and water at the same time as excluding potential pathogens. The gastrointestinal immune system must be able to respond to pathogens yet be unresponsive (tolerant) to commensal organisms and food proteins. Any stimulus which permit back-diffusion of luminal contents or bacteria may create a positive feedback loop which contributes to loss of oral tolerance and chronic inflammation\textsuperscript{25,26,27,28} (Figure I below).

Immunological tolerance to commensal intestinal flora may be compromised in inflammatory bowel disease\textsuperscript{29}. Inappropriate entry into the mucosal compartment of otherwise contained luminal entry could, at least in principle, lead to other pathology. Reports of associated conditions range from arthropathies\textsuperscript{30} to autism\textsuperscript{31}.

Despite the idiopathic nature of inflammatory bowel disease, it is apparent that much of the pathophysiology, tissue damage and symptomatology of these disorders are due to inappropriate or exaggerated immune reactions. Studies on cytokine deficient mice have demonstrated that exposure of mucosal surfaces to the normal gut flora can trigger epithelial responses which upregulate inflammation\textsuperscript{32,33,34}. A variety of pathogenic bacteria and their products have been shown to have direct effects on epithelial ion transport and permeability\textsuperscript{35,36}. Interestingly, the administration of \textit{Lactobacillus} from birth prevented the development of spontaneous colitis in IL-10 gene-deficient mice\textsuperscript{37}. However, the lactobacilli were not able to treat the experimental colitis once established. Instead, the probiotic compound, VSL\#3 containing a mixture of bifidobacteria lactobacilli and \textit{Streptococcus thermophilus} was able to normalise barrier integrity, reduce mucosal proinflammatory cytokines and improve the histological appearances of colitis in IL-10 deficient mice\textsuperscript{38}.
The normal mucosal immune system

The induction of T cell responses requires recognition of antigen in association with class II major histocompatibility complex (MHC) proteins and specialized antigen presenting cells such as dendritic cells, macrophages, B cells and endothelial cells. Peyer's patches transports antigens across the small intestine into the lymphoreticular system.

Alternatively, antigens may cross the mucosa between the epithelial cells, by persorption (the passage of macromolecules or small particles by kneading between epithelial cells), villous uptake and active uptake by endocytosis or by receptor-mediated mechanisms.

Figure II

After an antigen-presenting cell has taken up antigen, it is processed and presented to a T cell possessing a receptor for the antigen in combination with IL-1 and IL-6. The T cell then becomes activated and secretes IL-2 and interferon gamma (IFNγ), which in turn activates macrophages (figure II).

However, antigen exposure on its own leads to tolerance or apoptosis and additional signals from costimulatory molecules are required for T-cell activation. Antigen recognition by T cells induces the expression of the costimulatory molecule CD40 ligand (CD40L) on the T-cell surface. CD40L then engages another costimulatory molecule CD40 on the antigen-presenting cell and stimulates the expression of the costimulatory molecule B7 (also on the surface of the antigen-presenting cell) and the secretion of cytokines that activate T cells. B7 then engages yet another costimulatory molecule CD28 on the surface of the T cell.
T cells can express CD40L on antigen recognition even without costimulation, but sustained expression of CD40L requires B7-CD28 costimulation, as well as antigen. Thus, the B7 and CD40 pathways stimulate each other. Several other costimulatory molecules have been discovered, including ICOS\textsuperscript{250} B7RP\textsuperscript{251}, B7-1\textsuperscript{252} B7-2\textsuperscript{253} and B7H\textsuperscript{254}.

Apart from IL-2 and IFN\textgamma, activated T cells produce IL-4, IL-5 and IL-6, which control the proliferation and differentiation of B cells into specific antibody secreting cells. B cells take part in the secondary, but not the primary immune response. After activation, the T cell expresses IL-2 receptors on the cell surface and the interaction of the IL-2 receptor with IL-2 is a critical event in T cell proliferation, differentiation and function\textsuperscript{255}. Antigen recognition by T cells also induces the expression of CD40 ligand which connects with CD40 on the antigen presenting cell to induce the expression of B7 molecules and cytokines which further activate T cells\textsuperscript{256}.

After antigen exposure, dendritic cells migrate to the regional lymph nodes to interact with T cells bearing the specific antigen receptor to initiate the immune response. Dendritic cells are able to induce primary T-lymphocyte responses to both soluble and particulate antigens. Although, the majority of colonic mucosal macrophages express class II proteins\textsuperscript{257}, they have been shown to suppress rather than enhance the induction of primary immune responses by dendritic cells\textsuperscript{258}. After B and T lymphocytes have encountered antigen in Peyer's patches, they home back to the mucosal lamina propria via the blood stream. Many of these "homing" B-lymphocytes produce IgA, which bind with secretory component synthesised by enterocytes and travel to the mucosal surface. IgA is the major immunoglobulin produced by the gut and is resistant to digestion. It prevents binding of antigen to the epithelial cell but does not activate complement and may therefore block triggering of non-specific biological amplification mechanisms by serum IgG\textsuperscript{259}. IgA antibody may also remove intact antigen which has entered circulation, removing it via the biliary system\textsuperscript{260}.

**Mediators of inflammation**

*Non-specific mediators of inflammation*

Prostaglandins cause vasodilatation, enhance vascular permeability and produce pain. Patients with inflammatory bowel disease have elevated mucosal and serum levels of, primarily, prostaglandin E2 (PGE\textsubscript{2})\textsuperscript{261,262}. Paradoxically, non-steroidal anti-inflammatory drugs which block prostaglandin synthesis may cause deterioration of the disease\textsuperscript{263,264}.
Leukotriene B4 (LTB4) is a potent chemoattractant for human neutrophils. LTB4 is present in high concentrations in rectal dialysates and is a major neutrophil chemotactic agent in the mucosa of patients with inflammatory bowel disease.

Platelet-activating factor (PAF) is a potent chemotactic factor for neutrophils, monocytes and eosinophils. PAF also enhances vascular permeability and is found in higher levels in the colonic mucosa of patients with inflammatory bowel disease compared with healthy controls.

Reactive oxygen metabolites are produced in excess in inflammatory bowel disease. These increase the mucosal permeability and activate neutrophils further.

Nitrous oxide (NO) is synthesised from L-arginine by a constitutive calcium-dependent NO synthetase (NOS). There are two forms of NO synthase: a constitutive calcium-dependent synthase found in endothelial cells and regulating neuronal activity, vascular permeability and gut motility. Macrophages and neutrophils contain a calcium-independent NO synthase which is induced by inducing agents such as lipopolysaccharide and cytokines.

In active but not quiescent ulcerative colitis, rectal dialysates have increased levels of nitrite. Broughton-Smith et al. found increased nitric oxide synthase activity in supernatants of homogenates from inflamed ulcerative colitis but not Crohn's disease mucosa. L-citrulline is a breakdown product of L-arginine by NO synthase and raised mucosal levels have been reported in active ulcerative colitis.

**Cytokines**

Cytokines are small, non-structural proteins with molecular weights ranging from 8 to 40,000 Daltons. Originally called lymphokines and monokines to indicate their cellular sources, it soon became clear that the term "cytokine" is the best description, since nearly all nucleated cells are capable of synthesizing these proteins and, in turn, of responding to them. There is no amino acid sequence motif or three-dimensional structure that links cytokines; rather, their biological activities allow us to group them into different classes. For the most part, cytokines are primarily involved in host responses to disease or infection, and any involvement with homeostatic mechanisms has been less than dramatic.

Many scientists have made the analogy of cytokines to hormones, but this is not an accurate comparison.
First, hormones tend to be constitutively expressed by highly specialized cells, but nearly all cells synthesize cytokines. Whereas hormones are the primary synthetic product of a cell (insulin, thyroid, adrenocorticotropic hormone, etc), cytokines account for a rather small amount of the synthetic output of a cell. In addition, hormones are expressed in response to homeostatic control signals, many of which are part of a daily cycle.

In contrast, most cytokine genes are not expressed unless specifically stimulated by noxious events. In fact, it has become clear that the triggering of cytokine gene expression is nearly identical to "cell stressors." Cytokines are produced in response to "stress," whereas most hormones are produced by a daily intrinsic clock.

Cytokines regulate cellular infiltration, tissue damage, ulceration, secretion, motility and fibrosis. Another way to look at some cytokines is their role in infection and/or inflammation. Some cytokines clearly promote inflammation and are called proinflammatory cytokines, whereas other cytokines suppress the activity of proinflammatory cytokines and are called anti-inflammatory cytokines. For example, IL-4, IL-10, and IL-13 may be labelled as pro-inflammatory as they are all potent activators of B lymphocytes. However, they can also be called potent anti-inflammatory agents by virtue of their ability to suppress genes for proinflammatory cytokines such as IL-1, TNF, and the chemokines.

Listing cytokines in various categories should be done with an open mind, as a cytokine's bioactivity is governed not only by its concentration, but also by the number of membrane receptors, the modulating activities of binding proteins that can enhance or suppress receptor binding, the presence of receptor antagonists, synergy with or inhibition by other cytokines and eicosanoids, and the activation and differentiation state of the responding cell. The net inflammatory response is the result of a balance of all of these factors.

There is evidence that severe inflammatory disorders may be mediated by cytokines. As outlined above, mice rendered specifically deficient of IL-2 develop inflammation of the bowel, with similarities to human ulcerative colitis, disruption of the mouse transforming growth factor-beta 1 (TGF-β1) gene results in multifocal inflammatory disease. Severe intestinal inflammation is also observed in IL-10 knockout animals but this has not been reported in IL-4, IL-13, IL-4/IL-13 knockouts.
**Pathophysiological Effects of Cytokines**

Interleukin-1 (IL-1) and IL-2 are key immunoregulatory cytokines that amplify the inflammatory response by activating a cascade of immune cells. IL-1α and IL-1β and tumour necrosis factor alpha (TNFα) are secreted by activated macrophages and stimulate the production of cytokines, arachidonic acid metabolites, and proteases by intestinal macrophages, neutrophils, and smooth muscle cells, fibroblasts, and epithelial cells. In contrast to TNF, IL-1 can induce IL-2 and IL-2 receptors on T lymphocytes.

Following antigen and cytokine stimulation, T helper (Th1) lymphocytes secrete IL-2 and interferon γ (IFN-γ), which stimulate a predominantly cell-mediated immune response mediated by cytotoxic T lymphocyte subsets, macrophages, and natural killer cells. IFN-γ enhances the expression of major histocompatibility complex class II molecules on macrophages; B-lymphocytes; and dendritic, endothelial, mesenchymal, and epithelial cells, which increases antigen-presenting function of these cells. In contrast, IL-4, IL-5, and IL-10 produced by Th2, lymphocytes drive a predominantly humorally mediated hypersensitivity response through stimulation of immunoglobulin IgG1, IgA, IgE synthesis and activation of eosinophils.

Recruitment of circulating effector cells into the inflammatory focus is important in amplifying the immune response. Induction of adhesion molecules and their ligands on endothelial and immune cells by IL-1, TNF, and IFN-γ dramatically increases the ability of neutrophils, monocytes, and lymphocytes to adhere to blood vessels. IL-1 further stimulates migration of effector cells into an inflammatory focus and TNF induced production of chemotactic molecules such as the chemokine family of cytokines (IL-8, gro, monocyte chemotactic and activating factor, etc), TGF-β, leukotriene B4 and platelet activating factor by lamina propria immunocytes and mesenchymal cells.

In addition to their proinflammatory activities, cytokines have immunosuppressive properties. IL-1 and TNFα induce protective prostaglandins and cortisol. Transforming growth factor β (TGF-β) suppresses lymphocyte proliferation, IL-4 downregulates macrophages, IL-4, and IL-10 inhibit Th1 lymphocyte proliferation and cytokine production and IL-1 receptor antagonists are induced by IL-1, TGF-β and IL-4.
Cytokines and intestinal permeability

Mucosal epithelial cells are in dynamic equilibrium with the surrounding cells. Cytokines are important mediators of this interaction. For example, IL-1, IL-3, and TNFα have been found to indirectly stimulate epithelial ion secretion\textsuperscript{281,282,283,284}, an effect mediated by prostaglandins from subepithelial cells. The effects appear to be modulated by the presence of myofibroblasts\textsuperscript{285}. IFN-γ and IL-4 have no direct affect on baseline ion transport in vitro but both dramatically reduce the ability of the cells to secrete chloride in response to secretagogues\textsuperscript{286,287}. IL-10 appears to exert an anti-secretory influence on gut epithelium \textit{in vitro}\textsuperscript{288}.

Tight junctions form a circumferential seal at the luminal pole of adjacent epithelial cells and regulate paracellular permeability\textsuperscript{289}. These junctions express a high degree of plasticity and may be regulated by cytokines\textsuperscript{290}. For example, addition of IFN-γ to the basolateral surface of T84 monolayers causes a significant increase in paracellular permeability\textsuperscript{291}. IL-4 and IL-13, have broadly similar functions, both increasing epithelial permeability\textsuperscript{292}. Certain cytokines have been shown, in contrast to the pro-inflammatory molecules, to prevent or reverse impaired permeability. IFN-γ induced increases in T84 permeability can be prevented by concomitant treatment with transforming growth factor β\textsuperscript{293} or IL-10\textsuperscript{294}. It is unlikely that epithelia would be exposed to a single cytokine and immune mediated alterations in epithelial permeability are probably due to a combination of multiple mediators. TNFα alone increases endothelial permeability\textsuperscript{295,296,297} but the effect is enhanced by co-treatment with low dose IFN-γ\textsuperscript{298}.

Molecules other than cytokines may also contribute to signalling cascades which result in altered epithelial permeability. For instance, tissue and cell culture studies have shown that nitric oxide can regulate ion transport and epithelial permeability\textsuperscript{299,300} and may be the final mediator of IFN-γ evoked disruption of epithelial barrier function\textsuperscript{301}. Insulin-like growth factors have also been found to increase permeability across confluent T84 monolayers\textsuperscript{302}. Intra-epithelial lymphocytes produce at least two molecules which modulate epithelial permeability, a small molecule <30 kDa that causes a rapid (within four hours) 30% drop in barrier function and a larger molecule with a slower onset but with a more profound effect on epithelial barrier function (reduced by 90%)\textsuperscript{303}. Bacterial infection of HT-29 cells result in increased epithelial prostaglandin (E\textsubscript{2} and F\textsubscript{2} α) synthesis\textsuperscript{304} and transfer of supernatant from the infected cells to naive epithelial monolayers evokes a transient increase in electrolyte transport\textsuperscript{305}.  

38
**Cytokines and regulation of Fibrosis**

Intestinal cytokines and growth factors contribute to the fibrosis seen in Crohn's disease indirectly by amplifying the inflammatory response and directly via IL-1 and TNFα, which stimulate proliferation of intestinal smooth muscle and fibroblasts. TGF-β and insulin like growth factor-1 stimulate proliferation of fibroblasts and induce collagen synthesis by fibroblasts and smooth muscle cells. Fibroblasts derived from strictured segments of resected Crohn's disease tissues produce significantly more type III collagen than fibroblasts from unstimulated or normal segments on TGF-β stimulation.

**Cytokines and the systemic response to inflammation**

The systemic response to inflammation is mediated by cytokines derived from the inflamed mucosa or circulating activated immunocytes. Systemic effects of IL-1, IL-6 and TNFα include fever, anorexia, leukocytosis, normochromic anaemia, thrombocytosis, induction of the, repair response and stimulation of the hypothalamic/pituitary/adrenal axis.

In vivo responses to IL-1 and blockade of this molecule in experimental colitis confirm the central regulatory role of IL-1 in inflammatory bowel disease. Long-term injection of IL-1 produces epithelial cell necrosis, oedema, neutrophil infiltration and goblet cell depletion. Systemic effects include periportal hepatic inflammation and leukocytosis in peripheral blood. Blockade of IL-1 by recombinant IL-1 receptor antagonist does not completely abolish but attenuates both the local and systemic effects in acute and chronic experimental colitis. IL-1 may also affect the gut motility. It has been demonstrated that IL-1 stimulation of corticotrophin-releasing hormone not only induces cortisol secretion but also increases colonic motility.

Intravenous infusion of TNFα produces shock, diarrhoea, extravasation of neutrophils and erythrocytes in the small and large intestine accompanied by necrosis of epithelial and endothelial cells. Over a 10-day period both acute and chronic intestinal inflammation is induced with bile duct proliferation, periportal inflammation, anaemia and leukocytosis.

IFN-γ for 3 days induces class II major histocompatibility complex antigens on small intestinal and colonic epithelial and endothelial cells. High systemic doses of IFN-γ produce epithelial necrosis on villous tips. The majority of patients given IL-2 or IL-4 infusions develop diarrhoea, the mechanism of which is unknown.
Immunological abnormalities in inflammatory bowel disease

The balance between Th1 and Th2 phenotypes of T lymphocytes determines the characteristics of the chronic inflammatory process. It has been proposed that when the mucosal immune system in patients who develop Crohn's disease is exposed to an initiating antigenic stimulus, it mounts a dysregulated and excessive Th1 T-cell response characterized by increased expression of interferon, interleukin (IL)-2, IL-12, and IL-18 followed by subsequent increased production of the proinflammatory cytokines TNF and IL-1, and then NF-κB as well as a compensatory increase in the Th2 mediated anti-inflammatory cytokine IL-10 and transforming growth factor β. In ulcerative colitis, the pattern of cytokine expression is different with a relatively decreased Th1 response characterized by an increased expression of IL-4, IL-5, IL-6, IL-10, and IL-13.

Interleukin-2 knockout mice develop colonic inflammation characterised by an excessive Th1 response when reared in a conventional environment with normal bacterial flora but remain healthy when reared in a sterile or pathogen-free environment. This observation suggests that exposure to one or more antigens present in the normal microflora is necessary to trigger colitis. IL-2 deficient mice given anti-TGFβ developed colitis and oral tolerance in normal mice is at least partly mediated by TGFβ. Thus, TGFβ may be important in the downregulation of inflammatory mucosal responses in both normal and pathologic states, and dysregulation of this factor may be implicated in Crohn’s disease.

Humoral immune system

In inflammatory bowel disease, there is an expansion of the B cell population to a greater extent than the T cell population. In normal mucosa, dimeric IgA is the predominant type of immunoglobulin secreted by cells, whereas in inflammatory bowel disease, monomeric IgA is secreted in greater amounts and there is dramatic local overproduction of IgG, particularly in ulcerative colitis, increased amount of IgA1 at the expense of IgA2 and a reduced secretion of secretory IgA.

In active inflammatory bowel disease, the mucosal macrophage population is increased and often appear in clusters. The macrophages express receptors for IL-2 and have been shown to produce greater amounts of IL-1, TNFα, IL-6 and IL-8 than controls when stimulated. Other functions such as ability to undergo respiratory burst and capacity to release oxygen radicals are increased.
In active inflammatory bowel disease, especially ulcerative colitis, there is an infiltrate of neutrophils in the mucosa. Neutrophil activation leads to degranulation with release of tissue-damaging lysosomal enzymes, oxygen radicals and inflammatory mediators\textsuperscript{329}.

Several studies have focused on phagocytosis and respiratory burst activity of granulocytes in ulcerative colitis with conflicting results. While some found defective granulocyte functions\textsuperscript{330}, thus permitting a contribution to the ongoing chronic inflammation in this way, others did not\textsuperscript{331}.

**Cell mediated immunity**

In inflammatory bowel disease there is a polyclonal\textsuperscript{332} increase in the T lymphocyte population although there is no change in the ratio of CD4\textsuperscript{+} to CD8\textsuperscript{+} cells\textsuperscript{333}. The T cells are activated particularly in Crohn's disease\textsuperscript{334} and serum levels of the soluble IL-2 receptor are increased\textsuperscript{335,336}. In ulcerative colitis, there is also indirect evidence of increased local T cell activity. The expression of MHC class II antigens by enterocytes is upregulated by interferon-\(\gamma\)\textsuperscript{337} - a product of activated CD4\textsuperscript{+} T cells. This expression correlates with T cell expression of the CD7 marker of "immunostimulation"\textsuperscript{338}. It has been reported that T cells isolated from the lamina propria of patients with active inflammatory bowel disease express IL-2 receptor on a greater proportion of cells than healthy controls\textsuperscript{334,339}. However, another group found no difference\textsuperscript{340}.

In the lamina propria there are many memory T lymphocytes (CD 45RO) but few naive T cells (CD 45RA)\textsuperscript{341,342}. CD29, a marker of memory T cells, is not expressed in the lamina propria in high density indicating that lamina propria T cells differ from 'classical' memory T cells\textsuperscript{343}. This is supported by functional studies which show that lamina propria T cells do not proliferate after stimulation with antigen but rather provide helper function for immunoglobulin synthesis\textsuperscript{344}. The lamina propria T cells express CD4 and are so called "T helper cells" whereas the majority of the intraepithelial lymphocytes are "cytotoxic T cells" expressing CD8\textsuperscript{345}.

Many T cell mediated phenomena have been investigated \textit{in vivo} including skin reactivity to recall antigens\textsuperscript{346}, lymphocyte proliferation to lectin mitogens\textsuperscript{347}, cytotoxic function\textsuperscript{348}, allogenic and autologous mixed lymphocyte reactivity\textsuperscript{349,350} and suppressor cell function\textsuperscript{351,352,353}. No consistent defect has been found.
**Epithelium**

The normal colonic epithelial cell does not express class II antigens. However, in inflammatory bowel disease and infectious colitis, the expression is upregulated and probably represent a non-specific response to inflammation\(^354\). A similar response may be induced *in vitro* by stimulation with IFN\(\gamma\)\(^355\) and to a lesser extent TNF\(\alpha\) suggesting that cytokines are involved in the antigen processing by intestinal epithelial cells\(^356,357\).

It has been suggested that enterocytes are capable of processing and presenting antigen to lymphocytes\(^358\) and that the epithelial cells in inflammatory bowel disease preferentially stimulate helper/inducer T cells rather than suppressor T cells\(^359\).

