The Development and Use of a New Animal Model of Inflammatory Bowel Disease

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Inflammatory bowel disease (IBD) is of unknown aetiology. A clinically relevant animal model of IBD is essential for our understanding of intestinal inflammation and for evaluating drug efficacy and mode of action.

A new animal model for IBD was induced in rabbits by a single intracolonic instillation of trinitrobenzene sulphonic acid (TNB) in a 25 per cent ethanol solution. This produced a dose-dependent inflammation and ulceration that persisted for up to six weeks. A detailed histopathological study showed that the model had many features of IBD.

Inappropriate activity of the matrix metalloproteinase enzymes is implicated in tissue destruction; in IBD there is consistent expression of the matrix metalloproteinases, which are not present in normal colon. The temporal and spatial distribution of the matrix metalloproteinases - collagenase, stromelysin, and gelatinase - and their inhibitor, tissue inhibitor of metalloproteinase, were investigated in the model by means of immunolocalisation. The findings suggest that it is the differential expression of these enzymes that is significant in ulcer formation and fibrosis in IBD.

The effect of methylprednisolone on the pathogenesis of the model was examined using: immunoassay of arachidonic acid metabolites, computer-aided image analysis of the macroscopic appearance, and histological assessment. Methylprednisolone did not modify the macroscopic damage or influence arachidonic acid metabolism, but did reduce neutrophil infiltration. These results suggest that neutrophils are not responsible for the damage observed in this model.

Experiments to investigate the response of the model to a second challenge with TNB suggest that a type IV hypersensitivity reaction is not the underlying immunological mechanism.

The new model should prove a valuable tool for further studies in the pathophysiology and treatment of inflammatory bowel disease.
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# Abbreviations

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<tr>
<td>AMP</td>
<td>Adenosine 5’-monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>B</td>
<td>Binding</td>
</tr>
<tr>
<td>B0</td>
<td>Maximum binding</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guerin</td>
</tr>
<tr>
<td>C</td>
<td>Centigrade</td>
</tr>
<tr>
<td>C(3-5)a</td>
<td>Complement-derived anaphylatoxins</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge-coupled device</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster differentiation number</td>
</tr>
<tr>
<td>CL</td>
<td>Collagenase</td>
</tr>
<tr>
<td>cm</td>
<td>Centimetre</td>
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<tr>
<td>CNP</td>
<td>A mixture of 1,1,1-trichloroethane and tetrachloroethane</td>
</tr>
<tr>
<td>DNCB</td>
<td>2,4-dinitrochlorobenzene</td>
</tr>
<tr>
<td>dpm</td>
<td>Disintegrations per minute</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELAM</td>
<td>Endothelial leukocyte adhesion molecule</td>
</tr>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EU</td>
<td>Enzyme units</td>
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<td>F(ab)</td>
<td>Monovalent antibody fragment</td>
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<td>Fibroblast</td>
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<tr>
<td>g</td>
<td>Gram</td>
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<td>γ-aminobutyric acid</td>
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<td>HETE</td>
<td>Hydroxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>HLA</td>
<td>Human major histocompatibility complex</td>
</tr>
<tr>
<td>HPETE</td>
<td>Hydroperoxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>HRE</td>
<td>Hormone response element</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
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</table>
5-HT ....................... 5-hydroxytryptamine
IBD .......................... Inflammatory bowel disease
ICAM ......................... Intercellular adhesion molecule
IFN ............................ Interferon
Ig .............................. Immunoglobulin
im ............................. Intramuscular
ip ............................... Intraperitoneal
Il ............................... Interleukin
kDa ......................... Kilodalton
l ............................... Litre
LIMP .......................... Large inhibitor of metalloproteinase
LP ............................. Lamina propria
LPS ............................ Lipopolysaccharide
LT .............................. Leukotriene
M ............................... Molar
MΦ ............................. Macrophage
mM ............................. Millimolar
MPO ........................... Myeloperoxidase
mRNA ........................ Messenger ribonucleic acid
n ....................... Number in group
NADP+ ......................... Nicotinamide adenine dinucleotide phosphate (oxidised)
NADPH ......................... Nicotinamide adenine dinucleotide phosphate (reduced)
ng ............................ Nanogram
NS ............................. Non-specific
NSAID ........................ Non-steroidal anti