The epithelium is both target and source of cytokines. Epithelial derived cytokines have the potential to play an atocrine role or to influence adjacent non-epithelial cells. For example, cytokines induce epithelial expression of a number of proteins, including IL-6, IL-8, chemokines, TGF-\(\beta\), class I and II major histocompatibility complex antigens, manganese-dependent superoxide dismutase, and secretory component and acute phase reactants\(^360\). IL-6 is a major inducer of tissue inhibitor of matrix metalloproteinase and much of the mucosal damage in inflammatory bowel disease may be due to the release of metalloproteinases\(^361\). In contrast, TGF-\(\beta\) appears to be the central mediator of epithelial cell restitution following in vitro injury.

**Cytokines in inflammatory bowel disease**

It has been proposed that luminal antigens stimulate a local Th1 immune response, characterised by a T helper (CD4) pattern of lymphokines (IL-2, IFN-\(\gamma\) and IL-12, TNF-\(\alpha\)) in Crohn's disease and a Th2 profile (IL-4, IL-5, and IL-10) in ulcerative colitis (figure V below).

This concept is supported by observations of increased tissue concentrations of IL-4 and IL-10 in ulcerative colitis but not in Crohn's disease\(^362\) and the increased lamina propria secretion of IFN\(\gamma\) in Crohn's disease and IL-5 in ulcerative colitis\(^363\).
T helper cells, modulate cellular immunity through the secretion of multiple cytokines, which in turn modulate numerous effector functions including: immunoglobulin secretion, complement activation, neutrophil chemotaxis, and macrophage activation. In patients with inflammatory bowel disease, mucosal T suppressor (CD8) and T helper cells are present in the normal proportions but have an increased level of activation. The importance of CD4 cells was highlighted by a report of complete remission in a patient with Crohn's disease who became infected with the human immunodeficiency virus.

The Th2 response of ulcerative colitis is a humorally mediated hypersensitivity response leading to activation of eosinophils. It has been suggested that this Th2 response is primarily aimed at combating parasitic intestinal infection. This may explain why there is a lower risk of inflammatory bowel disease in regions of the world with higher prevalence of parasite infestations. The mucosal immune system in these parts of the world may be primed towards an immunoregulatory Th2 response rather than a cytotoxic Th1 response. Although Th2 cells and IL-4 were initially thought to be involved in intestinal immunoregulation, the recent description of apparently Th2-mediated murine inflammatory bowel disease indicates that excessive Th2-type responses may be pathogenic in their own right.

The levels of IL-1, IL-6 and IL-8 are increased in inflammatory bowel disease and infectious colitis. IL-1 is predominantly secreted by macrophages within the lamina propria. In contrast, IL-6 and IL-8 can be produced by activated epithelial cells as well as macrophages. Circulating monocytes, isolated from patients with inflammatory bowel disease secrete IL-1, IL-6 and IL-8.

Secretion of IL-2 and IFN-γ, by stimulated lymphocytes in long-term culture is lower in inflammatory bowel disease than control tissues. However, in vivo levels of mRNA are increased in Crohn's disease but not in ulcerative colitis. IL-2 receptor expression is increased on lamina propria mononuclear cells from patients with Crohn's disease and is accompanied by increased serum levels of soluble IL-2 receptor. Tissue and serum levels of IL-2 receptor are lower in ulcerative colitis than in patients with Crohn's disease, but remain higher than in healthy controls. In Crohn's disease, the increased IL-12 release can induce in vitro the activation of tissue metalloproteinases, inducing extracellular matrix degradation, thus leading to tissue damage.
Current anti-inflammatory therapy in inflammatory bowel disease

Several strategies can be used to inhibit the pro-inflammatory effects of cytokines associated with intestinal inflammation. Many of the drugs currently used in therapy of intestinal inflammation may block cytokine synthesis or secretion. Some of these agents, such as corticosteroids or mesalazine, inhibit synthesis of almost all cytokines, whereas others are more specific.

**Glucocorticoids**

Glucocorticoids alter the regulation of protein synthesis by forming of a complex with cytoplasmic receptors inside target cells. Binding of the receptor complex may up- or downregulate gene transcription. For example, lipocortin, an enzyme responsible for the release of arachidonic acid and inhibition of phospholipase A2, is stimulated. Glucocorticoids inhibit IL-1 and IL-8 synthesis by monocytes and IL-6 production by monocytes, endothelial cells and fibroblasts. Dexomethasone has been shown to diminish secretion of interleukin-1 (IL-1) by colonic biopsies from patients with active ulcerative colitis cultured in vitro. Glucocorticoids inhibit transcription of the IL-2 gene in T cells and attenuate the IL-2 mediated receptor activation of these cells. These drugs also inhibit the production of IFN-γ mRNA in human T cells and the adjuvant effect of IFN-γ in processing and presentation of antigens. Glucocorticoids upregulate expression of IL-6R on human epithelial and hepatoma cell lines.

**5-ASA**

Several groups have reported that 5-ASA (5-aminosalicyclic acid) reduces the production of IL-1β by cultured colonic cells obtained from patients with active ulcerative colitis. 5-ASA significantly reduced the IFN-γ induced increased trans-epithelial flux of mannitol (a marker of paracellular permeability) across HT-29-19A monolayers. This effect may be due to the 5-aminosalicylic acid competing for the IFN-gamma receptor or to the disruption of arachidonic acid metabolism. Sulphasalazine has been shown to inhibit IL-2 production of cultured splenocytes, whereas 5-ASA and sulphapyridine failed to affect production of this cytokine. Sulphasalazine inhibits TNFα production in human mononuclear cells and inhibits the binding of TNFα to its receptors.
5-ASA inhibits formation of leukotrienes and prostaglandins and acts as a scavenger of reactive oxygen metabolites. 5-ASA also decreases expression of HLA-DR molecules on the HT29 colonic adenocarcinoma line, by impairing the binding of IFNγ to its receptor on colonic epithelial cells. NF-κB is a final intracellular signal conduction pathway, which activate the production of IL-1, IL-2, IL-12, and TNF-α, cell-surface receptors, transcription factors, and adhesion molecules (including ICAM-1). 5-ASA compounds are potent nonselective inhibitors of NF-κB.

Treatment with topical antisense oligonucleotides to the p65 subunit of NF-κB in a mouse model and in IL-10 deficient mice has demonstrated a therapeutic benefit but as yet no human trials of selective inhibitors have been carried out.

**Cyclosporine A**

The main effect of cyclosporin A is to prevent T cell activation, IL-2 transcription and production. T helper cells are mainly affected whilst cytotoxic/cytotoxic T cells (CD8+) are relatively resistant to cyclosporin A, perhaps owing to a less IL-2 dependent or alternative activation pathway. The drug inhibits the effect of peptidyl-prolyl isomerase (PPIase) on its cytosolic binding protein - cyclophilin. PPIase is important for the folding of proteins in their native conformations and may be involved in the regulation of intracellular signalling events in T cells. The release of other cytokines such as IFNγ and IL-4 is also affected. After renal transplantation the drug has been shown to reduce the capacity of peripheral blood mononuclear cells to produce IL-6.

**Anti-TNF therapy**

One possible means of abrogating the Th1 T-cell response in Crohn disease is the blockade of the effects of TNF-α. TNF-α is involved in the inductive phase of the Th1 T-cell response by means of its ability to synergize with interferon-γ in the feedback enhancement of interleukin-12 production. In addition, TNF-α is involved in the effector phase of the Th1 T-cell response by means of its capacity to act as a pro-inflammatory cytokine that directly mediates mucosal inflammation. In the context of Crohn disease, one important component of TNF-α-mediated inflammatory activity is the activation of enzymes in the lamina propria, such as collagenase, and the release of substances that increase intestinal permeability and thus increase the exposure of T cells in the lamina propria to mucosal antigens that induce further adverse immune responses.
Clinical trials of the use of Infliximab in humans with Crohn's disease have clearly shown that anti-cytokine-based therapy is well tolerated and produces excellent clinical responses in some patients\cite{409,410,411}. Administration of anti-TNF-α addresses the effector phase of the Th1 T cell response more than it does the inductive phase.

Thus, the inflammatory process of Crohn's disease may still eventually shift to other effector cytokines and become resistant to anti-TNF α therapy, as has been the case in the treatment of some patients with rheumatoid arthritis\cite{412}. The recent study by Rutgeerts et al sheds some light on the issue of waning effect to infliximab\cite{413}. Five hundred seventy-three patients with active Crohn's disease who had responded to a single 5 mg/kg induction dose of infliximab were randomized to receive placebo alone or repeated doses of infliximab. Re-treatment with infliximab every 8 weeks proved more effective than placebo for maintaining remission. This study also demonstrated that a 3-dose induction regimen with infliximab (dosing at 0, 2, and 6 weeks) is more effective at inducing remission than a single induction dose (40% remission at week 10 after a 3-dose induction compared with 28% remission after a single-dose induction)\cite{414}.

Infliximab has also been studied for the closure of fistulas in Crohn's disease\cite{415}. Complete closure of all fistulas for at least 4 weeks occurred in 55% of patients given infliximab compared with 13% of the placebo-treated group.

Infliximab, contains approximately 75% human protein and 25% mouse protein\cite{416}. The murine portion of the antibody is the variable or antigenic recognition region. The formation of antibodies to the murine protein has been reported in between 36 – 68% of patients \cite{417}. The development of antibodies may be associated with a loss of response to infliximab\cite{418} and patients with antibodies also develop infusion reactions more frequently\cite{419}. Acute infusion reactions have been reported in 17% of patients compared with 7% treated with placebo infusions\cite{419}. In addition, a syndrome of delayed hypersensitivity characterized by myalgia, arthralgia with fever, rash, pruritus, facial, hand, or lip oedema, dysphagia, urticaria, sore throat and headache has been reported in up to 25% of patients retreated with infliximab after a drug holiday of 2–4 years\cite{420}.

Autoantibodies including antinuclear antibodies have been reported in 34 - 50% of patients and anti–double-stranded DNA antibodies in 9%\cite{421}. 

46
Rarely, these patients may develop features of drug-induced lupus. Non-Hodgkin's lymphoma has been reported in 3 patients with Crohn's disease and at least 4 patients with rheumatoid arthritis\textsuperscript{422,423}. Serious infections may reported after treatment with infliximab includes septicaemia, tuberculosis, histoplasmosis, listeriosis, and aspergillosis\textsuperscript{424,425}.

Another monoclonal antibody, CDP571 with less mouse antigen has been engineered. The molecule contains approximately 95% human protein and 5% murine protein, and appears less immunogenic than infliximab\textsuperscript{426}. Antimurine antibodies occurred overall at a rate of 5.3% and anti-double stranded antibodies in 5.3\%\textsuperscript{427}. The risk of infusion reactions may also be reduced and has been reported in 12.7\% of patients compared with 7.7\% of placebo-treated patients\textsuperscript{428}.

There are two distinct cell-surface TNF receptors, called p55 and p75. Soluble receptors consisting of only the extracellular, ligand-binding domain are present in body fluids and may be involved in regulating TNF activity\textsuperscript{429}. Etanercept is a genetically engineered human protein consisting of two identical chains of the TNF-receptor p75 monomer fused with the Fc domain of human IgG\textsuperscript{430}. Etanercept was constructed to neutralise soluble TNF recepts but a placebo-controlled trial in 43 patients with active Crohn's disease failed to demonstrate efficacy\textsuperscript{431}. Onercept, another genetically engineered recombinant human TNF-receptor has been compared with CDP571. At 2 weeks, 18\% of patients receiving onercept and 67\% of patients receiving CDP571 had attained clinical remission\textsuperscript{432}.

CNI-1493 is a small molecule which appears to block TNF gene expression by inhibiting intracellular signaling pathways that lead to translational activation of the TNF gene\textsuperscript{433}. A small randomized dose-finding study of CNI-1493 administered intravenously daily for 12 days in active Crohn's disease was recently reported by Hommes\textsuperscript{434} et al. At 2 weeks, 8 of 9 patients had a clinical response and 4 of 9 patients were in remission.

Thalidomide also inhibits TNF biosynthesis by enhancing degradation of TNF mRNA in macrophages, as well as inhibiting the Th1 polarising cytokine IL-12\textsuperscript{435}. Two uncontrolled pilot studies have been conducted to treat active inflammatory and fistulizing Crohn's disease. Vasiliasuksas et al.\textsuperscript{436} used thalidomide 50–100 mg/day for 12 weeks and reported response rates of 67\% and remission rates of up to 33\%. Ehrenpreis et al.\textsuperscript{437} used a higher dose of thalidomide (200–300 mg/day) for 12 weeks in patients with active inflammatory and fistulizing CD.
Eighty percent of patients with fistulizing disease had fistula closure, and 50\% of patients with active inflammatory disease had a clinical response.

**Future therapy to manipulate the mucosal immune response**

The increasing knowledge of the mechanism involved in chronic inflammation has led to the development of specific therapies that mechanistically target individual inflammatory pathways in inflammatory bowel disease. Many new therapies are under evaluation including; (1) recombinant proteins, (2) monoclonal antibodies; (3) nucleic acids in the form of anti-sense oligonucleotides and (4) gene therapy. The different therapeutic approaches, which are under investigation, are discussed below.

**Anti-Interferon-γ, Interferon-α and Interferon-β**

The Th1 T-cell response is characterized by increased expression of interferon-γ. The administration of monoclonal antibodies to interferon-γ could therefore theoretically have an effect in Crohn's disease. However, in animal model studies, anti–IFN-γ antibodies were less effective than anti–IL-12 antibodies for the reversal of experimental colitis in a mouse model\[438\]. A phase II study of anti–IFN γ - antibody therapy in patients with Crohn's disease is underway.

In contrast to interferon-γ, interferon-α and interferon-β are both produced by virally infected cells to induce resistance to further infection. Recombinant IFN-α-2a, IFN-α-2b, and IFN-α-n are used to treat chronic hepatitis B and hepatitis C infections, HIV-related Kaposi's sarcoma and multiple melanoma whilst IFN-β is used to treat multiple sclerosis. Uncontrolled studies of IFN-α-2a, IFN-α-2b in patients with Crohn's disease have reported response rates of up to 50\% (P values ranging between 0.08 - 0.26)\[439,440,441,442,443\] and up to 93\% in patients with ulcerative colitis\[444,445\]. Interferon-β has been used in a small placebo-controlled study comprising 18 patients with ulcerative colitis. A response rate of 50\% was reported in the interferon arm compared with 14\% in the placebo group (P=0.14)\[446\].

**Anti–IL-2 receptor antibodies (daclizumab and basiliximab)**

Interleukin-2 is a key immunoregulatory cytokines that amplifies the early inflammatory response by activating a cascade of immune cells. A genetically engineered human anti–IL-2 receptor antibody (Daclizumab) blocks the binding of IL-2 to its receptor. A pilot study of daclizumab in patients with refractory ulcerative colitis reported a beneficial effect in 4 of 5 patients\[447\].
**IL-10 (rHuIL-10)**

Interleukin-10 and the anti-cytokine interleukin-12 may be used to modify the Th1 response seen in Crohn's disease. These agents act by inhibiting interleukin-12 synthesis or function which indirectly inhibits Th1-mediated inflammation. IL-10 suppresses the production of IL-2 and IFN- by T-helper cells and decreases IL-12 production by macrophages. IL-10 knock out mice spontaneously develop chronic enterocolitis. The administration of IL-10 to mice weanlings completely prevented enterocolitis, whilst mice with established colitis had some improvement of their disease.

A phase II dose-response study of 46 patients with refractory Crohn's disease reported that recombinant IL-10 (rHuIL-10) for 7 days was beneficial. Unfortunately, these preliminary results have not been confirmed in subsequent larger placebo-controlled trials in patients with Crohn's disease or ulcerative colitis. Development of systemic administration of rHuIL-10 by intravenous or subcutaneous routes for the indications of ulcerative colitis and Crohn's disease has been discontinued because of a lack of efficacy in these controlled trials.

**IL-11**

IL-11 is produced by mesenchymal cells and has an effect on the barrier function of the intestinal mucosa. Recombinant human IL-11 has been evaluated in Crohn's disease. Sands et al. found no benefit in 76 patients with Crohn's disease given recombinant human IL-11. A larger subsequent study comprising 148 patients did detect a response when IL-11 was given once weekly rather than divided into two smaller weekly doses.

**Anti-interleukin-12**

Increased production of IL-12 is part of the process of polarization toward a Th1 immunologic response characteristic of Crohn's disease. Studies in animal models have demonstrated that antibodies to IL-12 can prevent or treat intestinal inflammation. A phase II trial with a human anti-IL-12 antibody that has been genetically modified in its variable region so that it has a high affinity for human IL-12 in patients with active Crohn's disease is currently underway.
Anti–IL-18

IL-18 is a recently described cytokine found in epithelial cells and activated macrophages. Patients with Crohn's disease have increased mucosal levels\textsuperscript{464}. The cytokine may act synergistically with IL-12 to drive the development of a Th1 immune response by inducing interferon\textsuperscript{465,466}. In a mouse model, anti–IL-18 antibodies were effective in the reducing the experimental colitis\textsuperscript{467}. A monoclonal antibody to human IL-18 has been created\textsuperscript{468} but no human trials are underway as yet.

Anti-CD4 therapies; cM-T412, MAX.16H5 and BF-5

cM-T412 is a genetically engineered IgG1 antibody to CD4 containing approximately 75% human protein and 25% murine protein. In a small phase I study, seven of 8 patients with Crohn's disease and 3 of 4 patients with ulcerative colitis had endoscopic and histologic remission with a mean remission duration of 11 months and 12 months respectively\textsuperscript{469}. Unfortunately, CD4 counts remained persistently low in all patients\textsuperscript{470}. In another small phase I study by Stronkhorst et al\textsuperscript{471}, 9 of 12 patients with Crohn's disease reached remission. Similarly, CD4 counts dropped significantly compared with baseline. Although there have been no opportunistic infections, concern remains over toxicity from sustained CD4 lymphopenia.

MAX.16H5 is another monoclonal antibody to CD4. In a small study by Emmrich et al\textsuperscript{472}, three patients with Crohn’s disease and seven with ulcerative colitis were treated. All three patients with Crohn's disease and four of seven patients with ulcerative colitis responded. Circulating CD4 cells were significantly depleted but the effect only lasted 24 hours\textsuperscript{473}.

BF-5 is a CD4 specific murine monoclonal antibody. In a phase I dose-finding study\textsuperscript{474} four out of eleven patients with Crohn’s disease improved. There was no significant decline of CD4 counts.
Growth factors (epidermal growth factor, fibroblast factor 7, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor and growth hormone)

Several growth factors have been implicated in inflammatory bowel disease including transforming growth factor (secreted by Th3 cells and providing negative feedback on the differentiation of naive T helper cells to Th1 or Th2 subtypes), epidermal growth factor, keratinocyte growth factor-1, and human growth hormone.

Epidermal growth factor (EGF), stimulates cell proliferation in the gastrointestinal tract. Sinha et al, reported on 23 patients with left sided ulcerative colitis treated with EGF enemas. In the active treatment arm, 82% patients attained remission compared with 8% in the placebo arm.

Fibroblast factor 7, is expressed in the gastrointestinal tract where it is a potent stimulant of epithelial cells. The amount and distribution of fibroblast factor 7 in the gastrointestinal tract is altered in patients with inflammatory bowel disease. Animal studies of a recombinant human fibroblast factor 7 (rHuKGF) and keratinocyte growth factor-2 (repifermin, a homolog of fibroblast factor 7) have been encouraging.

A phase II study of repifermin in patients with active ulcerative colitis has recently been carried out. An initial case report by Vaughan et al in 1999, suggested that the administration of human granulocyte colony-stimulating factor (filgrastim), was beneficial in closing perianal fistulas in Crohn's disease. Two subsequent pilot studies of human granulocyte colony-stimulating factor and human granulocyte-macrophage colony-stimulating factor (sargramostim) also reported an effect in treating patients with fistulating disease. A phase II trial with sargramostim in patients with active Crohn's disease is underway.

Growth hormone has been used in Crohn's disease to reverse the catabolic process associated with inflammation. A placebo-controlled trial of recombinant human growth hormone (somatropin) in 37 patients with active Crohn's disease demonstrated a greater decrease in the mean CDAI score for somatropin-treated patients compared with placebo. Surprisingly, the proportion of patients entering remission was not reported.
Anti-adhesion molecules (natalizumab, LDP-02 and isis 2302)

A variety of therapeutic approaches have been used to inhibit lymphocyte trafficking in patients with inflammatory bowel disease including monoclonal antibodies to α4 integrin (natalizumab) and α4β7 integrin (LDP-02); and antisense to intercellular adhesion molecule-1 (ICAM-1). Natalizumab is a recombinant IgG antibody which has been constructed against human α4 integrin. The α4 integrin is expressed on almost all lymphocytes and usually exists in combination with either a β1 or β7 subunit.

α4 Integrin interacts with vascular cellular adhesion molecule 1 and mucosal addressin cellular adhesion molecule (Mad-CAM-1), respectively to mediate circulating leukocyte homing into the gut mucosa. Two placebo-controlled trials have been conducted in patients with Crohn's disease. In the first study of 30 patients clinical remission occurred in 39% of patients in the natalizumab group compared to 8% in the placebo group. In a second larger study, Ghosh et al., reported on 244 patients with active Crohn's disease. After 6 weeks, 29% of patients receiving a single dose of natalizumab 3 mg/kg, 46% of patients receiving 2 doses of 3 mg/kg, and 31% of patients receiving 2 doses of 6 mg/kg had reached clinical remission. However there was a marked placebo effect as 27% of placebo-treated patients were also in remission. An uncontrolled pilot study suggested that natalizumab may also be of benefit in patients with active ulcerative colitis.

LDP-02 is a recombinant IgG1 humanized monoclonal antibody against α4β6 integrin. A phase II study in ulcerative colitis demonstrated endoscopic improvement in 1 of 5 patients who received 0.15 mg/kg and in 3 of 5 patients who received 0.5 mg/kg intravenously compared with 2 of 8 (25%) who received placebo.

Isis 2302 is a 20 base nucleotide designed to inhibit the intracellular RNA translation of ICAM-1 to reduce its production. An early phase II placebo-controlled dose finding study reported a short-term efficacy in patients with Crohn's disease. Seven of 15 patients treated with Isis 2302 improved compared with 1 of 5 patients treated with placebo. Unfortunately, two subsequent larger trials, the first involving 75 patients with Crohn's disease and the second with 299 patients, failed to demonstrate an effect.
Gene therapy

Gene therapy for inflammatory bowel disease is another exciting prospect. Adenoviral gene transfer methodology\textsuperscript{405} has been used to introduce the gene encoding TGF-\(\alpha\)\textsuperscript{406} and an NF-\(\kappa\)B super-repressor protein into gut epithelial cells in vitro\textsuperscript{407} resulting in a diminution of the production of pro-inflammatory molecules. The therapeutic implication of this is obvious, and has already been used to correct the ion transport defect in cystic fibrosis transgenic mice\textsuperscript{408}.

However, the critical issues remain whether genes can be effectively introduced to the gut epithelium. After all, the genes would have to be targeted at the longer-lived crypt stem cells, rather than villous enterocytes. There may however be some cause for optimism. Using an adenoviral construct encoding a marker protein, systemic administration resulted in detectable levels of the marker protein in murine colonic crypt cells. However, this successful transfer into colonic crypt cells only occurred in immunosuppressed mice\textsuperscript{409}.

Recent animal studies have suggested that local administration of IL-10 to the colon via genetically engineered lactococcus bacteria that are orally administered may result in high colonic mucosal concentrations of IL-10 and potentially better efficacy\textsuperscript{500}.

Coyle et al have shown significantly enhanced efficiency of adenovirus mediated gene delivery to intestinal epithelia when CaCo-2 monolayers were treated with IL-1\(\beta\), a cytokine which is known to increase expression of integrins which are involved in tight junction formation\textsuperscript{501}. 


Ulcerative colitis - an autoimmune disease?

If ulcerative colitis is an autoimmune condition it should fulfil the following five criteria associated with autoimmune disease502:

a) circulating disease-specific auto-antibodies
b) an association with other autoimmune disorders
c) an association with one or more HLA haplotypes
d) a prominent lymphocytic infiltrate at the site of active disease, with induced epithelial expression of HLA class II antigens.
e) corticosteroid responsiveness.

Many of above features are indeed recognised in ulcerative colitis:

a) Circulating antibodies directed against antigens present on colonic epithelial cells were described in 1959503. The antibodies are usually directed against dietary, bacterial antigens of the IgG isotype504. However, they lack sensitivity and specificity for inflammatory bowel disease and the titres are not related to disease activity. There are also reports of serum and tissue auto-antibodies against colonic epithelial cells specific to ulcerative colitis505,506,507,508. However, serum containing high titres of colonic antibodies does not damage colonocytes in vitro experiments509, casting doubt on their relevance.

A tissue bound IgG antibody has been described in patients with UC510,511. The antibody recognises a 40 kD-protein found on colonic epithelium, gallbladder and skin512. However, another group was unable to detect any tissue bound autoantibody. In a subsequent paper Halstensen found that colonic enterocytes expressed the 40 kD antigen apically with increasing intensity in a distal direction and that activated complement often co-localised with this antigen apically on the surface epithelium in active ulcerative colitis but not in Crohn's disease513.

Antineutrophil cytoplasmic antibodies (ANCA) were first described in ulcerative colitis 514 but were subsequently also described in Crohn's disease. pANCA has been found in 60-80% of patients with ulcerative colitis515 and a positive test has been reported to have a 65% sensitivity and 85% specificity for ulcerative colitis516. pANCA has also been described in 20-30% of patients with Crohn's disease517,518 and in 6% of patients with other diarrhoeal diseases519.
As these autoantibodies are probably unrelated to disease activity, disease extent and persist after colectomy, they are thought to be a genetic marker of colitis, rather than be a causative factor. Although some have correlated pANCA titres with disease activity, this has been refuted by others.

It has also been proposed that a positive pANCA in patients with ulcerative colitis may identify those at risk of pouchitis following ileo-anal anastomosis. This has also been refuted by more recent studies.

b) Patients with ulcerative colitis have an increased risk of other autoimmune disorders such as thyroid disease, diabetes mellitus and pernicious anaemia. Autoantibodies to lymphocytes, ribonucleic acid and smooth muscle, gastric parietal cells and thyroid gland have also been described.

c) There are differences in HLA class II gene expression as outlined in the section on genetics. For example, HLA-DR2 has been associated with pANCA expression while HLA-DR4 has associated with ulcerative colitis patients not expressing pANCA. Reports of an association between Crohn's disease and specific HLA-DQ antigens and between ulcerative colitis and HLA-DR antigens lends some support to the concept that the antigenic make-up, expressed on enterocytes, may predispose an individual to inflammatory bowel disease.

d) Histological examination of the inflamed colon reveals a marked increase in plasma cells. A lymphocytic infiltrate is present in inflammatory bowel disease but the absolute numbers of intraepithelial lymphocytes is normal and the CD4/CD8 ratio is unchanged. HLA class II antigens are induced on epithelial cells which renders the cell capable of behaving as an antigen-presenting cell.

e) Finally, both ulcerative colitis and Crohn's disease do respond to corticosteroids.
Chapter 3

THE COLONIC FLORA

As outlined in the immunological section above, it has been suggested that intestinal pathogens or luminal contents produced by bacteria, may be the direct or indirect trigger of an abnormal immune response. For example, *E.coli*, *Listeria* and Streptococcal antigen has been demonstrated in macrophages and giant cells beneath mucosal ulcers adjacent to fistulae, near abscesses and within the lamina propria, granulomas and mesenteric lymph nodes. These antigens may then perpetuate the intestinal inflammation by chronically stimulating resident and recruited lamina propria cells. The circumstantial evidence linking the intestinal microflora with inflammatory bowel disease can be summarised as follows:

1) There are similarities between the clinical picture of ulcerative colitis and the infective colitides associated with the closely related organism Shigella, Salmonella species and invasive *E.coli*, verocytotoxin producing *E.coli*, and the enteroaggregative group of *E.coli*. Infection with *Entamoeba histolytica* and *Campylobacter jejuni* infection may also present similarly.

2) Histological examination of the colonic mucosa from patients with ulcerative colitis at an early stage may be identical to that of infectious diarrhoea.

3) Idiopathic inflammatory bowel disease may follow attacks of infectious diarrhoea and a high proportion of patients returning from the tropics with chronic diarrhoea are found to have inflammatory bowel disease.

However, genetic factors are also clearly implicated. There is evidence for a genetically determined abnormality in mucosal barrier function as asymptomatic relatives of patients with inflammatory bowel disease have enhanced mucosal permeability following exposure to aspirin. About 10% of asymptomatic relatives of patients with Crohn's disease have abnormal mucosal permeability. The technology to specifically target a mutation in the interleukin genes of pluripotent murine embryonic stem cells has lead to the development of "immunological knockouts". Interleukin-2 deficient and interleukin-10 deficient mice have been shown to develop a chronic enterocolitis, which is attenuated in a germ-free environment or by antibiotics.
HLA-B27 transgenic mice develop colitis and arthritis which is not seen in genetically identical mice, lacking the transgene or when the mice are bred under germ free conditions. There are several possible ways that a luminal pathogen could give rise to inflammation of the colonic mucosa. A pathogen may directly damage the mucosa or induce a harmful immune response which indirectly causes damage. The possibilities are discussed below.

**Direct damage by an infective agent?**

In spite of the circumstantial evidence linking microorganisms with ulcerative colitis, no specific pathogen has been identified. It has been suggested that the resident normal luminal bacteria may induce a chronic intestinal inflammation in genetically susceptible hosts. A number of bacteria have been investigated:

**Diplostreptococcus**

Bargen suggested that diplostreptococci were prevalent in faeces from patients with ulcerative colitis. This theory was soon refuted when other investigators found the organism in a variety of other conditions. The characteristics of the organism isolated by Bargen were highly variable suggesting that many species of faecal streptococci were isolated.

**Bacteroides necrophorum**

Dragsteadt et al cultured this organism from colons removed from patients with ulcerative colitis and found serum agglutinin antibodies against this organism in patients with ulcerative colitis but not healthy controls. The organism is one of a large number of gram-negative anaerobic rods in the intestinal tract. Even now it can be difficult to differentiate these organisms by biochemical tests, growth on bile salts, susceptibility to antibiotics and analysis of volatile acid fermentation products by gas-liquid chromatography. These methods were not available to earlier workers and it is unlikely that isolates of *B. necrophorum* contained a single taxonomic group. Indeed, according to Dragsteadt's own illustrations, there was a mixed assortment of morphotypes.

**Clostridium difficile**

Following the identification Clostridium difficile as the cause of pseudomembranous colitis, a number of authors reported the detection of Clostridium difficile cytotoxin in the stool of patients with relapses of ulcerative colitis. These early reports have not been confirmed in later studies. There is no role for routine screening or treatment of *Clostridium difficile* in ulcerative colitis.
**Cell wall deficient bacteria**

Some bacteria can replicate by means of the L-cycle, named after the Lister Institute. This cycle results in a cell-wall deficient variant, which requires hypertonic conditions for growth. L-forms of bacteria have been isolated from patients with both ulcerative colitis and Crohn's disease more frequently than from controls. L-forms may cause persistent infection and lead to the formation of granulomas. The relevance of this finding to ulcerative colitis is unknown.

**Saccharomyces cerevisiae**

Several studies have reported the presence of antibodies to the brewers yeast Saccharomyces cerevisiae in a proportion of patients with Crohn's disease\(^5\). Antibodies to this yeast has been reported to be between 50-70% sensitive and 80-90% specific for the disease\(^5\). Antibodies appear to be associated with terminal ileal disease and with a younger age at diagnosis\(^5\). The most accepted hypothesis for this association is that an increased intestinal permeability leads to a higher immune stimulation by yeast antigens \(^5\). However a recent study by Vermeire et al failed to show any significant association between intestinal permeability and anti-Saccharomyces cerevisiae antibodies\(^5\). The likely explanation proposed by the authors is that there is a cross reactivity with another putative antigen involved in the immunopathogenesis of Crohn's disease.

**Escherichia coli**

A wealth of circumstantial evidence links *Escherichia coli* (*E. coli*) with ulcerative colitis and *E. coli* is the only organism which is still actively investigated in ulcerative colitis. The evidence available is considered in the next section.
Indirect damage by interference with colonic organic ions?

One of the main functions of the large intestine is to salvage energy from dietary carbohydrate which has neither been digested nor absorbed in the small intestine. This occurs through a process known as fermentation in which anaerobic bacteria breakdown carbohydrate to short chain fatty acids (SCFA). SCFA's are the major energy source for colonocytes. SCFA's block the osmotic cathartic effect of carbohydrates because they are absorbed in the colon by a concentration dependent mechanism resulting in improvement in salt and water absorption. Faecal SCFA levels are elevated in mild ulcerative colitis and become progressively lower with increasing disease severity. Short chain fatty acid enemas have been shown to have an effect in ulcerative proctitis.

Hydrogen is an important product of fermentation which has to be safely and effectively disposed of. The hydrogen levels in the colon are kept low by losses in breath and flatus and by hydrogen utilising bacteria such as methanogenic, acetogenic and sulphate reducing bacteria. Sulphate reducing bacteria produce hydrogen sulphide, an agent that is potentially damaging to the colonic epithelium by reducing absorption of SCFA's and destroying the disulphide bridges in mucus. Fermentation experiments have shown that colonic sulphate reducing bacteria outcompete methanogenic bacteria for hydrogen.

However the luminal pH can also influence the outcome of competition between methanogenic, acetogenic and sulphate reducing bacteria. Acidic conditions select for acetogenesis as the route of hydrogen disposal. The stools of patients with ulcerative colitis have a high 24 hour weight, high sodium and chloride concentrations and a low pH. Faecal lactate levels gradually increases with increasing disease severity. This may be exacerbated by an increase in intraluminal oxygen concentration caused by mucosal haemorrhage which favours facultative anaerobic strains such as lactobacilli and streptococci which are lactic acid producers. Infusion of lactic acid causes mucosal changes resembling those of ulcerative colitis in rats. It has therefore been suggested that lactate may play a role in the mucosal damage seen in ulcerative colitis.
Indirect damage by bacterial products?

Systemic distribution of bacterial products could explain the extraintestinal manifestations seen in ulcerative colitis and there is evidence that bacterial products can cross the altered mucosal barrier. For example, endotoxaemia has been documented in 88% of patients with active ulcerative colitis and 94% of those with active Crohn's disease. Chadwick et al has reviewed the influence of luminal bacterial and bacterial products on extraintestinal inflammation.

a) Lipopolysaccharide (LPS) consists of lipid A, core polysaccharide and polysaccharide side chains. Lipid A is a phosphoglycolipid with the same basic structure in all gram-negative LPS with a backbone of the disaccharide beta-glucosaminyl-1,6glucosamine. Lipid A is a potent toxin which may activate complement and macrophages, induce prostaglandins and interferon and has mitogenic effects. The role of LPS in the pathogenesis of intestinal inflammation is unknown. The polysaccharide components of LPS show considerable heterogeneity and are involved in bacterial replication as well as adhesion and colonisation. Patients with severe ulcerative colitis may have an endotoxaemia. However, the frequency of portal bacteraemia in patients with ulcerative colitis has been disputed. Tolerance to circulating endotoxin may be affected by acute phase proteins and antibodies to LPS.

b) Peptidoglycan polysaccharide polymers (PGPS) and their monomer subunits are components of the cell walls of gram-positive and gram-negative bacteria. Intramural injection of streptococcal PGPS in to the caecum of mice produces chronic granulomatous lesions resembling Crohn's disease. Green was able to induce "exacerbations" of the colitis by intravenous injection of PGPS, 20 days after the caecal injection. Lichtman et al injected rat ankle joints with peptidoglycan-polysaccharide-polymer (PG-PS) and then induced bacterial overgrowth by creating self-filling blind loops. The bacterial overgrowth reactivated arthritis in previously sensitised joints, which was prevented by metronidazole or an endotoxin-neutralising agent.
c) **Formyl-methionyl-leucylphenylalanine** (FMLP) is probably the most potent member of the F-met peptide family. These peptides arise from the amino-terminals of bacterial precursor proteins and are potent inflammatory agents. FMLP is a potent neutrophil chemotactic agent and bacterial F-met peptides may induce experimental colitis. FMLP also affects mucosal permeability and results in intestinal release of leukotrienes. F-met peptides induce adhesion molecules on the surface of neutrophils and superoxide production and release of lysosomal enzymes from neutrophils.

Circulating polymorphonuclear leukocytes from patients with Crohn's disease and ulcerative colitis have increased numbers of surface receptors for FMLP.

**A harmful immune response triggered by molecular mimicry?**

It has been suggested that an infecting agent may cause indirect damage to the colonic mucosa by "molecular mimicry", triggering a destructive immune response. According to this concept a close similarity between a host antigen and an antigen possessed by an infecting agent may result in an immune response to the microbe damaging host tissue. The best model is the cardiac valvular damage following acute rheumatic fever. Cross reactivity between *E.coli* antigens and intestinal epithelial cell antigens has been well documented by Cooke and Lagercrantz. Anti-colon antibodies may be induced by immunisation with *E.coli* in Freund's adjuvant without the development of colitis. Immunological cross-reactivity has also been described between *Klebsiella nitrogenes* epitopes and the HLA B27 antigen and between anti-mitochondrial antibodies found in primary biliary cirrhosis and antigens on gram-negative bacteria.
Antibiotics to treat ulcerative colitis

Regardless of the precise pathogenesis, should a microbial pathogen be involved in the aetiology of inflammatory bowel disease one would expect antibiotics to have an effect. Antibiotics may have a beneficial effect in one of three ways. Firstly, a non-specific action which reduces the number of antigenic triggers in the intestinal lumen. Secondly, a specific action against a specific organism which is etiologically involved. Finally, antibiotics might have an action unrelated to the drugs antibacterial spectrum (for example, the immunosuppressive action of metronidazole). There have only been a few trials of antibacterial agents in inflammatory bowel disease and the evidence for a beneficial effect is limited.

Sulphasalazine is the most widely used antibacterial agent in ulcerative colitis. It is effective in the maintenance of remission and in mild and moderately active disease. The drug consists of 5-aminosalicylic acid (5-ASA) linked by a diazo bond to sulphapyridine. However the elegant studies of Azad Khan and Truelove demonstrated that 5-ASA was the active moiety and not the antibiotic component, which acts as a carrier molecule to achieve delivery of 5-ASA to the colon. It has been suggested that Sulphasalazine may also affect the faecal flora by increasing the prevalence of sulphonamide resistant E.coli. There are little clinical data available relating changes in intestinal microflora to the use of sulphasalazine. In vitro work has suggested that the intact sulphasalazine molecule may have anti-inflammatory actions not shared by 5-ASA alone.

Tetracycline formed an integral part of the Oxford 5-day intensive regimen for severe acute ulcerative colitis. This protocol used 60 mg/day, tetracycline 250mg four times daily, parenteral vitamins ('Parentrovite') and a rectal infusion of hydrocortisone 100mg. As the remission rate of about 73% was very similar to that achieved by the same group using no additional antibiotic, the Oxford workers concluded that the addition of tetracycline offered no additional advantage.

Vancomycin was the first antibiotic to undergo a randomised controlled trial in ulcerative colitis. The antibiotic is not systemically absorbed when administered orally and has activity against gram-positive organisms. In a trial by Dickinson, 32 patients with ulcerative colitis and 7 with Crohn's disease received oral vancomycin 500mg six hourly or placebo for seven days in addition to routine medical therapy.
Although there was no overall difference between the groups, when those with ulcerative colitis were considered separately, there was a trend towards a reduction in the need for operative intervention in those receiving vancomycin.

**Metronidazole** is used in Crohn's disease but there is little experience with its use in ulcerative colitis. A controlled trial examined the use of intravenous metronidazole in severe ulcerative colitis, as an adjunct to the Oxford regimen, but found no additional benefit from the drug. Metronidazole has also been found to be ineffective when given orally in less severe disease. An effect has however been demonstrated when treating ileo-anal pouchitis and one trial found it superior to sulphasalazine when used for maintenance therapy. In contrast, metronidazole has a definite role in the treatment of Crohn's disease, particularly for peri-anal disease.

**Ciprofloxacin** has been studied by Turunen et al. She reported a 21% treatment failure rate at 6 months in those maintained on ciprofloxacin compared with 44% with placebo. However there are a number of methodological problems with the study. For example, although all patients were said to have active disease, 50% were not taking steroids prior to randomisation. There were 16% more smokers in the group randomised to receive Ciprofloxacin. This difference may be important as smoking has a beneficial effect in colitis. Finally, there was no difference in clinical, endoscopic or histological findings in the two groups at 6 months.

**Tobramycin** was used in a randomised, double-blind, placebo-controlled trial undertaken by Burke in 1988. 84 patients were randomised to treatment with tobramycin or placebo, as an adjunct to their normal therapy. After three weeks therapy, 74% of patients in the tobramycin group had achieved complete symptomatic remission, compared to 43% in the placebo group. The benefit of oral tobramycin in active ulcerative colitis proved short-lived as 50% of patients had relapsed at one year follow up.

If the normal luminal flora would act as opportunistic pathogens, one would expect broad range antibiotics to have a beneficial effect. In contrast, broad range antibiotics have been disappointing in the treatment of active ulcerative colitis. This would suggest that ulcerative colitis is not an infectious disease or alternatively is caused by a specific pathogen or defined sub-group of the gut flora is involved in perpetuating the mucosal inflammation. **E. coli** is the main candidate organism as tobramycin, with activity against **E. coli**, is the only antibiotic which have been demonstrated to have a beneficial effect in ulcerative colitis.


Chapter 4

E. COLI AND ULCERATIVE COLITIS

Classification of pathogenic E. coli

*Escherichia coli* is a gram-negative rod of the family enterobacteriaceae. It is a common intestinal commensal where they constitute about 3% of the flora as a whole. Salmonella and Shigella are close relatives. *E. coli* is the major intestinal anaerobe occurring at densities of $10^6$ cells per gram of faeces. It however constitutes a minor component of the total intestinal flora which largely consists of obligate anaerobes reaching up to $10^{11}$ cells per gram of colonic contents.

While most *E. coli* are benign commensals or opportunistic pathogens, they may also be dangerous pathogens. *E. coli* are a leading cause of diarrhoea, surgical wound infection, secondary pneumonia, peritonitis, neonatal septicaemia and urinary tract infections.

Three classes of surface antigens have been extensively explored and form the basis of the serotyping of *E. coli*. The O antigens are the polysaccharide component of the lipopolysaccharide in the outer layer of the cell wall. The K antigens are capsular polysaccharides and the H antigens which are flagellar proteins. Analysis of O, K and H antigens has made it possible to distinguish many hundreds of serotypes.

Serotyping of *E. coli* does not provide a reliable basis for analysing populations. For example, Ochman et al. estimated that on average 76% of the total genetic diversity of the species may occur within strains of the same O serogroup. Multilocus enzyme electrophoresis will detect nearly all amino acid sequence variations in *E. coli* and provides a more exact means of genotyping the species.

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<th>Table I Classification of E. coli associated with gastrointestinal pathogenicity.</th>
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<td>♦ Enteropathogenic <em>E. coli</em> (EPEC)</td>
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<td>♦ Enterocaggregative <em>E. coli</em> (EAaggEC)</td>
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The property of adherence by *E. coli* to epithelial cells is associated with gastrointestinal pathogenicity. The pathogenic types of *E. coli* may be classified according to Table.
**Enteropathogenic E. coli**

EPEC were the first group of *E. coli* to be described in association with diarrhoeal disease and remain one of the major causes of infantile diarrhoea in developing countries. The disease is characterised by fever, malaise, vomiting and diarrhoea with little or no macroscopic blood.

EPEC infection is associated with both structural and biochemical damage to the small intestinal microvillus membrane. Polotsky et al., first documented a characteristic ultrastructural "attaching-effacing lesion" in the small intestine. The bacteria adhere to the enterocyte membrane, which adopts a cupped, or pedestal formation. In addition, the microvilli become effaced and the cellular cytoskeleton becomes disrupted with condensation of filamentous actin at the site of bacterial attachment. It has recently been shown that both enteropathogenic and enterohemorrhagic *E. coli* bind to the host cell membrane by injecting their own receptor. This injected receptor activates signalling pathways in the infected cells, reorganising the cytoskeletons forming "pedestals" thus inducing a sequence of events capable of binding the bacterium to the infected cells.

The accumulation of high concentrations of microfilaments in the apical enterocyte cytoplasm beneath attached bacteria formed the basis for the highly sensitive "fluorescein actin staining test" (FAS test) described by Knutton.

About 80% of EPEC strains adhered to HEp-2 cells, a property rare in ETEC, EIEC or non-pathogenic strains of *E. coli*. This adherence could either be localised or diffuse. Localised adherence is plasmid mediated and associated with the carriage of a plasmid of approximately 60kDa. Localised adherence is also associated with a plasmid-encoded 94kDa outer membrane protein. Nine out of 10 volunteers who ingested a plasmid carrying strain developed marked diarrhoea, compared with mild diarrhoea in two out of 10 who received a plasmid free variant. Furthermore, ingestion of the plasmid carrying strain resulted in the development of antibodies to the 94kDa protein. The single volunteer who did not develop diarrhoea was the only one who had antibodies to the protein before ingestion.

Klapproth et al., suggested that soluble products of enteropathogenic *E. coli* could lead to suppression of lymphocyte activation and lymphokine production.
**Enteroaggregative E.coli**

Vial et al\(^2\)\(^2\)\(^2\), reported a new group of enteroaggregative *E.coli* (EAggEC) on the basis of a distinct aggregative pattern of adherence to HEp-2 cells. Using genetic probes, infection with EPEC, ETEC, EIIEC and EHEC were excluded. Most of the *E.coli* with an aggregative adherence pattern carried a plasmid in the range 55-65Mda. A 1 kilobase fragment from the plasmid of EAggEC strain 17-2 has been shown to have 89% sensitivity and 99% specificity for EAggEC identification\(^\text{623}\). In a report by Cravioto et al\(^6\)\(^2\)\(^4\), *E.coli* with an aggregative adherence pattern were isolated more often in Mexican infants with persistent diarrhoea than in controls. A third of children with aggregative *E.coli* had blood in their stools and aggregative *E.coli* was the most common pathogen isolated in those with bloody diarrhoea.

**Enterotoxigenic E.coli**

Enterotoxigenic *E.coli* (ETEC) is a common cause of acute watery diarrhoea\(^6\)\(^2\)\(^5\) in developing countries\(^6\)\(^2\)\(^6\) and of traveller's diarrhoea\(^6\)\(^2\)\(^7\),\(^6\)\(^2\)\(^8\).

ETEC strains adhere to mucosal surfaces using fimbrial adhesins. A large number of adhesins or colonisation factor antigens (CFA) have been described\(^6\)\(^2\)\(^9\),\(^6\)\(^3\)\(^0\) including CFA I, II, III and IV. Two forms of CFA/III have been described\(^6\)\(^3\)\(^1\) one of which shares epitopes with CFA/I\(^6\)\(^3\)\(^2\) and share identical N-terminal amino acid sequences. However, many ETEC strains do not carry any of these adhesins and further colonisation factors probably remain to be characterised.

ETEC may produce heat labile enterotoxins (LT) or heat stable enterotoxins (ST) or both. Heat labile toxin comprises an A subunit of approximately 25.5 kDa and 5 receptor binding B subunits of approximately 11.5 kDa each\(^6\)\(^3\)\(^3\),\(^6\)\(^3\)\(^4\). The toxin is related to but not identical to cholera toxin\(^6\)\(^3\)\(^5\),\(^6\)\(^3\)\(^6\),\(^6\)\(^3\)\(^7\),\(^6\)\(^3\)\(^8\). The A subunit stimulates adenylyl cyclase on small intestinal cells, increasing intracellular cAMP resulting in secretion of water and electrolytes\(^6\)\(^3\)\(^9\). The B subunit attaches to ganglioside receptors, binding toxin to epithelial cells, and delivering the enzymatically active A subunit into the cell cytoplasm. The heat stable enterotoxin (ST) is smaller (5 kDa)\(^6\)\(^4\)\(^0\) than heat labile enterotoxin. Heat stable enterotoxin I (STI) is methanol soluble\(^6\)\(^4\)\(^1\) and causes intestinal distension in the "suckling mouse assay", by stimulating guanylate cyclase\(^6\)\(^4\)\(^2\),\(^6\)\(^4\)\(^3\),\(^6\)\(^4\)\(^4\). Two forms, STIa and STIb have been described. STII is methanol insoluble, active in the pig jejunal loop but negative in the suckling mouse assay.
**Enterohaemorrhagic E.coli**

Haemorrhagic colitis is characterised by bloody diarrhoea, without fever. Outbreaks were first reported in 1983 and have subsequently been reported in other countries.

The aetiology was unknown until Konawalchuk et al., showed that culture filtrates of some E.coli strains, isolated from infants with diarrhoea, produced a cytotoxic effect on Vero cells. This was shown to be due to cytotoxins, termed vero-cytotoxins (VT). There has been one case-report of a case of ulcerative colitis precipitated by a verocytotoxin producing strain of E.coli. Karmali et al., first reported the association between VTEC and HUS; a disease characterised by acute renal failure, microangiopathic haemolytic anaemia and thrombocytopenia. Most VTEC strains belong to the O157 serotype. The two toxins that have been described (VT-1 and VT-2) are identical to Shiga toxin I and II.

Both VT-1 and VT-2 comprise A and B subunits. The A-subunit carries the biological activity of the toxin, and the B-subunit mediates specific binding and receptor mediated uptake of the toxin. The receptor for VTI and VT2 is the same as for Shiga toxin: a globotriosyl ceramide containing a galactose-\(\alpha\)-(1->4) galactose-beta-(1->4)-glucose ceramide.

Enterohaemorrhagic E.coli are also adhesive to epithelial cells, and produce small intestinal lesions similar to the attaching-effacing lesion produced by enteropathogenic E.coli.

**Enteroinvasive E.coli**

Enteroinvasive E.coli (EIEC), a cause of bloody diarrhoea, was first described in 1967. Enteroinvasive E.coli is biochemically similar to Shigella species and carries a large virulence plasmid 140-160kDa in size. The invasive capacity of the organisms can either be confirmed in the "Sereny test" whereby bacteria are inoculated in to a guinea-pig conjunctival sac and invasive strains produce a purulent keratoconjunctivitis or by tissue culture methods. Only a limited amount of information is available on adhesion by this group of pathogenic E.coli. Attachment to calf ileal brush border of a K-12 strain transformed with the invasion plasmid was inhibited by a number of sugars including mannose and glucose. In the presence of both mannose and wheat germ lectin (which specifically binds N-acetyl glucosamine), adhesion was reduced by 90%.

67
Quantitative differences in *E. coli* from patients with ulcerative colitis

*E. coli* have been implicated in the aetiology of ulcerative colitis and a number of differences have been described in the coliform flora of patients with ulcerative colitis:

Quantitative assessments of the faecal flora have been conflicting. The proportion of coliforms to total viable bacteria in the stool of patients with ulcerative colitis may be increased\(^6,6^3,6^4\). Furthermore, Gorbach\(^6,6^5\) and Giaffer\(^6,6^6\) have reported that the number of faecal coliforms increase as the disease improved. Hartley et al\(^6,6^7\), reported that patients with quiescent colitis maintained on sulphasalazine had 8-fold lower counts of *E. coli* compared with those not taking the drug. However, others have not found any quantitative difference in the microflora\(^6,6^8,6^9\) and it is possible that diarrhoea itself may change the relative proportions of faecal organisms\(^6,7^0,6,7^1,6,7^2\). Indeed, there are three main recognised effects of diarrhoea;

1) Increase in certain coliform species that are uncommon in the normal flora, such as *Enterobacter*, *Proteus*, *Klebsiella* and *Pseudomonas*. Such strains may gain prevalence while the usual *E. coli* population is suppressed. This may result in a net increase in the total coliform count.

2) The anaerobic strains that normally predominate the flora may decline below the coliform count.

   In very brisk diarrhoea, the obligate anaerobes may fall to low or even unrecordable levels.

3) Retrograde contamination of the jejunum by pathogens may persist for several weeks following the acute episode.
Qualitative differences in *E. coli* from patients with ulcerative colitis

Several qualitative differences have been noted between *E. coli* isolated from patients with ulcerative colitis and controls. Cooke et al. found an increased prevalence of serotypes considered in other contexts to be pathogenic. Patients with ulcerative colitis are more likely to carry haemolytic or necrotoxic strains. *E. coli* isolated from patients with ulcerative colitis was found to have a greater ability to degrade mucins and to cause dilatation of rabbit ileal loops. Haemolysin producing strains were isolated more frequently in patients in relapse than in remission, but their presence appeared to follow rather than precede the relapse. Giaffer et al. found *E. coli* haemolysin production in 24% of patients with ulcerative colitis, 18% of patients with Crohn's disease and 11% of healthy controls.

Toxin producing strains of *E. coli* have occasionally been isolated from patients with ulcerative colitis. Giaffer et al. also looked for verocytotoxin producing strains but did not find any in the 216 strains tested. However, extract from 5 strains of *E. coli* (4 from patients with Crohn's disease and 1 strain from a patient with ulcerative colitis) produced a distinct cytopathic effect on Vero cell monolayers. Patients with ulcerative colitis have circulating, agglutinating antibodies to a greater number of *E. coli* O antigens and in higher titres than controls. The Kunin antigen, common to the enterobacteraceae, is present in high concentration in *E. coli* O14 and antibodies to this antigen cross-react with a goblet cell antigen. However, there is no correlation between the presence of anti-colon antibody and clinical severity, extent or duration of disease.

Lymphocytes exposed to an *Escherichia coli* lipopolysaccharide extract may exhibit cellular cytotoxicity to colonic epithelial cell. Humoral antibodies to certain *Escherichia coli* serotypes cross react with colonic epithelium. Cytotoxicity for colonic epithelial cells may be induced in lymphocytes from normal volunteers following incubation with lipopolysaccharide from *Escherichia coli* O119.

Lobo analysed a number of *E. coli* strains, isolated from patients with ulcerative colitis for the following; enteroadhesive factor production, production of adhesin for diffuse adhesion, heat-labile enterotoxin-1 (A and B subunit), production, heat-stable enterotoxin-1 and -2 production, verocytotoxin-1 and -2 production, enteroaggregative gene probe, attaching-effacing gene probe. He was however unable to detect any difference in prevalence between the strains isolated from patients with colitis, Crohn's disease, patients who had undergone restorative proctocolectomy and healthy volunteers.
**Adhesive properties of *E.coli* isolated from patients with ulcerative colitis**

Dickinson et al\(^6\) reported that 35% of patients with active ulcerative colitis and 27% with inactive colitis harboured at least one *E.coli* that was adhesive to HeLa cells, compared with 5% of controls. Burke et al\(^6\) suggested that as HeLa cells are a neoplastic cell line, unrelated to the gastrointestinal tract, the enzymatic stripping methods used in the manipulation of the cell line, may result in the expression of non-physiological receptors for bacteria, rendering them less suitable for the study of adherence. Instead he proposed the buccal epithelial cell adhesion assay described by van Houte\(^6\), Ofek\(^6\) and further modified by Candy\(^6\).

Using this method, Burke et al\(^6\) reported that 86% of *E.coli* strains isolated patients with inflammatory bowel disease were adhesive compared with 27% of strains isolated from patients with infective diarrhoea and none of the *E.coli* isolated from healthy controls. The *E.coli* adhered as readily to buccal epithelial cells from other sources as to buccal epithelial cell from their own host\(^6\).

Using the buccal epithelial adhesion assay, Giaffer et al isolated adhesive *E.coli* in 68% of patients with ulcerative colitis, 62% with Crohn's disease and 6% of normal controls. Subsequently , Shen et al\(^6\) reported increased adhesion of *E.coli* isolated from patients with ulcerative colitis to HT29 cells as well as the extracellular matrix proteins; plasminogen, thrombospondin, vitronectin and fibrinogen. However, Hartley et al found that *E.coli* isolated from biopsies in patients with ulcerative colitis, were only marginally more adhesive to Hep-2 cells than those from controls.

The distinction between mannose-resistant adhesion (adhesion not inhibited by the presence of D-mannose) and mannose-sensitive adhesion is thought to be important. The adhesion of *E.coli* from patients with ulcerative colitis is mannose-resistant, as is that of enteropathogenic *E.coli*. Adhesion which is inhibited by D-mannose (mannose-sensitive adhesion) is characteristic of type I fimbrial adhesion. Fimbriae are produced by most *E.coli* in culture\(^6\) and appear to promote inter-bacillary adhesion and increase the rate plasmid transfer\(^6\), possibly by stabilising mating pairs\(^6\).

As with the fimbrial adhesins of pathogenic *E.coli*, the adhesin associated ulcerative colitis is hydrophobic. This has been demonstrated using a salting out method in which the lowest molar concentration of ammonium sulphate resulting in autoagglutination of a bacterial suspension, is determined.
The lower this is, the higher the surface hydrophobicity of the bacterial cell. *E. coli* from patients with ulcerative colitis have a higher surface hydrophobicity than controls, and the buccal epithelial cell adhesion index correlates inversely with lowest molar dilution of ammonium sulphate causing autoagglutination.

Restorative proctocolectomy with the formation of a pelvic reservoir is a common surgical approach in ulcerative colitis. Acute pouchitis is a common complication which resembles active ulcerative colitis. Lobo et al, studied the *E. coli* flora of 24 patients with acute pouchitis, He found that patients with pouchitis did carry adhesive strains but the degree of adhesiveness was inversely related to the degree of pouchitis and the functional outcome.

**SUMMARY TO INTRODUCTION**

It is likely that a combination of genes result in an increased susceptibility to ulcerative colitis. As most individuals with a family history of inflammatory bowel disease do not develop the disease, an environmental trigger is necessary to develop the disease.

The environmental trigger is likely to affect the intestinal epithelium. The NOD2 gene probably encode for “pattern-recognition receptors”, recognising different components of microbes and thus providing another line of defence against invasion. Thus the discovery of the NOD2 gene may link a genetic predisposition with the enteric flora.

The enteric bacterial flora is the common driving force in all animal models of inflammatory bowel disease. If the normal enteric flora gives rise to the mucosal inflammation in ulcerative colitis, broad spectrum antibiotics should have a beneficial effect. However, in clinical trials, broad range antibiotics, with the exception of tobramycin, have proved ineffective.

The lack of beneficial effect suggests either that ulcerative colitis is not an infectious disease or is caused by a specific pathogen or defined sub-group of the gut flora. Tobramycin has activity against *E. coli*, one of the main micro organisms which have been linked to ulcerative colitis. A number of qualitative differences have been described between *E. coli* isolated from patients with ulcerative colitis and normal individuals. *E. coli* have been found to be more adhesive to cell surfaces. Adhesion to the epithelial surface is an important preliminary step following which other virulence factors may take effect.
E. coli isolated from patients with ulcerative colitis has been reported to have a greater ability to degrade mucin and be more likely to be haemolytic or necrotoxic.

Dickinson and Burke have established that patients with inflammatory bowel disease carry more adhesive strains of E. coli than patients with infective diarrhoea or healthy controls. However, it was uncertain if the level of adhesiveness altered with time or remained stable. Until development of the REP-PCR assay, the cost of tracking a large number of wild E. coli using serotyping had been prohibitive. With this assay, we set out to follow up E. coli isolates over time to establish if the level of adhesiveness changed.

Burke et al, demonstrated a higher remission rate with the use of tobramycin in patients with active ulcerative colitis. However, after on year follow up half the patients who initially responded had relapsed. Lobo et al suggested that this may be because the gene which encodes adhesiveness is carried on a 98 MDa transferable plasmid. The adhesiveness may then quickly transfer to other strains of E. coli. An initial objective was therefore to search for the 98 MDa plasmid amongst other strains of E. coli isolated from other patients with inflammatory bowel disease.

Another possible reason for the high relapse rate in the study of tobramycin was that the original colonic E. coli flora was quickly able to re-establish itself. In the hope of inducing a lasting remission, we attempted to bring about a lasting change in the colonic flora by introducing a non-pathogenic strain of E. coli into the colon of patients with active ulcerative colitis.
Chapter 5

METHODS AND LABORATORY TECHNIQUES

General methods in the isolation of *E. coli*

Upon arrival at the laboratory, samples were processed as soon as possible. Approximately 0.5g of faeces was removed with a sterile swab and homogenised in sterile saline. Serial, ten-fold dilutions were made in sterile saline (up to $10^{-5}$) and 100 ml aliquots of the dilutions and original suspension were spread onto CLED agar plates. The plates were incubated overnight at 37°C. After incubation, plates with confluent growth were discarded. Colonies were selected from plates with discrete, well-spaced colonies. The colony morphology of different strains of *E.coli* can vary and to eliminate selection bias, a marker pen was used to make 20 random spots on the back of the plate. The 20 coliform colonies closest to the spots were then removed using sterile toothpicks and cultured on Fluorocult, Indole and Iso-Sensitest agar at 37°C over night.

The plates were incubated overnight at 37°C. Isolates with both a positive Fluorocult and indole reaction were identified as *E.coli*. The first 10 isolates from each faecal sample that were confirmed to be *E.coli* were used for PCR analysis and stored for future use suspended in 15% glycerol broth in 1.5mL Nalgene cryovials (Merck) and kept at -70°C. Isolates with only one positive test, were investigated further using API 20E identification strips (Biomérieux, Marcy-L'Etoile, France).

**Culture media used**

Cystine-lactose-electrolyte deficient agar (CLED, Unipath, Code CM301) is a non-inhibitory diagnostic agar used in the primary isolation of *E.coli*. After overnight incubation, colonies of lactose fermenting *E.coli* are approximately 1.25mm in diameter, yellow and opaque while non-lactose-fermenting strains are blue.

Indole agar (IDM34, Mast Ltd, Bootle UK) was used for performing the indole test. After overnight incubation, colonies are touched with a swab moistened with DMACA reagent (p-dimethyl-amino-cinnamaldehyde in 10% v/v concentrated hydrochloric acid). Indole positive organisms then appear with a blue colour and negative strains are pink.
Fluorocult ECD agar (E.coli direct, Merck Ltd, Poole, Dorset, UK.): was used in the preliminary identification of E. coli. According to the manufacturers, the test is 95.6% specific and 96.5% sensitive for E.coli by screening for organism with the enzyme -D-glucuronidase. Apart from a few strains of Salmonella and Shigella, E coli is the only species belonging to the Enterobacteriaceae with the enzyme. The enzyme splits a substrate in the agar (4-methylumbelliferyl-D-glucuronide), to form 4-methylumbelliferone, which fluoresces in long wave UV light (photograph I). The agar also contains a bile salt mixture to inhibit non-intestinal Enterobacteriaceae.

Iso-Sensitest agar (Unipath, Code CM471) was used in the routine culture of E.coli strains. It is a semi-defined media originally developed for antimicrobial susceptibility tests. Batch-to-batch variation and cation content are minimal and growth of most bacteria is supported without further supplementation.

Luria Bertani broth (LB broth) was used as a general-purpose liquid medium for the growth of E.coli. Luria Bertani broth was prepared by the media laboratory of the Dept of Microbiology, University of Leeds.
Methods used in PCR typing of *E.coli* isolates

**Background**

Because of the lack of a simple, and accurate typing system, few studies have attempted to prospectively monitor the human coliform flora. As "wild strains" of *E.coli* do not have any phenotypic markers such as pigmentation, resistance to antibiotics or the ability to grow under restrictive conditions, a new method had to be devised to quickly and accurately characterise many different *E.coli* isolates.

Serotyping mainly of the O and H antigens is expensive and time consuming. Complete O:H:K serotyping has only been carried out to a very limited extent in a few laboratories. It is not known how many different serotypes of *Escherichia coli* exist. Other methods of typing have been used. Phage typing has been used to a limited extent to subdivide serotypes. Biotyping differentiates epidemiologically different clones of *E.coli* on the outcome of fermentation tests and other biochemical reactions. Without serotyping, biotyping is insufficient for differentiating different clones of *E.coli*. Colicin based typing uses the sensitivity to a series of known colicins or the varying capacity of strains to produce colicins. Colicin based typing is not sufficiently discriminating to distinguish different bacterial strains and species.

**Development of a typing system for *E.coli***

Amplification-based DNA fingerprinting of bacteria is used in the identification of a wide range of bacteria. There are several variations of the technique, using random or specific primers to amplify unspecified regions of the chromosome to develop a polymorphism in the length of restriction fragments.

Ann Buckingham at the department of Microbiology, Leeds University, investigated different ways of typing of *E.coli*. Initially amplification of variable regions between the C- and N- termini of the *hag* gene (encoding for the flagellin protein) of *E.coli* was attempted. Unfortunately, amplification using *hag*-PCR proved unreliable with less than half of all *E.coli* serotypes amplifying. Subsequently, amplification of the *lacZ* gene, encoding the enzyme β-galactosidase was attempted in the hope that the product could be used to differentiate different coliform bacteria. Unfortunately, only a limited number of different profiles were produced suggesting that the *lacZ* gene was highly conserved. Sequence comparisons of the *lacZ* gene from other species have supported this.

75
In 1990, novel palindromic sequences were discovered in the chromosomes of *E.coli* and *Salmonella typhimurium* called Enterobacterial Repetitive Intragenic Consequence sequence (ERIC)\(^{699}\). These sequences are 136 bp long and are located in the non-coding transcribed regions of the chromosome. It has been shown that the generation of primers matching the consensus sequence for ERIC sequences may allow generation of strain specific genomic fingerprints\(^{700}\). As ERIC sequences are confined to enterobacterial species, ERIC primers were developed in attempt at typing *E.coli*. Unfortunately, profiles were only generated in two thirds of the *E.coli* serotypes and the profiles produced were rather indistinct with many bands. A technique, using Randomly Amplified Polymorphic DNA (RAPD), for typing different strains of Listeria has been developed at the Department of Microbiology at University of Leeds. This technique was tried in the typing of *E.coli*. Unfortunately, this technique also proved unsuitable.

In 1982, the presence of a 183 bp sequence in the histidine transport region of *Salmonella typhimurium* was reported\(^{701}\). This sequence contains a long inverted repeat which when transcribed could form a stable stem-loop structure. These elements, found in many bacterial chromosomes were designated Repetitive Extragenic Palindromic sequences (REP)\(^{702}\).

Versalovic et al\(^{702}\), designed oligonucleotide primers for this sequence and the primers REP1R-I and REP2-I were found to generate multiple amplimers of different sizes with different species and strains within species groups. The *E.coli* chromosome is estimated to have over 500 REP sequences and REP-PCR was thought to be a promising method for typing *E.coli*. An added attraction of REP-PCR based typing was that purified DNA was not always required for REP-PCR and it is sometimes possible to use whole-cell lysate preparations. As the our *E.coli* typing system was to be used for the identification of a large number of *E.coli*, any technique requiring extracted and purified DNA would be inappropriate.

**Typing of known serotypes**

Ann Buckingham refined the REP-PCR typing of *E.coli*. Originally, two primers REP1R-I and REP2-I were used in combination, however too many bands were produced causing difficulties in interpretation of each profile. On testing combinations of the primers alone and together, it was discovered that the primer REP1R-I produced the most satisfactory profile.
REP-PCR was used to type 128 isolates of *E. coli* with known H and O serotypes available from the Department of Gastroenterology. Every serotype has three designations, the primary antigen with which the serotype is identified and two other antigens with which it is associated. This is known as the O: H: K serotype. The serotype profile of an *E. coli* isolate is not necessarily stable and may change during its passage from host to host and from one location to another, thereby obscuring its clonal origin.  

The K serotypes were not attempted as it was felt that if all the H and O serotypes produced a differential pattern, this would be sufficient confirmation of the success of the method. The adhesiveness of the strains was not measured as the aim was to validate the REP-PCR typing as a means of differentiating strains. Serotypes with commonly associated antigens were assigned into clonal groups to determine if similar serotypes (i.e. related phenotypes) had similar REP-PCR patterns (figure 1). The profiles for each of the serotypes involved were compared to the profile of the serotype designated by the common antigen. Common H serotypes shared an average of 30% of their bands with their different O antigens. Common O serotypes shared an average of 23% suggesting that the H antigen has a greater impact on the REP-PCR profile. The 128 strains of *E.coli* screened using REP-PCR provided 127 different profiles. Only two serotypes, (O73: H31 and H31: O3) sharing the same H antigen, produced an identical REP-PCR profile.

**Figure 1** Example of the REP-PCR patterns obtained from 10 related serotypes of *E. coli*  
(reproduced with permission from Ann Buckingham)
**Using REP-PCR to type “wild strains” of E.coli isolated from faeces**

REP-PCR was used to type the *E.coli* from the faeces of normal individuals working in the department of Gastroenterology at the General Infirmary at Leeds. Three healthy subjects produced faecal samples from which five isolates of *E.coli* was isolated and analysed using REP-PCR (Figure II). The strains were different in each subject and could not be matched to any recognised profiles from the 128 serotypes analysed by REP-PCR. This confirmed previous work in which it had been noted that faeces contain a few, predominant serotypes of *E.coli* and that these serotypes differ from person to person.

**Figure II**  REP-PCR of *E.coli* derived from three healthy subjects

*republished with permission from Ann Buckingham*

1. *BglI* ladder
2. mutaflor pattern
3. blank
4. subject 1 (only isolate)
5. subject 2 isolate a
6. subject 2 isolate b
7. subject 2, isolate c
8. subject 2, isolate d
9. subject 2, isolate e
10. subject 3, isolate a
11. subject 3, isolate b
12. subject 3, isolate c
13. subject 3, isolate d
14. *BglI* ladder

The isolate from subject 1 is unique and different to all other isolates on the gel above. Four of the five isolates from subject 2 are evidently the same strain as the REP-PCR profiles are identical (lanes 5, 6, 7, 8). However, isolate 2 (lane 9) has a markedly different profile and represent a different isolate. Subject 3 have two different *E.coli* isolates. Both isolate a and b (lanes 10 and 11) are identical whilst isolates c and d (lanes 12 and 13) are identical and different to isolates a and b.
Using REP-PCR to type the non-pathogenic *E. coli* Nissle 1917 (Mutaflor)

The technique was used to type the *E. coli* from a patient suffering from ulcerative colitis who had received Nissle 1917 for two days. The profiles of the *E. coli* isolates all had the Nissle 1917 REP-PCR profile (figure III).

**Figure III** Rep-PCR profile from patient with ulcerative colitis given Nissle 1917 until 2 days before sampling

1. 123 ladder  
2. Blank  
3. Standard; mutaflor profile  
4. colony 1  
5. colony 2  
6. colony 3  
7. colony 4  
8. colony 5  
9. BglII ladder

It was concluded that REP-PCR typing would be more reliable and more discriminative than serotyping strains of *E. coli* and considerably more convenient than other PCR based techniques as extracted and purified DNA was not required.
**REP-PCR protocol used in the study**

PCR reagents were purchased in bulk to minimise batch-to-batch variations. The same PCR machine (Triple block Omnigene, Hybaid, Teddington Middlesex, UK) was used throughout the study. Reactions were set up in a clean room reserved for PCR work and at least one negative control was included in every run to detect any contamination of reagents. The primers were designed by Fraser Lewis at the Department of Histopathology at the Infirmary in Leeds.

**Template preparation.** Isolates were grown overnight on Iso-Sensitest agar. Approximately 3-4 colonies of each isolate were selected with a sterile toothpick and emulsified in 50μl sterile, ultrapure, water. This was heated at 95°C for 5 minutes to lyse the cells and then frozen at -20°C until required. Frozen crude template was always used within 2h of preparation.

**Reactions.** Primer REP1R-I (5'-IIIICGICGICATCIGGC-3') was prepared by the Institute of Pathology, University of Leeds, and stocks kept at -70°C in liquid ammonia. The reaction mix (25μl final volume) consisted of 200μM dNTP's (Pharmacia), 1.5U of 'Super-Taq' DNA polymerase (HT Biotechnology, Cambridge, UK) 1xPCR reaction buffer (Promega, Madison, Wisconsin, USA), 25pmol of primer, 10% dimethyl sulfoxide (DMSO), 1.5mM Mg2+ and 2.5μl of just-thawed crude template DNA. Reactions were performed in 0.5ml Safe-Lock tubes (Eppendorf, Hamburg, Germany). The mixture was overlaid with 4 drops of mineral oil, and tubes were run through the following PCR amplification program (in box):

<table>
<thead>
<tr>
<th>PCR amplification</th>
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<tbody>
<tr>
<td>3 min at 95°C for initial denaturation</td>
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<tr>
<td>30 seconds at 90°C for denaturation</td>
</tr>
<tr>
<td>1 min at 40°C for annealing during 30 cycles</td>
</tr>
<tr>
<td>8 min at 65°C for extension</td>
</tr>
<tr>
<td>16 min at 65°C for final extension</td>
</tr>
<tr>
<td>4 min 30°C to allow tubes to cool</td>
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After amplification, the oil layer was removed from each tube and 8μl of loading dye was added. 20μl of the resulting mixture was run on a 2% w/v agarose gel (UltraPure Electrophoresis grade, Gibco BRL, Paisley, Scotland) with TAE electrophoresis buffer. γ DNA cut with BglI was used as the molecular weight marker. DNA bands were visualised by ultra-violet transillumination, after staining with 0.05% ethidium bromide.
Interpretation of REP-PCR profiles. A positive control was included to aid comparison. Variations in the intensity or 'shape' of the bands were not taken into account when analysing the gels. As the amount of DNA added to each PCR reaction was unquantified, the relative yield of amplimers could not be taken into account.

When analysing the gel patterns, the guidelines of Woods et al\textsuperscript{705} and Reboli et al\textsuperscript{706} were followed. The absence of up to two bands from a profile was allowed, when all other visible bands matched, before isolates were considered different by visual inspection.

**Method for isolation of plasmid DNA**

A modified version of the "rapid method" of isolating plasmid DNA as described by Bennet\textsuperscript{707} was used. This method is in turn, a further modification of the original method described by Birnboim\textsuperscript{708}. Cells were grown overnight in Luria Bertani broth at 37°C with shaking. Following incubation, 3 ml of overnight culture was harvested by centrifugation in a 1.5ml polypropylene tube in a microcentrifuge (Micro Centaur MSE Scientific Instruments, Crawley, Surrey) at 13.000 rpm for 2 minutes. Pellets were resuspended in 100 µl of 50mM glucose, 10mM EDTA and 25mM Tris.HCl pH 8.0 (Solution 1) containing 1g/l lysozyme. The suspension was left at ambient temperature for at least 15 minutes to allow digestion of the cell walls.

Cells were completely lysed by the addition of 200 µl freshly prepared 1% alkaline sodium dodecyl sulphate (SDS) in 0.2M NaOH (Solution 2) which was mixed with the cell suspension by inversion. The lysis mixture was left at room temperature for 5 minutes. The mixture was colourless and transparent at this point due to the solubilisation of the cell proteins and lipids by the SDS and the fragmented chromosomal DNA molecules were denatured by the alkaline conditions.

The solution was neutralised by adding 150 µl of 3M sodium acetate, pH 4.8 (Solution 3) and mixing by inversion. The sodium acetate precipitates the SDS and the denatured chromosomal DNA, which are then removed by centrifuging in a microcentrifuge at 13.000 rpm for 5 minutes. The plasmid DNA is present as supercoiled, covalently closed circles which do not separate completely in the denaturing conditions employed. The chromosomal and linear DNA strands are unlikely to re-anneal because the strands will have parted and may not locate their complementary strands.
Following centrifugation 400 µl of the cleared supernatant were removed to a clean microcentrifuge tube. The plasmid DNA was precipitated by adding 1 ml of ethanol, mixing by inversion and leaving the tube at -70°C for twenty minutes, or -20°C overnight as convenient. The precipitated plasmid DNA was pelleted by spinning at 13,000 rpm for 2 minutes. The microcentrifuge tubes were arranged with their hinges outermost in the centrifuge.

The pellets are translucent and feathery at this stage and orienting the tubes in this way means they can be easily located and are less likely to be disturbed when discarding the supernatant in the next step. The pellets were resuspended in 400 µg of 50mM Tris.HCl, pH 8.0 containing 100 mM sodium acetate (Solution 4) by brief vortexing or gently pumping the mixture with a pipette. Proteins were removed by extraction with an equal volume of Tris-saturated phenol pH 8.0. The phases were briefly vortexed and separated by centrifuging for 2 minutes. The top, aqueous layer was then removed to a clean tube taking care that none of the organic phase was carried over with it. 2 µl of RNase A (Boehringer-Mannheim UK, Diagnostics and Biochemicals Ltd, Lewes, Sussex) were added and the tube left at 37°C for at least 20 minutes. The RNase was prepared by dissolving this enzyme in 10 mM Tris.HCl pH 7.5 and 15mm NaCl, to give a final concentration of 10g/l.

This solution was boiled for exactly 5 minutes and then left to equilibrate at room temperature. The RNase digests the RNA which is extracted along with the DNA during the first part of the plasmid isolation procedure. Digesting the RNA reduces the intensity of the RNA band, when viewed under UV illumination. The RNA band runs ahead of the DNA and the tracking dye (loading buffer) during agarose gel electrophoresis.

To remove the RNase and any remaining proteins, an equal volume of a 25:24:1 mixture of phenol:chloroform:isoamyl alcohol (Sigma Chemical Co., Poole, Dorset) was added and the mixture vortexed. The layers were separated by centrifuging for two minutes and the top aqueous layer was removed to a clean tube. One ml of ethanol was added and mixed by inversion and the plasmid DNA precipitated as before. The precipitates were harvested by centrifugation and the supernatant removed by aspiration or careful decanting. The pellets were brought down to the bottom of the tube by washing, without mixing, in 200 µl diethyl ether and centrifuging immediately for 15 seconds. Most of the ether was then carefully tipped or aspirated off and any remaining was allowed to evaporate.
The evaporation process was speeded up by holding the tubes under a vacuum. After drying, the pellets were dissolved in 10 µl of sterile distilled water and then mixed with 5 µl of 40% (w/v) sucrose, 0.5M EDTA and 0.5% (w/v) bromophenol blue (loading buffer). The plasmid DNA was separated by electrophoresis in a 0.8% (w/v) agarose gel made and run with E buffer. This comprised 40mM Tris with 2mM EDTA, adjusted to pH 7.9 with glacial acetic acid.

Uncut plasmid DNA from strains NCTC50192 and NCTC50193 and Nissle 1917 SK22 were also run on the gels as standards as required. A non-adhesive *E. coli* strain SC13 (O1:H7) from the Division of Enteric Pathogens, Central Public Health Laboratory, London was used as a negative control. This strain is known to harbour a 98 MDa plasmid. An H4 or H5-electrophoresis tank (Gibco BRL Life Technologies Ltd, Paisley, Scotland) was used and gels were run at 100-115V for 2-3.5 hours.

Following electrophoresis, the agarose gel was submerged in a 0.5mg/l solution of ethidium bromide and left in the dark at room temperature for 20 minutes. After this, the ethidium bromide solution was replaced with tap water and the gel shaken at room temperature for a further 20 minutes. The gel was soaked in water after being stained with the ethidium bromide in order to remove this agent from the agarose in those places where it was not bound to the DNA.

The gel was photographed under UV light using a Polaroid CU-5, 88-46 camera (magnification 0.65x) and Polaroid 665 or 667 film. The 665 film produces both a positive print and a permanent negative.

The ultra-rapid method of Bennett was further modified by lysing the cells at 37° instead of room temperature. The higher temperature increased the lysozyme activity and allowed the digestion step to be shortened. The cells were then lysed with plasmid Solution 2. In addition, instead of allowing 20 minutes for the DNA to precipitate at -70°C, this was reduced to 15 minutes without any detrimental effect on the plasmid yield. The phenol extraction of proteins was carried out as usual although the RNase digest and phenol:chloroform:isoamyl alcohol washes were both omitted. One millilitre of ethanol was added to the top aqueous layer, following its separation from the phenol and the tube was stored at -20°C overnight.
Method for curing plasmids by heat stress

*E. coli* were subcultured onto two isosensitest agar plates. One plate was incubated at 37°C and the other at 42°C, a temperature which should cause the bacterium physiological stress. After each overnight incubation, the resulting growth was subcultured on to two fresh isosensitest plates and these were returned to the appropriate incubator to allow overnight growth to occur. Comparison of the cultures from different temperatures was done to increase the likelihood of obtaining bacteria cured of their plasmids. The overnight growths from the fifth subcultures were used for plasmid isolation.

As there was no difference in the appearance of the colonies grown at 37°C and 42°C, the growth from the 42°C incubation was subcultured further at the higher temperature of 44°C. Again there was little difference in the appearance of the overnight growth and the 44°C plates was subcultured again onto fresh isosensitest agar and incubated overnight at the higher temperature of 46°C. Once again, there was little difference in appearance between this overnight growth and that of the cultures stored at 4°C.

The fresh overnight growth from the 46°C incubation was subcultured onto CLED agar and now incubated overnight at 48°C. The resultant growth was now poor and the colonies smaller than previously, indicating that the cells had been adequately stressed. The colonies were subcultured onto isosensitest agar before being incubated a second time at 48°C overnight. The cultures grown at 37°C and the final cultures grown at 48°C were used to inoculate 10ml quantities of Luria Bertani broth. After overnight growth, with shaking, at 37°C these cultures were used for plasmid isolations as described previously. Two of the stored cultures originally grown at 48°C, were also subcultured onto isosensitest and CLED agars. These were incubated overnight at 55°C.

Using a dry sterile swab, the growth covering approximately one quarter of a plate was transferred to 1 ml of plasmid solution 1 (see earlier) in a 1.5 ml eppendorf tube. The resuspended cells were harvested by centrifugation for one minute and the resulting pellet resuspended in 100 µl of freshly prepared plasmid solution 1 containing 1 g/l of lysozyme. The plasmid isolation procedure was then resumed as described previously, except that the lysozyme digest was incubated at 37°C for 25 minutes. This was done to increase the effectiveness of this step, as each tube contained more than the usual amount of cells. The plasmid DNA was separated on a 0.8% (w/v) agarose gel made with E buffer. After staining with ethidium bromide as before, the gel was photographed and documented, as detailed earlier.
Assessment of adhesiveness

Background

Colonisation of mucosal surfaces by bacteria is an important preliminary step as in order for the pathogen to exert an effect, it must be able to adhere to the intestinal mucosa.

Figure I. The surface energy theory of bacterial adhesion

The surface energy theory of bacterial adhesion of Derjaguin-Landau-Verwey-Overbeek attempts to explain the mechanism of bacterial adhesion and the necessity of bacterial surface appendages for adhesion to occur. The net negative surface charges of cells and bacteria, as well as diffuse ion clouds in the area, repulse adhesion (figure I). While at 15 nm. there is little repulsion, as bacteria approach 10 nm. maximum repulsion occurs. The magnitude of attractive as well as repulsive forces increases with the diameter of the approaching body. Bacterial fimbriae, which are of a much smaller diameter, allow the bacteria to reach receptors on the cell surface. Once adherent, the normal flow of body fluids, does not wash away the bacteria.

Bacterial adhesins

The term adhesin is a class designation of any structure leading to bacterial adhesion to a cell or tissue. Adhesion may also occur because of a non-specific characteristic, such as hydrophobicity, but more frequently it is due to a specific receptor-lectin interaction by means of surface hair-like appendages termed fimbriae or pili. Fimbriae are not the only means of bacterial attachment and bacterial-cell adhesion may also be facilitated by adherence pedestals (similar to fimbriae) and “afimbrial adhesins”, such as polymers, polysaccharides, lipoteichoic acid and high molecular weight proteins. Most studies of E.coli adhesiveness have been carried out in urinary tract infection. A study by Foxman et al evaluated patients with cystitis and found that all virulence factors examined, (type 1 fimbriae, P-fimbriae, S-fimbriae, afimbrial adhesin, haemolysin, aerobactin and cytotoxic necrotising factor) were all commonly expressed among isolates from patients with cystitis. Apart from type 1 fimbriae, these adhesins were rarely found in E.coli isolated from stool.
**Mannose sensitive fimbrial adhesins**

Type 1 fimbriae adhere to mannose residues of glycoproteins and agglutinate guinea pig red cells whose surface is rich in mannose. Therefore, treating the bacteria with a mannose solution prevents agglutination of these cells and the fimbriae are said to be mannose sensitive. Other fimbriae have been classified as mannose resistant since mannose does not prevent haemaglutination.

Type 1 fimbriae are found in most enterobacteriaceae, frequently on non-pathogenic as well as pathogenic *E.coli*. There are a number of potential host targets for type 1 fimbriated bacteria, including human buccal cells, renal tubular cells of the kidney, epithelial cells of the bladder, lung and intestine, as well as vaginal cells. It has been shown that *E.coli* with type 1 fimbriae adhere to mucin coating urothelial cells. As the mucin is shed, however, adherent *E.coli* are also shed, thus, creating an effective defence mechanism. Type 1 fimbriated bacteria are commonly implicated in urinary tract infections in catheterised patients.

**Mannose resistant fimbrial adhesins**

Mannose resistant adhesins include P-fimbriated *E.coli*, the rare S, G and M fimbriae, as well as some afimbrial strains of *E.coli*. P-fimbriated *E.coli* are implicated in more than 95 percent of pyelonephritis in children and between 50 to 90 percent of pyelonephritis in adults. P blood group antigens are the specific urothelial receptors for P-fimbriae, thus the reason for the name. However, P-fimbriae is not the only virulence factor important in the aetiology of acute pyelonephritis as P-fimbriated *E.coli* often also have other proposed virulence factors, such as bacterial haemolysin, type 1 fimbriae and aerobactin, and are commonly resistant to serum bactericidal activity.

In ultrastructural studies, P-fimbriae have been found to be heteropolymeric structures composed of a rigid stalk containing the major protein linked end to end with a flexible tip fibrillum consisting of 4 proteins, with papG, the receptor binding adhesin, at the tip of the fimbriae. While the tip protein is the most important adhesion, binding to a secondary binding site on cell membranes occurs via the fibrillum papE (to which the tip protein is attached), which adheres to fibronectin.
In vitro measurement of bacterial adhesion

There are different methods to investigate adhesiveness in vitro. Early assessments used haemagglutination which detects the ability of bacteria to bind red blood cells together. Subsequently more direct methods of measuring adherence of bacteria to epithelial cells have been described including enterocytes, colonocytes, tissue culture cells such as HeLa and HEp-2 cells, tissue sections (human and animal), brush border preparations, and organ culture. To assess adhesiveness, we used the Buccal Epithelial Adhesion Assay as used by Burke, based on the assay of Gibbons and van Houte and Ofek and further modified by Candy.

Isolates were coded and assayed blindly, without knowledge of their source. They were grown for 18 hours at 37°C on CLED agar. Bacteria were washed using phosphate buffered saline, pH 7.2, and the concentration adjusted to approximately 1x10^8 bacteria/ml.

Buccal epithelial cells were obtained by gently scraping the buccal mucosa of healthy laboratory workers with a sterile wooden spatula and suspended in phosphate buffered saline. The cells were washed four times and resuspended at approximately 1x10^5 cells/ml in phosphate buffered saline in which D-mannose had been dissolved (28 mmol/l) to inhibit mannose-sensitive adherence by type 1 fimbriae. 0.5 ml of each of the bacterial and buccal epithelial cell suspensions was mixed for 30 minutes on a rotary roller.

The buccal epithelial cells were then washed four times over a 5µm millipore filter (Millipore, Watford, UK) with phosphate buffered saline. In the original description of this assay, the bacteria remaining free at the end of incubation period were removed by centrifugation. However, Burke found that filtration was easier to carry out and correlated well with differential centrifugation. An impression smear was then made from the filter on to a clear glass slide. This was air dried, fixed in methanol for two minutes and stained by Gram's method. A control without added bacteria was always included.

Inspecting 100 non-overlapping buccal epithelial cells in at least 10 high power fields assessed the degree of mannose resistant adhesion. The number of cells with more than 50 adherent Gram-negative rods was recorded, and the control cell count was subtracted from this to give the adhesion index expressed as a percentage to form the buccal epithelial adhesion index (BECAI). A control cell count of over 10% was taken to imply oral contamination, and the experiment repeated with a different buccal epithelial cell source.
As adhesive and non-adhesive control strains we used a recognised mannose resistant adhesive enteropathogenic *E.coli* E851/71 (O142:H6) and a Hep-2 non-adhesive *E.coli* SC13 (O1:H7), from the Division of Enteric Pathogens, Central Public Health Laboratory, London.

Photograph 2 and 3 illustrates the buccal epithelial cell adhesion assay. Photograph 2 shows the appearance of the epithelial cells after 30 min incubation with non-adhesive *E.coli* and photograph 3 show the appearances after incubation with an adhesive *E.coli* isolate. The adhesive strain (E851/71) had a mean adhesive score of 15% and the non-adhesive standard (SC13), a mean of 0%.

**Photograph 2 and 3**

*Buccal cells incubated with non-adhesive and adhesive E.coli respectively*

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**Definition of adhesiveness**

The different cell lines used as models for assessing bacterial adherence to intestinal epithelial cells may give different results depending on the adherence assay used. For example Adlerberg et al\(^7\) found a strong adherence of type 1 fimbriated *E.coli* to cultured, detached, HT-29 cells. In contrast, Neeser et al\(^7\) found no binding of type 1-fimbriated *E.coli* when the HT-29 cells were cultured in confluent cell layers. Also the culture conditions may greatly affect the receptor expression. In post-confluent cell cultured, Caco-2 cells and glucose deprived HT-29 cells achieve a differentiated phenotype expressing disaccharidases and other enzymes\(^7\) including receptors for a number of colonization factor antigens\(^7\).
In the original studies by Gibbons and Ofek, adhesiveness was regarded as a continuous variable. Subsequently, Candy arbitrarily classified adhesive strains as having a buccal epithelial cell adhesion index of > 25%. Giaffer et al, did not use an absolute cut-off value and instead regarded a strain as adhesive if it exceeded the adhesive index of the non-adhesive control strain by 2 standard deviations.

In an earlier study of *E. coli* adhesiveness, an arbitrary index of adhesion of to buccal epithelial cells of >25% was taken to differentiate between adhesive and non-adhesive strains. However, adhesiveness appears to be a continuous feature or microorganisms rather than a feature, which is, either present or absent. Instead, “adhesiveness” is likely to be a continuous variable, the net-result of bacterial surface charge, bacterial size, hydrophobicity, specific receptor-lectin interactions, membrane polysaccharides, and high molecular weight proteins. In view of this, we decided to use the buccal epithelial adhesive index (BECAI) in the results rather than treating “adhesiveness” as a feature which is either present or absent.

**Validation of the buccal epithelial adhesion assay**

We found that a major determinant of the buccal epithelial adhesion index was the origin of the donor buccal cells. Others have recognized this inherent problem of the buccal epithelial cell assay. Hartley et al studied the adhesiveness of *E. coli* isolated from patients with ulcerative colitis but reportedly had to abandon the assay, as he could not find a suitable donor whose buccal epithelial cells gave reproducible and consistent results. Burke compared the adhesive score of 5 different *E. coli* isolates when measured using buccal epithelial cells obtained from 5 different donors. He found that the adhesive index varied between 10 and 50% depending on which epithelial cells were used.
I approached many buccal cell donors and tested the adhesive index before suitable sources were found. The figure below shows the adhesive index (BECAI) of our adhesive and non-adhesive control strains when buccal epithelial cells from 3 different healthy donors were tested.

**Figure II.** E.coli adhesiveness when measured using buccal cells from three different donors

![Adhesiveness vs. buccal cell donor](image)

The assays were repeated 26 times over the period of a week. The mean adhesiveness of our positive *E.coli* control strain was 14.8% (95% CI 13.7 – 16.0%) when buccal cells from donor 1 were used. However, this fell to 2.5% (CI 1.1 – 3.9) when cells from donor 2 were used and 3.3% (CI 1.3 – 5.2) when donor 3 was used.

The mean adhesiveness score for our negative control strain was less than 1% for all donors (donor 1; 0.3%, donor 2; 0.5% and donor 3; 0.6%). Buccal epithelial cells from donor 1 were used in the studies of *E.coli* adhesiveness over time and *E.coli* adhesiveness in different extent of disease. This buccal epithelial cell donor unfortunately left the department and instead, buccal epithelial cells from donor 3 were used in the plasmid experiments and in the Mutaflor study. This resulted in a different mean adhesiveness score of *E.coli* isolated from patients with inflammatory bowel disease in the different studies.
IS A 98MDA PLASMID RESPONSIBLE FOR THE ADHESIVENESS OF E. COLI?

Background
Studies by Dickinson have showed that the adhesive Escherichia coli of colitics do not represent one single strain\textsuperscript{747}. Neither do they possess the other pathogenic properties held by classical pathogens\textsuperscript{748}.

Plasmid mediated adhesion of Escherichia coli is well documented\textsuperscript{749,750,751}. Lobo et al, suggested that a transferable plasmid may explain the difficulty in identifying a single strain of organism and possibly account for the short lasting effect of tobramycin therapy. He analysed 105 E.coli isolates from 28 patients with ulcerative colitis, 33 E.coli isolated from patients with Crohn's disease and 14 E.coli isolated from healthy 14 volunteers and reported that strains with a 98 MDa plasmid were more adhesive than strains without it\textsuperscript{752}. The median adhesive index in strains with the large plasmid was 38% (range 0 – 97%) compared with 0% (range 0-87%) for strains without the 98 MDa plasmid. This finding was irrespective of whether the E.coli strain had been isolated from a patient with ulcerative colitis or Crohn's disease, and irrespectively of whether their disease was active or quiescent.

Methods
One hundred different E.coli isolates from 37 patients with ulcerative colitis were screened for the presence of a plasmid of approximately 98 MDa. A further thirty different isolates from healthy controls were also examined (table I). The specimens were coded so that the origin of the samples was not known to the person performing the analysis.
Table I Details of patients and controls

<table>
<thead>
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<th>Patients</th>
<th>Controls</th>
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</thead>
<tbody>
<tr>
<td>Patients</td>
<td>37 patients with evidence of active UC</td>
</tr>
<tr>
<td>Median age</td>
<td>46 (IQR 33 – 67)</td>
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<tr>
<td>Sex ratio</td>
<td>19 male : 18 female</td>
</tr>
<tr>
<td>Smokers</td>
<td>5</td>
</tr>
<tr>
<td>Median number of years since diagnosis</td>
<td>10 (interquartile range 2 – 17)</td>
</tr>
<tr>
<td>Proctitis</td>
<td>5</td>
</tr>
<tr>
<td>Colitis limited to left hemi-colon</td>
<td>18</td>
</tr>
<tr>
<td>Colitis involving the transverse colon</td>
<td>4</td>
</tr>
<tr>
<td>Total colitis</td>
<td>10</td>
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<tr>
<td>Mean number of previous admissions</td>
<td>0.97 (SD 1.3 interquartile range 0 - 1)</td>
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<td>Topical steroids used</td>
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<td>Aminosalicylates</td>
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<td>Azathioprine</td>
<td>4</td>
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<td>Systemic steroids</td>
<td>10</td>
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</tbody>
</table>

Plasmid curing by heat stress

Plasmid curing experiments were attempted to determine if subjecting the organisms to stress would affect the prevalence of the 98 MDa plasmid and if the genes encoding adhesiveness in *E. coli* from patients with ulcerative colitis were plasmid-encoded. Bacteria usually lose their plasmids during physiological stress. Four *E. coli* isolates were subjected to heat stress in an attempt to cure the isolates of their plasmids.

Statistical analysis

The Mann-Whitney test was used in the analysis. The Mann-Whitney U test is the non-parametric analogue of the two sample t test and is used when data in the two groups is skewed as in this case.
Results

**Plasmid carriage and adhesiveness**

Of the 100 *E.coli* isolated from patients with ulcerative colitis, 32 appeared to contain a plasmid of approximately 98MDa. Of these 32 isolates, 19 had adhesive indexes of 0. In the healthy controls, a similar proportion (11/30 isolates) contained a plasmid in the 98MDa range. Ten of these 11 isolates had adhesive indexes of 0.

Photograph I below shows the plasmid profile of 6 strains of *E.coli* isolated from patients with ulcerative colitis together with the Nissle 1917 strain (MTF). Strain 3 and 5 appear to have a plasmid in the 98 MDa range. As a positive control we used *E.coli* 190.1. This Hep-2 strain of *E.coli* (SC13) was obtained from the Division of Enteric Pathogens, Central Public Health Laboratory, London.

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Photograph I
The buccal epithelial adhesive index of the 100 different strains of *E.coli* isolated from patients with ulcerative colitis and the 30 *E.coli* isolates from our controls are plotted on the "scattergram" plot below. Of the 100 different *E.coli* isolates from patients with UC, the mean buccal epithelial adhesive index was 5.65 (median 0, IQR 0 — 9, SD 9.58) for *E.coli* with a 98 MDa plasmid and 6.12 (median 2 IQR 0 — 9, SD 8.9) for *E.coli* without the 98 MDa plasmid. Of the 30 isolates from healthy controls, the mean adhesive index was 1.36 for the 11 (median 0 IQR 0 — 15, SD 4.5) *E.coli* with a large plasmid and 1.0 (median 0, IQR 0 — 4, SD 1.3) for the 19 isolates without the large plasmid.

![Adhesiveness of E.coli isolated from colitics and controls with and without 98 MDa plasmid](image)

The proportion of strains carrying the large plasmid between strains isolated from patients with ulcerative colitis and healthy controls was not significant (chi-squared statistic; P=0.66). The difference in adhesiveness between strains with (mean BECAI 4.56, median 0, SD 8.7 IQR 0-8.5) and without the 98 MDa plasmid (mean 5.1, median 2, SD 8.2, IQR 0-8) was not significant. (Mann Whitney test, P=0.17).

**Plasmid curing experiments**

The plasmid isolations of adhesive *E.coli* heat stressed at 42°C, 44°C, 46°C and 48°C were all identical to those which had been incubated at the normal, lower temperature of 37°C. When the cultures were heat-stressed at 55°C, there was no growth after overnight incubation. The number of bacteria which are cured by heat treatment can be less than 10% of the total population.
Three *E. coli* isolates (labelled 1, 3 and 4), with a high adhesive index (BECAI 44, 43, 36) and harbouring a 98 MDa plasmid were chosen from patients with ulcerative colitis. Photograph II shows the plasmid profile of the three *E. coli* isolates together with our positive control strain 190.1.

As "wild strains" of *E. coli* have no selection marker, a large number of single colonies would have to be individually screened at a range of different temperatures to identify those organisms which were cured of their plasmid. In view of this, no further attempts at plasmid curing was undertaken.

**Discussion**

Dr Lobo and his co-workers reported that a plasmid of approximately 98MDa was more common in *E. coli* isolated from patients with ulcerative colitis than healthy controls. Lobo et al also reported that those strains which contained a 98MDa plasmid, were more adhesive than those without and that there was no difference in the prevalence of a 98 MDa plasmid amongst *E. coli* strains isolated from patients with active or quiescent disease.

In plasmids of this large size, there is little separation by electrophoresis and plasmids which appear to run closely together on the electrophoresis gel may be of very different size. We found a large plasmid in a similar proportion of patients with colitis and healthy controls. Strains with the large plasmid were not significantly more or less adhesive than strains without this plasmid. It therefore appears unlikely that the presence of the large plasmid, alone enhances adhesiveness.
However, it is possible that the presence of a 98 MDa plasmid confers some component of adhesiveness which requires a second constituent to become expressed. If this is the case, one may expect the adhesiveness to be dichotomised into two groups – non adhesive strains, which only harbour the 98MDa plasmid and an adhesive group containing both the 98 MDa plasmid as well as another putative gene encoding a protein necessary for the expression of adhesiveness. However, the range of adhesiveness did not appear dichotomised and was similarly distributed amongst strains isolated from patients with and without a large plasmid.

Another possible reason for the difference between my findings and those reported by Lobo et al. is that although, Lobo et al had examined over a hundred isolates from several patients, multiple isolates were obtained from a few stool samples without attempting to differentiate the different isolates. As faecal samples usually only contain a few dominant *E.coli* strains, it is likely that many of the isolates analysed in Dr Lobo’s study were in fact the same *E.coli* isolate.

Patients were recruited from the Mesalazine arm of the “Mutaflor study”. As their disease was active they were treated with steroids and aminosalicylates. However, in addition to standard medical therapy, the patients were also given oral gentamicin for one week. Gentamicin, is part of the standard treatment of active ulcerative colitis at Leeds General Infirmary and was also part of the research protocol in the “Mutaflor study”.

To model the effect of recent “antibiotic stress” on *E.coli*, we subjected *E.coli* to “heat stress”, hoping to study the effect on their plasmids profile and degree of adhesiveness. Unfortunately, we were unable to provoke the curing of plasmids in any of the three different *E.coli* isolates subjected to a range of different temperatures. It appears unlikely that a plasmid of approximately 98 MDa encodes for adhesiveness in *E.coli* isolated from patients with ulcerative colitis.
DO PATIENTS WITH IBD RETAIN THE ADHESIVE *E. coli* OVER TIME?

**Introduction**

Dickinson and Burke originally reported that patients with ulcerative colitis harbour more adhesive *E. coli* than healthy controls. This chapter describes a prospective study following the *E. coli* flora in patients with inflammatory bowel disease over a 12 months period using REP-PCR electrophoresis to monitor *E. coli* isolates and the buccal epithelial cell adhesion assay to assess adhesion. The objective was to determine if patients with ulcerative colitis retain adhesive *E. coli* strains over a one year period.

**Methods**

Thirteen patients with quiescent ulcerative colitis and 8 with quiescent Crohn's disease were invited to take part. The control group comprised 12 healthy volunteers. Four faecal samples were collected over a 12 month period from each subject. For comparison, 25 patients with active ulcerative colitis were asked to produce a single faecal sample. All patients accepted the invitation and gave written consent. The study was approved by the local research ethics committee.

**Statistical analysis**

The Mann-Whitney and the Kruskal-Wallis tests were used in the analysis. The Mann-Whitney U test is the non-parametric analogue of the two sample t test and is used when data in the two groups is skewed. As shown in the graph below, the data is skewed. The Kruskal-Wallis test is an alternative non-parametric test, used when more than two groups are compared.

**Results**

Over the one year follow up period, 151 different *E. coli* isolates were isolated from 13 patients with quiescent ulcerative colitis, 56 different isolates of *E. coli* from 8 patients with quiescent Crohn's disease and 84 isolates from the 12 healthy volunteers with an average age of 38 years. In addition, another 36 different isolates were cultured from 25 patients with active ulcerative colitis (Table I).

**Table I** Patient characteristics and number of different isolates as determined by REP-PCR
There was no significant difference between the mean adhesive scores in patients with quiescent ulcerative colitis (mean adhesive score 22, IQR 9-30), active ulcerative colitis (mean 21, IQR 8-28) and quiescent Crohn's disease (mean 17.5, IQR 8.5-23) (Kruskal-Wallis test P=0.63). However, the healthy controls had a significantly lower mean adhesive score (mean 9.98, IQR 0-16) when analysed using the Kruskall-Wallis test (P<0.0001).

In the graph below, E851 is the adhesive control strain and SC13 the non-adhesive control strain.
**Adhesiveness of the permanent E. coli isolates**

Four faecal samples were collected over a 12 month period from patients with quiescent ulcerative colitis. Ninety percent of all *E. coli* isolates (136/151) were repeatedly isolated throughout the year. The adhesive scores of these 136 different "permanent isolates" were compared at the beginning and towards the end of the study. When the isolate was not found in the 12 months sample, the strain isolated from the 9 month sample was used. The mean interval between the first and last sample was 10.8 months.

**Graph II**
The mean adhesive score was 23 (IQR 5.5-32) at the beginning of the study and 22 (IQR 8-32) at the end of the follow up. This difference in mean adhesive score was not significant (Mann-Whitney U test, P=0.7494). See graph II above.
**E. coli and extent of ulcerative colitis**

To search for a link between extent of colitis and adhesiveness of the *E. coli*, single stool samples from the 9 patients with pancolitis (21 isolates), our 14 patients with left sided ulcerative colitis (20 isolates) and the 15 patients with colitis limited to the rectum (22 isolates) were analysed.

<table>
<thead>
<tr>
<th>Adhesive score and location of disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adhesive index (BECAl)</td>
</tr>
<tr>
<td>Extensive UC</td>
</tr>
<tr>
<td>Left sided UC</td>
</tr>
<tr>
<td>Proctitis</td>
</tr>
<tr>
<td>E851</td>
</tr>
<tr>
<td>SC13</td>
</tr>
</tbody>
</table>

There was no significant association between the mean adhesiveness and the extent of colitis (graph above). The 21 isolates obtained from patients with extensive colitis (colitis beyond the splenic flexure) had a mean adhesive score of 22.43 (IQR 8-33.5) compared to 10.85 (IQR 0-15.5) in the 20 isolates from patients with left sided colitis and the mean adhesive index of 23.64 (IQR 0-37) in the 22 isolates from patients with proctitis.

The difference in mean adhesive scores of isolates from patients with extensive colitis, left sided colitis and proctitis was not significantly different when analysed by the Kruskall-Wallis test (P>0.05, table). See graph III below (E851 is our adhesive control strain and SC13 is our non-adhesive control strain).

<table>
<thead>
<tr>
<th>Graph III</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extensive UC vs</td>
<td>P&gt;0.05</td>
</tr>
<tr>
<td>Left sided UC</td>
<td></td>
</tr>
<tr>
<td>Extensive UC vs</td>
<td>P&gt;0.05</td>
</tr>
<tr>
<td>Proctitis</td>
<td></td>
</tr>
<tr>
<td>Left sided UC vs</td>
<td>P&gt;0.05</td>
</tr>
<tr>
<td>Proctitis</td>
<td></td>
</tr>
</tbody>
</table>
Discussion

Bacterial association with mucosal cells is complex and the cell adhesion is only the final step in the process of mucosal colonisation. First, the organism must compete with the indigenous flora and establish itself within the lumen. Then, if the host's defence mechanisms do not interfere with mucosal association, penetrate the mucus gel and overcome the repulsive forces that exist between two negatively charged bodies. Finally the organism must achieve a position close enough to allow surface-to-surface binding\textsuperscript{754}.

The demonstration of adhesive properties does not necessarily imply in vivo pathogenicity. Indeed the possession of an adhesin can on occasion be detrimental to the bacterium. The bacteria may attach to receptors in the mucus gel which if shed rapidly will remove the entrapped bacterium with it\textsuperscript{755}. Strains of E.coli, thought not to be pathogenic, have been found to possess an adhesin that results in a localised adherence in vitro\textsuperscript{756}. They presumably lack other virulence attributes suggesting that the possession of an adhesin alone is insufficient to cause disease.

However, adhesion to the epithelial surface is an important preliminary step following which other virulence factors may take effect. In neonatal diarrhoea in pigs caused by enterotoxigenic E.coli, adhesion to the surface of the intestinal tract is recognised as an important feature in the pathogenesis of infection. Removal of the plasmid encoding for the fimbrial mannose resistant adhesin K88 resulted in the loss of its diarrhoea producing capacity\textsuperscript{757} and to colonise the intestinal mucosa\textsuperscript{758}. Immunisation against adhesins and the passive administration of anti-adhesin antibodies can prevent enterotoxigenic E.coli from causing diarrhoea in animals\textsuperscript{759,760}.

In man, Satterwhite and colleagues\textsuperscript{761}, demonstrated that toxin production alone in enterotoxigenic E.coli was insufficient to produce disease in all cases. Using a laboratory derived strain that retained the ability to produce toxin but lacked the mannose resistant mucosal adhesive property they confirmed by oral challenge that the parent strain caused diarrhoea but the mutant strain did not. The importance of adhesion as an \textit{E.coli} virulence factor is also recognised in the urinary tract\textsuperscript{762}. A genetically cloned, adhesive and haemolytic E.coli, triggered the release of more inflammatory mediators but the adhesiveness alone did not confer a pathogenic role.
Dickinson et al\textsuperscript{763} reported that 35\% of patients with active ulcerative colitis and 27\% with inactive colitis harboured at least one \textit{E. coli} that was adhesive to HeLa cells, compared with 5\% of controls. However, HeLa cells may be unsuitable as a model of adherence to intestinal mucosal surfaces as the neoplastic cell line, is unrelated to the gastrointestinal tract and the enzymatic stripping methods used in the manipulation of the cell line, may result in the expression of non-physiological receptors for bacteria\textsuperscript{764}.

\textit{Potential bias with the experimental design}

In this study, we chose to isolate \textit{E. coli} from stool samples rather than from mucosal biopsies. It is possible that the organisms isolated from faeces have a different, perhaps lower, adhesiveness than those in close contact with the mucosa. Furthermore, mucosal biopsies allows immediate culture of the mucosa associated \textit{E. coli} flora whilst samples sent by post may not be viable after 2-3 days in transit. However, three-monthly sigmoidoscopy with mucosal biopsies is difficult to justify when the disease is in remission. Furthermore, Burke compared the serotype and plasmid profile of 10 \textit{E. coli} colonies isolated from 8 patients with ulcerative colitis and found that the \textit{E. coli} detected in the faeces were also represented in the mucosal associated flora. He also found that the adhesiveness was similar for strains isolated from biopsies and from the faeces\textsuperscript{765}.

In the study of \textit{E. coli} adhesiveness and extent of ulcerative colitis, patients with both active and quiescent disease were included. Had the degree of adhesiveness been different in patients with active and quiescent disease been different, this approach may have been flawed. However, similarly to Burke, we found that \textit{E. coli} isolated during remission and active colitis had a comparable adhesiveness. Notwithstanding this, we found that patients with active ulcerative colitis had on average fewer distinct \textit{E. coli} isolates compared to patients with quiescent disease (mean 1.4 vs. 2.9). Hartley et al reported that the use of sulphasalazine was associated with an 8-fold reduction in enterobacteria count (predominantly \textit{E. coli}) compared with those not taking the drug\textsuperscript{766}.

Unfortunately, in our cohort all patients with ulcerative colitis and half of patients with Crohn’s disease were taking an aminosalicylate such as Mesalazine, and it was not possible to correlate the number of distinct \textit{E. coli} isolates with the aminosalicylates taken.

To avoid postal delays patients were asked to produce their stool sample on the day of their visit to the outpatient clinic and the samples were processed immediately. Patients who did not bring a stool sample to the outpatient department were given advice to send a sample by first class post on the following Monday to avoid postal weekend delays.
The 12 healthy volunteers were friends and relatives of hospital workers who had not been treated with antibiotics for the preceding 6 months. As the colonic flora may change when the patient is hospitalised, only outpatients with active ulcerative colitis were asked to provide the samples.

As the isolation and characterisation of large numbers of *E. coli* is costly both in terms of consumable costs and time, we decided to approach a smaller number of patients and isolate all the major *E. coli* isolates from these patients stool. The alternative would have been to recruit a larger number of patients and only analyse the single “dominant” *E. coli* isolate from each case. We decided against the latter approach as it may have introduced a bias into the studies.

“Less dominant” *E. coli* (*E. coli* isolated in smaller numbers from stool) may also be qualitatively different and have a different degree of adhesiveness and behave differently over a one year follow up period compared to the “dominant isolates”.

Using the buccal epithelial cell adhesion method\(^7\), Burke et al\(^7\) reported that *E. coli* strains isolated patients with active inflammatory bowel disease were more adhesive compared with strains isolated from patients with infective diarrhoea and healthy controls (table IV below).

<table>
<thead>
<tr>
<th>Table IV Adhesiveness of <em>E. coli</em> isolated from different patient groups (from Burke 1988)</th>
<th>100-</th>
<th>80-</th>
<th>60-</th>
<th>40-</th>
<th>20-</th>
<th>0-</th>
</tr>
</thead>
<tbody>
<tr>
<td>UC relapse</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UC remission</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crohn's disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infectious diarrhoea</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^7\) Burke et al, 1988.
However, Burke and Axon did not have the REP-PCR assay to allow accurate identification of different *E.coli* isolates. Instead a random colony was chosen from each patient with inflammatory bowel disease. As a result, a mixture of “dominant” and “minor” strains of *E.coli* is likely to have been used in their experiments on adhesiveness. Furthermore, there were no safeguards against the repeated inclusion of identical *E.coli* isolates obtained from different patients. In spite of these methological differences, our findings are comparable. The adhesiveness of *E.coli* isolated from patients with Crohn’s disease, active and quiescent colitis was similar. In contrast, healthy people appear to carry less adhesive *E.coli*.

To conclude, the one-year follow up study of the “permanent” flora in patients with quiescent colitis confirmed that the adhesive property is not a transient effect. Nor was the adhesiveness related to the extent of colonic inflammation as strains isolated from extensive, left-sided colitis and proctitis was similar.
Introduction
The randomised study by Burke demonstrated that tobramycin, with activity against Gram-negative bacteria such as *E. coli*, conferred a significant benefit in the short term when given orally as an adjunct to systemic or topical steroids in active ulcerative colitis. Unfortunately, the benefit was short-lived and may reflect short-term changes in the gut flora as the relapse rates were identical in the two groups at one year follow-up. These findings suggest that treatments aimed at modifying the gut flora for a longer period may be of therapeutic value.

A physician with ulcerative colitis reported a novel method of inducing a lasting change in the faecal flora. After antibiotics, a heterologous faecal flora was administrated in the form of a faecal enema. After this treatment the patient had a prolonged remission. In her own MD thesis, Cooke described the administration of selected "benign" strains of *E. coli* to 14 patients with ulcerative colitis, with the subsequent improvement in 10. However some patients had been pre-treated with neomycin and others were taking corticosteroids and so the reason for improvement could not be clearly discerned. Such an approach has been used with success in chronic diarrhoea from *Clostridium difficile* infection. Lactobacilli have also successfully been used to replace normal flora after the use of antibiotics and to treat cases of relapsing pseudomembranous colitis.

A prospective study was carried out to determine if induction of a prolonged change in the coliform flora provides more lasting protection against relapse. The primary objective was to compare time to relapse and rate of relapse in patients treated with a non-pathogenic strain of *E. coli* compared with mesalazine.
Methods

Patients

120 consecutive patients with an exacerbation of ulcerative colitis were asked for written consent to take part in this study. 116 patients accepted the invitation. The study was approved by the local research ethics committee and by the Medicines Control Agency. Eligible patients were aged between 18 and 80 years, had clinically active ulcerative colitis (defined as four or more liquid stools a day for the last seven days with or without blood), with at least erythema on sigmoidoscopy as well as histological confirmation of active ulcerative colitis. At each assessment, a clinical symptom index was calculated. This index was a modification of an index first described by Rachmilewitz.

The following haematological indices were measured on trial entry, on remission and on relapse; haemoglobin, white cell count, platelet count, plasma viscosity, C-reactive protein, albumin and erythrocyte sedimentation rate.

Randomisation

The randomisation was managed by the department of pharmacy at Leeds General Infirmary. To ensure that both treatment arms were allocated patients of equal severity, the randomisation was stratified into (1) mild colitis and proctitis, (2) moderate colitis and (3) severe colitis according to the criteria by Truelove and Witts. Once patients had consented to partake in the trial, they were supplied with either active mesalazine (Asacol formulation) and dummy probiotics or dummy asacol and active probiotic from the hospital pharmacy. Neither the patient, nor the physician would know to which treatment the patient had been allocated. Active asacol was given at a dose of 800 mg tds, as recommended in the BNF and ABPI Data Sheet for patients with active disease.

The active probiotic consisted of a non-pathogenic strain of *E. coli* (serotype O6:K5:H1) named "Nissle 1917" (Mutaflor®, Ardeypharm GmbH, Herdecke, Germany) at a dose of two capsules bd (2.5x10^10 viable bacteria per capsule). The enteric coated capsule protects the bacteria from gastric juices and releases the *E. coli* in the terminal ileum. All patients were also given a one week course of oral gentamicin 80mg tds, at entry into the study, to suppress their native *E. coli* flora. In addition to the trial medication, patients with mild proctitis were treated with hydrocortisone acetate enemas 1 application bd, patients with mild or moderate disease extending further than the sigmoid colon without systemic disturbance were treated with prednisolone 30 mg/day and patients with symptoms of a severe attack of ulcerative colitis received prednisolone 60 mg/day as inpatients.
Patients were managed in a stepped approach reflecting the current practice at The General Infirmary at Leeds. Those receiving topical therapy who continued to be symptomatic after four weeks treatment, were given 30 mg of prednisolone, orally. Patients who had not responded to four weeks of 30mg prednisolone, were admitted to hospital and treated with 60 mg of prednisolone.

**Trial withdrawals**

The randomisation code was only broken once the final patient had left the study. Patients not in remission after a maximum of twelve weeks were excluded from the further trial, as was any patient who deteriorated clinically. Remission was defined as general well-being with the passage of no more than three formed stools per day, a rectal mucosa without erythema, granularity or friability as well as histological confirmation of inactive disease. The individual trial codes were broken prematurely in two cases of serious adverse events. In both cases, the patients had been hospitalized with active colitis and randomised to the Mesalazine arm of the study. The first patient developed sudden abdominal pain and was found to have perforated diverticulum and laparotomy. The second patient, with severe emphysema, developed respiratory failure whilst in hospital.

**The follow-up phase**

Once the patient was generally asymptomatic and passed no more than three normal stools a day, a sigmoidoscopy was performed to confirm quiescent ulcerative colitis. The patient then entered the follow-up phase of the study. The doses of *E. coli* and mesalazine were reduced to 2 *E. coli* or placebo capsules od and 1 mesalazine or placebo capsule tds. Mesalazine was chosen at a dose of 1.2g/day as this is the usual dose prescribed at the hospital and within the range currently recommended in the BNF and ABPI Data Sheet for maintenance of remission. It is the usual dose prescribed at the hospital and the dose used in previous trials of ulcerative colitis. The dose of hydrocortisone acetate enema was reduced to one application per day and then stopped after two weeks. The prednisolone was slowly withdrawn over four months. Patients were reviewed monthly in the outpatient clinic for the first two months and then every other month until relapse or for a maximum of 12 months. Patients were given a telephone number to contact the investigator at the first sign of relapse. Patients were asked to report any deterioration in general well being or an increased stool frequency, rectal bleeding or mucus. Patients were then re-assessed by sigmoidoscopy and histology to confirm the relapse. The mucosal appearances were scored according to Baron (Table II).

**Table II - Baron’s scoring system**

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>normal mucosa</td>
</tr>
<tr>
<td>2</td>
<td>mucosal granularity</td>
</tr>
<tr>
<td>3</td>
<td>granularity and contact bleeding</td>
</tr>
<tr>
<td>4</td>
<td>spontaneous bleeding, friability and frank ulceration</td>
</tr>
</tbody>
</table>

107
Statistical analysis

A two stage, conditional, intention to treat analysis was carried out. In the first stage, the number of patients recruited and achieving remission was compared in the two groups. In the second stage, the number of patients remaining in remission for twelve months was compared in the two groups.

Most clinical trials are "superiority trials" in which investigators are interested in finding out whether an innovative treatment is superior or inferior to standard therapy. This study was a "non-inferiority trial" with the primary objective of showing that the efficacy of \textit{E.coli} is not clinically inferior to mesalazine for maintaining remission. In accordance with the ICH-E9 guideline\textsuperscript{780}, one-sided tests were used for testing the null hypothesis that the treatment difference (\textit{E.coli} minus mesalazine) is less or equal to the lower equivalence margin of -20\%, versus the alternative that the treatment difference is greater than 20\%. In addition, two-sided 95\% confidence intervals were calculated. The p-values incorporated no stratification for initial severity.

The study was designed with a power of 80\% to exclude, with 90\% (one-sided) confidence, a difference greater than 20 percentage points in favour of mesalazine, concerning the primary efficacy parameter "relapse rate". Assuming a one year relapse rate of 20\%, to reach a statistical power of 80\%, at least 36 patients were required in each treatment arm. Inferential statistical comparisons of the secondary endpoints "remission rate", "time to remission" and "time to relapse" were to be interpreted in the exploratory sense.

Kaplan-Meier graphs were used to compare the groups, and log-rank tests assessed the statistical significance of the difference, incorporating the stratification factor. Adverse event rates were compared using Fisher's exact test. Data available were incorporated into analysis regardless of protocol compliance. All analyses were undertaken using SPSS for Windows release 6.0. Results

The study profile is summarised in figure I.
A total of 59 patients were randomised to receive mesalazine and 57 to treatment with *E. coli*. The median clinical activity index on study entry was 11 in the mesalazine group and 9 in the group given *E. coli*. The median sigmoidoscopy score was 4 in both groups on entry. Table III below, lists the demographic details at randomisation. The two groups were well matched for putative confounding factors such as age, sex, disease duration and extent, smoking, medication taken, and clinical activity index.

<table>
<thead>
<tr>
<th><strong>Table III - Baseline factors at randomisation</strong></th>
<th>Mesalazine, (median, iqr)</th>
<th>Mutaflor, (median, iqr)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age at randomisation</strong></td>
<td>1 (30,49)</td>
<td>39 (30,60)</td>
</tr>
<tr>
<td><strong>Years since diagnosis</strong></td>
<td>1 (1,8)</td>
<td>5 (2,12)</td>
</tr>
<tr>
<td><strong>Median activity index</strong></td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td><strong>Median mucosal score</strong></td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td><strong>Factor</strong></td>
<td><strong>Mesalazine n (%)</strong></td>
<td><strong>Mutaflor n (%)</strong></td>
</tr>
<tr>
<td>Sex male</td>
<td>32 (54%)</td>
<td>29 (51%)</td>
</tr>
<tr>
<td>Smoker</td>
<td>2 (3%)</td>
<td>1 (2%)</td>
</tr>
<tr>
<td><strong>Extent of disease</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proctitis only</td>
<td>7 (29%)</td>
<td>7 (30%)</td>
</tr>
<tr>
<td>Left colon involved</td>
<td>21 (36%)</td>
<td>15 (27%)</td>
</tr>
<tr>
<td>Subtotal colon</td>
<td>6 (11%)</td>
<td>6 (11%)</td>
</tr>
<tr>
<td>Total colitis</td>
<td>13 (22%)</td>
<td>18 (32%)</td>
</tr>
<tr>
<td><strong>Medication</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Topical steroids</td>
<td>3 (73%)</td>
<td>4 (77%)</td>
</tr>
<tr>
<td>Aminosalicylates</td>
<td>13 (22%)</td>
<td>7 (12%)</td>
</tr>
<tr>
<td>Azathioprine</td>
<td>28 (47%)</td>
<td>30 (53%)</td>
</tr>
</tbody>
</table>
The medications were well tolerated. The numbers of patients achieving remission were 44 (75%) in the mesalazine group and 39 (68%) in the E. coli group (table IV).

<table>
<thead>
<tr>
<th>Table IV: Number of patients at each stage of study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number randomised</td>
</tr>
<tr>
<td>Mild</td>
</tr>
<tr>
<td>Moderate</td>
</tr>
<tr>
<td>Severe</td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>Number reaching remission (% of those randomised)</td>
</tr>
<tr>
<td>Mild</td>
</tr>
<tr>
<td>Moderate</td>
</tr>
<tr>
<td>Severe</td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>Number relapsing (% of those in remission)</td>
</tr>
<tr>
<td>Mild</td>
</tr>
<tr>
<td>Moderate</td>
</tr>
<tr>
<td>Severe</td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>Number reaching end of study (% of those in remission)</td>
</tr>
<tr>
<td>Mild</td>
</tr>
<tr>
<td>Moderate</td>
</tr>
<tr>
<td>Severe</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

Testing the null hypothesis that the difference E. coli minus mesalazine is less or equal to the lower equivalence margin of -20% versus the alternative that the treatment difference is greater than 20% resulted in \( p=0.0508 \). The two-sided 95% confidence interval for the treatment difference ranged from -22.7% to +10.4%.

The mean time to remission was 44 days (median 42) for patients receiving mesalazine and 42 days (median 37) for those treated with E. coli \( (p=0.0092) \) (figure 2). The two-sided 95% confidence interval for the treatment difference was -11 to 7 days.
Slightly more patients than anticipated achieved remission (83 rather than the expected 72). Upon remission, the median clinical activity index and sigmoidoscopy scores were 1 in both groups. In the mesalazine group, 32 (73%) patients relapsed in the one year follow-up period compared with 26 (67%) in the E.coli group.

In the mesalazine group, 32 (73%) patients relapsed compared with 26 (67%) in the E.coli group. Testing the null hypothesis that the difference E.coli minus mesalazine is greater equal to the upper equivalence margin of 20% versus the alternative that the treatment difference is less than the upper equivalence margin resulted in p=0.0059. The two-sided 95% confidence interval for the treatment difference ranged from -26.4% to +14.2%. (figure 3).

The mean duration of remission in the mesalazine group was 206 days (median 175) and 221 days (median 185) in the E.coli group. The two-sided 95% confidence interval for the treatment difference was -37 to +66 days.

The median clinical activity and sigmoidoscopy scores on relapse were 7 and 3 respectively in both groups. There were no significant differences in haematological indices between the two groups on remission or on relapse.

In the mesalazine arm, 6 patients requested withdrawal from the trial, two patients violated the study protocol by prematurely stopping the medication and 2 patients suffered serious adverse events (perforated sigmoid diverticulum requiring laparotomy and respiratory failure in a patient with severe emphysema). A relationship with the study medication was thought unlikely. One patient was erroneously included into the trial as her histology did not confirm active colitis.
Table V  Non-serious adverse events of possible, probable or uncertain association with medication

<table>
<thead>
<tr>
<th></th>
<th>Mesalazine</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abdominal pain or bloating</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Headache</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Nausea</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Mouth ulcer</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>11</td>
</tr>
</tbody>
</table>

The groups were similar in the proportions of patients who reported non-serious adverse events of probable, possible or uncertain causality with the medication.

In the mesalazine arm there were 7 patients, one of whom reported 2 simultaneous adverse events. In the E. coli arm there were 9 patients, 2 of these reported two simultaneous adverse events (table V above). The overall compliance, as calculated by counting the returned tablets, was 82% in the mesalazine group and 81% in the E. coli group.

Discussion

In this study E. coli appeared to be as effective as mesalazine in maintaining remission of ulcerative colitis. Both the proportion of patients remaining in remission and duration of remission were similar in patients treated with mesalazine and E. coli. There was no difference in the remission or relapse rates in patients with proctitis, moderate disease or severe ulcerative colitis.

Instead of the expected 20% of patients relapsing over the twelve month follow-up period, 70% (58/83) of patients relapsed. This resulted in a slight loss of statistical power from 80% to 75%. Despite this, the one-sided 90% confidence-limit was for mesalazine to have a 19 percentage point advantage over E. coli. This was just within the limits for "equivalence" set by the study protocol.

The proportion of patients reaching remission and time to remission in the two treatment arms were similar. This was not unexpected as both treatment arms benefited from gentamicin and the potent anti-inflammatory properties of prednisolone, which were likely to be more effective in resolving the active inflammation than mesalazine or E. coli.

Possible reasons for the high relapse rates on mesalazine

Aminosalicylates have remained the main group of drugs used to prolong remission in ulcerative colitis. The first studies of maintenance therapy were very encouraging with only 12-30% of patients relapsing over one year. 781, 782

112
More recent studies have shown that the risk of relapse, over one year, may be much greater. Reports range from 74% with 4g/day of sulphasalazine, 69% with 3g/day of sulphasalazine, 53.3% with 2g/day of sulphasalazine vs. 50% with 1 mg/day of mesalazine, 53.5% with dipentum 1g/day vs. 49.1% with sulphasalazine 2g/day and 55% with 2g/day Balsalazide vs. 36% with 4g/day of balsalazide.

The wide range of reported relapse rates may be due to the varying methodology of the different trials. Most studies recruited patients after varying length of relapse, and then reviewed the patients at a few fixed time points during the year, commonly in different hospitals and by different physicians, using different definitions of relapse. In contrast, to eliminate the potential bias of patients of varying disease severities being recruited at different points in time we decided to follow the study population in a single outpatient clinic by a single physician.

In view of the high relapse rate it would have been of interest to have a placebo arm in the study. However, the use of a “no treatment arm” was considered unethical as a Cochrane systemic review of aminosalicylates did conclude that 5-ASA is significantly more effective than placebo.

A possible reason for the high relapse rate seen in our trial is that “Asacol”, which depend on an increased colonic pH for its release, is poorly suited for maintaining remission in ulcerative colitis. However, the formulation of aminosalicylate is unlikely to be important. In the latest systematic review from the Cochrane database, Sutherland concluded that there was no difference in efficacy between different formulations. In our study, “Asacol” was used at the maintenance dose recommended in the British National Formulary for the maintenance of remission. Another potential reason for our high relapse rate, is that a higher dose of “Asacol” is required for the maintenance of remission. Several studies have looked at the maintenance of remission with high dose aminosalicylates and have found no evidence of benefit compared with the use of standard maintenance doses. In a leading article, Riley concluded that the "results of high dose maintenance treatment have been particularly disappointing.”

Another proposed reason for the high relapse rate was our heterogenic study population which included patients with disease ranging from an exacerbation of proctitis to severe fulminant disease. However, there was no difference in the relapse rates between the different patient subgroups. In fact, a heterogenic group of patients including a mixture of those with low and high risk of relapse could be expected to have produced a lower one year relapse rate than found in our study.
Perhaps the most likely reason for our high relapse rate is the converse - our study population was “too homogenous”. Considering the early finding that about 10% of patients with ulcerative colitis will pursue a chronic continuous course.

Perhaps the most likely reason for our high relapse rates is that by recruiting patients following a recent exacerbation, we have selected for the 10% of patients suffering with chronic continuous form of the disease.

A final possible reason for the high relapse rates is that the epithelial barrier, once disrupted remains unstable for a prolonged period. A defective epithelial barrier allowing enteric bacteria and hydrophilic bacterial products access to the submucosa, may drive further proinflammatory pathways which further disrupt epithelial tight junctions and perpetuate inflammation.

**Potential effect of tobramycin**

In 1988, Burke found that one week of oral tobramycin, almost doubled the remission rate in patients with active ulcerative colitis receiving standard prednisolone therapy. Unfortunately, the benefit proved short-lived as 50% of patients had relapsed at one year follow up.

Notwithstanding this, an oral aminoglycoside has been part of the standard therapy for patients with active ulcerative colitis at the General Infirmary at Leeds since the publication of the study. Gentamicin was later chosen as this aminoglycoside has a similar activity to tobramycin but is cheaper and is available as a “stock ward drug”.

Lobo et al postulated that the short-lived benefit of a 7-day course of tobramycin in active ulcerative colitis was because it elicited only a transient change in the gut flora. Long-term aminoglycoside therapy is however undesirable, as it can give rise to diarrhoea and may select for antibiotic-resistant bacterial strains. An alternative method for inducing a long-lasting change in the colonic flora was reported by Bennet and Brinkman. By following a short course of antibiotics with administration of an enema of faeces from a healthy donor, they were able to induce remission in active ulcerative colitis.

In planning the study, we were hoping that by giving gentamicin, an “ecological niche” would be created into which our therapeutic strain of *E.coli* could become established and provide longer term remission. Naturally, it follows that those patients randomised to receive *E.coli* must also be given gentamicin.
However, the use of an aminoglycoside antibiotic, in addition to prednisolone and high dose aminosalicylates, had already been proven to almost double the chances of remission. Therefore, if those patients randomised to receive asacol were not given gentamicin, we would potentially have required nearly twice as many patients in the asacol arm of the study as in the *E. coli* arm.

As this would have biased the randomisation of the study, we decided to give antibiotics to both arms of the study population. This approach proved correct as the number of patients reaching remission was similar in both arms of the study (75% in asacol arm and 68% in *E. coli* arm of the study).

Finally, the primary aim of our study was to compare relapse rates over a one year period with the two therapies. In contrast, if the primary aim had been to compare remission rates the following four-way randomisation would have been appropriate; (1) no antibiotic + asacol, (2) gentamicin + asacol, (3) no antibiotic + *E. coli* and (4) gentamicin + *E. coli*.

**Potential beneficial effects of the non-pathogenic strain of *E. coli***

There are several possible mechanisms by which the non-pathogenic *E. coli* strain (Nissle 1917) may be having an effect.

(1) It may be blocking receptors in the intestinal mucosa and thus preventing adhesive, pathogenic bacteria from becoming established. Lactobacilli have been shown to adhere to the intestinal mucosa and reduce the binding of pathogens to intestinal mucus\(^ {797} \). Lactobacilli can also competitively inhibit the attachment of more pathogenic organisms to the mucosa\(^ {798,799} \), a phenomenon known as competitive exclusion.

(2) The *E. coli* strain used in the study does exhibit antagonistic activity against a variety of pathogenic and non-pathogenic enterobacteria, probably due to the production of bacteriocins or microcins\(^ {800,801} \). Strains of *E. coli* have been shown to protect against enteropathogens in vivo. One study tested the *E. coli* strains EM0 and JM105 for their ability to prevent infection by *Salmonella typhimurium*. Establishment of the non-pathogenic *E. coli* in the gut of germfree mice resulted in a significant increase in the number of surviving animals and a reduced translocation rate of *S. typhimurium*\(^ {802} \).
In vitro administration of Lactobacilli has also been shown to prevent the formation of biofilm and removes yeast and bacteria from silicon rubber voice prostheses\textsuperscript{803,804}. Although the precise mechanisms are unknown, the effect may be profound. Cell free supernatants of \textit{Lactobacillus rhamnosus} were shown to be bacteriostatic for enteropathogenic \textit{E. coli}, enterotoxigenic \textit{E. coli}, \textit{K. pneumoniae}, \textit{Shigella flexneri}, \textit{Salmonella typhimurium}, \textit{Enterobacter cloacae}, \textit{Pseudomonas aeruginosa}, \textit{Enterococcus faecalis} and \textit{Clostridium difficile}\textsuperscript{805}.

\begin{enumerate}
\item The growth and metabolic activity of \textit{E. coli} Nissle 1917 may have caused changes in the pH or chemical composition of the colonic lumen that are unfavourable to bacteria involved in the pathogenesis of ulcerative colitis or which promote repair and nourishment of the mucosa.
\item The \textit{E. coli} may have induced the host immune response by stimulating the production of mucosal immunoglobulins or cytokines.
\end{enumerate}

The gut microflora does contribute to the induction of oral tolerance\textsuperscript{806} and to the processing of food antigens in the gut. Probiotics have also been shown to modify the structure of potentially harmful antigens and reduce their immunogenicity, potentially dampening hypersensitivity reactions\textsuperscript{807}. The ability of probiotic bacteria to influence the mucosal immune system has been studied in vivo using Lactobacilli species. This ability appears to vary from one strain of Lactobacilli to another. Although many Lactobacilli have been shown to induce IgA secretion\textsuperscript{808}, few inhibit antigen-induced IgE secretion\textsuperscript{809}. In contrast to \textit{L. paracaei}, \textit{L. rhamnosus}, and \textit{Bifidobacter animalis}, only \textit{Lactobacillus plantarum}, induced the mucosal production of IL-6 after the passage through the stomach and small intestine\textsuperscript{810}.

In another study, eight different strains of Lactobacilli were used to study the mucosal immune response to a parenterally administered antigen\textsuperscript{811}. Only two lactobacilli strains (\textit{Lactobacillus reuteri} and \textit{Lactobacillus brevis}) induced expression of the proinflammatory cytokines; TNF\textsubscript{\alpha}, IL-2, and IL-1\beta. These two strains and \textit{Lactobacillus fermentum} also notably enhanced the IgG response against the parenterally administered antigen.
Chapter 9

DID PATIENTS WITH HIGH LEVEL OF COLONISATION WITH Nissle 1917 REMAIN IN REMISSION LONGER?

Introduction
The use of a non-pathogenic strain of *E.coli* (Nissle 1917, serotype O6:K5:H1) in the clinical study reported in the previous section, presented an opportunity to study the colonisation of patients with ulcerative colitis and gather information on the behaviour of the strain in vivo, during remission of colitis. Typing *E.coli* isolates using Rep-PCR allowed retrospective analysis of the faecal samples obtained to determine if patients with high level of colonisation with the therapeutic *E.coli*, remained in remission for longer than those in whom the organism could not be identified.

Methods
Patients taking part in the clinical “Mutaflor study” who had entered remission were studied. A total of 39 patients were maintained in remission on the *E.coli* strain Nissle 1917 (Mutaflor®, Ardeyparm GmbH, Herdecke, Germany) and 44 patients were given mesalazine during the remission phase of their colitis.

Blood and faecal samples
Patients were followed prospectively with up to 7 clinic visits per patient to determine the disease activity. There were a total of 457 visits at which the following measurements were recorded; activity of ulcerative colitis, presence of the therapeutic *E.coli* strain, number of dominant *E.coli* isolates, white cell count, platelet count, plasma viscosity, erythrocyte sedimentation rate and C-reactive protein level. Monthly stool samples were collected to identify the main colonic *E.coli* isolates and evaluated retrospectively. Most faecal samples were provided on the day of the hospital visit or sent by post within one week of the appointment. *E.coli* were isolated from faecal samples and typed using Rep-PCR as previously described.
**Rationale for PCR typing** of 10 *E. coli* colonies from each stool sample

To make certain that a particular strain is not present in a faecal sample, even in small numbers, every *E. coli* bacteria in the sample would have to be analysed. However, the analysis of up to $10^8$ cells per gram of faeces would be impossible. With the use of serotyping it has been shown that relatively few distinct faecal O-serotypes are present in the stool and one dominant strain can usually be identified in a greater number than others.

Vosti et.al\(^\text{812}\) found that examining 5 colonies could identify the dominant faecal O-serotype. Lidin-Janson et.al\(^\text{813}\) demonstrated that the probability of including at least one isolate of the dominant clone amongst 5 randomly selected colonies was 99.3%. Hedges et.al\(^\text{814}\) then recommended the analysis of 10 colonies to ensure the majority of dominant O-serotypes would be included. As the aim of the study was to search for the therapeutic trial strain amongst the 'majority' or 'dominant' strains present in the faeces, the isolation of 10 colonies from each faecal sample was thought to be realistic in terms of cost and workload.

**Assessment of adhesiveness**

The buccal epithelial cell adhesion assay\(^\text{768,769,692,815}\) was used to measure adhesiveness as described earlier.

**Statistical analysis**

The effect of the variables on "time to relapse" was investigated using a time-dependent model in which the factors were updated whenever new values were recorded. The following values were taken into account; white cell count (WCC), platelet count, C-reactive protein (CRP), plasma viscosity (PV) and erythrocyte sedimentation rate (ESR) and the number of different *E. coli* isolates. The number of different *E. coli* isolates was skewed and discrete and so was grouped together into three categories (1, 2 and 3 or more separate isolates). CRP was dichotomised into normal and raised levels. The ESR was positively skewed and so a log transformation was performed.

**Results**

Of the 44 patients maintained on Mesalazine, 32 (73%) relapsed compared with 26 out of 39 (67%) maintained on the Nissle 1917 strain of *E. coli*. Mean duration of remission in the mesalazine group was 206 days (median 175) and 221 days (median 185) in the *E. coli* group.
A total of 371 stool samples were collected at monthly intervals (median 38.5 days) in the two groups. In 52 faecal samples (14%), *E.coli* could not be identified. At 165 sample points (52%) a single dominant strain of *E.coli* was identified, at 89 sample points (28%) two separate isolates were found and in 65 (20%) stool samples, three or more separate isolates were identified.

Of the 39 patients randomised to receive the Nissle 1917 strain of *E.coli*, this therapeutic strain could be found amongst the dominant faecal strains in 29 patients on at least one occasion. However, none of these 29 patients consistently excreted the non-pathogenic strain.

Table I: Number of visits when Mutaflor could be identified in patients faeces and disease activity

<table>
<thead>
<tr>
<th></th>
<th>Visit 1</th>
<th>Visit 2</th>
<th>Visit 3</th>
<th>Visit 4</th>
<th>Visit 5</th>
<th>Visit 6</th>
<th>Visit 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTF seen</td>
<td>R</td>
<td>A</td>
<td>R</td>
<td>A</td>
<td>R</td>
<td>A</td>
<td>R</td>
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<tr>
<td>9</td>
<td>1</td>
<td>17</td>
<td>1</td>
<td>8</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>MTF not seen</td>
<td>17</td>
<td>-</td>
<td>14</td>
<td>4</td>
<td>12</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>No sample</td>
<td>12</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>5</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>38</td>
<td>1</td>
<td>33</td>
<td>5</td>
<td>25</td>
<td>5</td>
<td>15</td>
</tr>
</tbody>
</table>

R = ulcerative colitis in remission
A = ulcerative colitis reactivated

Table I above, outlines the proportion of faecal samples at which our trial strain of *E.coli* (Nissle 1917) could be isolated. 29 patients relapsed during the one year follow up period in the *E.coli* group. On relapse, a stool sample was obtained and *E.coli* isolated from 18 of these patients. In 6 of these 18 samples, our non-pathogenic strain of *E.coli* was isolated amongst the dominant faecal strains. Identification of the non-pathogenic trial strain of *E.coli* in the stool was not associated with a reduced risk of relapse of ulcerative colitis (risk ratio 0.977, 95% CI 0.441-2.168).

The non-pathogenic *E.coli* trial strain contains two plasmids. When analysing the plasmid profiles of the Nissle 1917 strain after isolation from faeces, a number of isolates were found with extra plasmid bands superimposed upon the normal plasmid band pattern (see photograph on the following page).
The plasmid profile gel in photograph I show the two “original” Nissle 1917 plasmids in position 1 and 2 as well as two new plasmids in position 3 and 4 present in 6 different Mutaflor isolates. The faint band between band 1 and 2 were thought to be different configurations of plasmid 2.

These extra bands probably represent instances when Nissle 1917 have gained plasmids while in the gastrointestinal tract. However, there was no difference in adhesiveness between strains with the "original" two plasmids and those with extra plasmids.

There was a trend for patients with three or more separate E. coli isolates in the first stool sample to have an increased risk of relapse compared with those in whom only 1 or 2 distinct isolates could be identified (risk ratio 1.304, 95% CI 0.998-1.704). In the group taking mesalazine, the dominant E. coli of the first faecal sample on remission was compared with those found in the stool sample on relapse. Relapse was usually associated with a change in the E. coli flora. In 12 patients all isolates were different on relapse compared with the prior remission sample, in 8 there were some new and some old isolates and 4 patients relapsed with the same flora as had been detected in remission (p=0.01, Fisher’s exact test).

The adhesiveness of all “wild” E. coli isolates cultured from the first remission sample was compared with those isolated on relapse. There was no statistically significant change in the adhesiveness of these “wild” E. coli isolates between remission and relapse. The median E. coli adhesion score was 3 in remission and 6 on relapse (p=0.14, Fisher’s exact test). Elevations in white cell count, platelet count, plasma viscosity, CRP and log(ESR+1) were all independently significant predictors of relapse within one month.
Even at the first visit in remission, a higher level of ESR was associated with an increased risk of subsequent relapse ($p=0.03$, 95% CI 1.06 - 1.95).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Risk Ratio</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>White cell count</td>
<td>1.012</td>
<td>1.003 to 1.022</td>
</tr>
<tr>
<td>Platelet count</td>
<td>1.004</td>
<td>1.001 to 1.007</td>
</tr>
<tr>
<td>Plasma viscosity</td>
<td>1.044</td>
<td>1.018 to 1.071</td>
</tr>
<tr>
<td>log(ESR + 1)</td>
<td>1.687</td>
<td>1.232 to 2.310</td>
</tr>
<tr>
<td>CRP (grouped into normal or elevated levels)</td>
<td>2.695</td>
<td>1.593 to 4.559</td>
</tr>
</tbody>
</table>

CRP and Log(ESR+1) were the best predictors of relapse and little was gained by the white cell count, plasma viscosity or platelet count (table II).

Discussion

The factors governing the fate of ingested coliforms are not known. Some workers have found the coliform flora to be relatively stable$^8$. Others have shown that a change in environment is associated with a change in the faecal coliform flora$^{8,16}$. Some strains appear to establish themselves in the bowel more readily than others do. There are also differences in the fate of single strains in different subjects suggesting that both the nature of the organism and the characteristics of the hosts are involved$^{8,19}$.

On tracking our non-pathogenic strain of *E.coli* we found the relapse rates to be similar in patients in whom the therapeutic strain could be identified amongst the dominant faecal flora and in those who the strain could not be found at all. This was a surprising as our clinical study had demonstrated a similar remission rate in both arms of our study. One may have predicted that Nissle 1917 to be more prevalent in faecal samples from patients with the longest remissions.

*Possible reasons for not finding a difference in the carriage of Nissle 1917 amongst patients relapsing early and those relapsing late*

In our study we chose to isolate *E.coli* from the faeces of patients with ulcerative colitis. However, our non-pathogenic strain of *E.coli* may have adhered or formed biofilms on the colonic mucosa and the *E.coli* isolated from faeces may not reflect those found in close proximity to the colonic epithelium.
By staining histological sections of colonic mucosa with a monoclonal antibody to *E. coli*, the organism was found in close proximity to the surface epithelium or ulcers in 35% of patients with Crohn's disease and in 26% of patients with ulcerative colitis but in none of 11 normal controls820.

The reason for the increased adhesiveness of *E. coli* of patients with inflammatory bowel disease may be to adhere more closely to the colonic epithelium. On the other hand, Burke compared the serotype and plasmid profile of 10 *E. coli* colonies isolated from 8 patients with ulcerative colitis and found that the *E. coli* detected in the faeces were also represented in the mucosal associated flora821. In our study, patients were assessed by rigid sigmoidoscopy on three occasions as part of our research protocol; a) on entry into the study to confirm active colitis, b) to confirm remission of colitis and c) to confirm reactivation of disease. In addition to these examinations, further regular flexible sigmoidoscopies would have been required to track the mucosal associated *E. coli* strains. This would have been difficult to justify both to patients and to our endoscopy department.

Another possible reason is that patients in whom our *E. coli* trial strain could not be identified were complying poorly with their trial medication. However, the compliance with medication, as calculated by counting the returned tablets, was no different in those that the therapeutic organism could be identified compared with those it could not be found (80% vs 81%).

Patients were advised to produce a faecal sample on the day they attended their outpatient appointment. In two thirds of cases, faecal samples were brought to the outpatient clinic. The remaining patients were asked to send the faecal samples by first class post on the first or second day of the week to minimise the duration of transit. If no viable bacteria could be isolated, the patient was contacted by telephone and a further sample requested. In this way, we attempted to minimise the potential bias of faecal samples being analysed after variable time intervals. Naturally, it was impossible to be certain that samples brought with the patient to hospital had actually been produced the same morning. The possible bias therefore remain that our non-pathogenic strain of *E. coli* was more sensitive to "long transfer times" than "wild" strains of *E. coli* and that this may account for the intermittent appearance of the Nissle 1917 strain in patients faecal samples.
A final possible reason is that insufficient numbers of Nissle 1917 were given to consistently detect the strain in faeces. Patients were given a total daily dose of $10 \times 10^{10}$ viable bacteria. Previous dose ranging studies by the pharmaceutical company had indicated that this is the maximum dose of Nissle 1917 which is likely to be tolerated without developing abdominal discomfort. In the \textit{E.coli} arm of the study, 8 patients withdrew because of abdominal discomfort compared with 6 from the Mesalazine arm. It is likely that the use of a higher dose of Nissle 1917 would have resulted in an increased withdrawal rate from the \textit{E.coli} arm of our study.

\textbf{\textit{E.coli} turnover and ulcerative colitis}

Gorbach et al\textsuperscript{822} reported that patients with severe colitis have increased numbers of faecal coliforms. In contrast Hartley et al\textsuperscript{666}, isolated \textit{E.coli} from mucosal biopsies in patients with quiescent colitis and found them less frequently and in lower numbers than patients with active disease. Although we found no evidence that changes in the adhesiveness of the \textit{E.coli} were associated with imminent relapse in the disease, patients in whom three or more different \textit{E.coli} isolates could be found, were more likely to suffer a relapse within the next year compared to those in whom only one or two different dominant strains were found (risk ratio 1.304, 95% CI 0.998-1.704).

This finding suggests that an increased turnover of the colonic \textit{E.coli} flora precedes rather than follows relapse. Our finding that most patients relapse with a different faecal coliform flora is consistent with this conclusion.
SUMMARY AND CONCLUSIONS

Our understanding of inflammatory bowel disease has been hampered by the absence of pathognomonic features, uncertain therapeutic end points and the lack of genetic or serological markers. In addition, the understanding of the enteric flora in colitis has been hindered by a lack of representative animal models and difficulty in tracking enteric organisms isolated from biopsies or faecal samples.

In spite of this, a wealth of circumstantial evidence has linked inflammatory bowel disease with the intestinal flora. Other researchers have proposed that inflammatory bowel disease is a rouge, self-perpetuating immune response caused by genetic factors. Until the discovery of the NOD2 gene in Crohn's disease, these opposing views were difficult to reconcile. Genes similar to NOD2 has been found in plants were they confer resistance to infection. NOD2 therefore provides a possible link between a genetic predisposition and the intestinal flora.

Yet, if inflammatory bowel disease were the reaction to a normal commensal intestinal flora, one would expect most broad-spectrum antibiotics to have a beneficial effect. In contrast, although many antibiotic regimens have been tried, partial successes have only been reported in Crohn's disease. The poor results with antibiotics suggest that ulcerative colitis is caused either by a specific pathogen or by a defined sub-group of the intestinal flora.

The *E. coli* flora in ulcerative colitis is unusual in several aspects. It is more adhesive, more able to degrade mucin and more likely to produce haemolysins than the corresponding flora in healthy controls. In addition, reducing the coliform flora with the use of tobramycin leads to a short-term improvement of the disease.

Earlier studies have showed that patients with ulcerative colitis do not harbour a single adhesive strain of *E. coli*. A plasmid, able to transfer pathogenic characteristics between different strains may explain why a single *E. coli* serotype has not been implicated. Lobo et al analysed the plasmid complement of *E. coli* isolates from patients with ulcerative colitis and found that *E. coli* harbouring a plasmid of approximately 98MDa size were more adhesive. Although, Lobo et al had analysed a large number of *E.coli* isolates, these had been isolated from relatively few patients without any means of detecting multiple samples of the same isolates.
Our REP-PCR assay allowed large numbers of wild *E. coli* strains to be followed over time. With this assay we were able to search for the large plasmid from more than 100 different *E. coli* isolates. A plasmid in the 98 MDa range was found to be equally prevalent in patients and controls. Furthermore, *E. coli* isolates harbouring the large plasmid were no more likely to be adhesive than isolates without the plasmid.

The one-year follow up study of the “permanent” flora in patients with quiescent colitis confirmed that the adhesive property is a stable characteristic. The adhesiveness was not related to the extent of colonic inflammation as strains isolated from extensive, left-sided colitis and proctitis had a similar degree of adhesiveness.

In the “Mutaflor study”, we were hoping to induce a longer period of remission by substituting the patient’s native *E. coli* flora with a non-pathogenic strain. Patients receiving *E. coli* fared no worse than patients receiving standard maintenance therapy. Paradoxically, on tracking our non-pathogenic *E. coli* we found the relapse rates to be similar in patients in whom our therapeutic strain was frequently isolated and in those who the strain could not be found at all. There was no evidence that changes in adhesiveness was associated with imminent relapse in the disease. Instead, the risk of relapse appeared to be highest in patients with the highest turnover of their *E. coli*.

Our studies have confirmed that the adhesiveness of the coliform flora is stable over time and that attempting to modify the *E. coli* flora may have a beneficial impact on the disease process. A better understanding of this relationship will require insight into the mechanisms behind fluctuations in *E. coli* flora and further investigation of the mucosa-associated *E. coli* flora of patients and healthy controls.
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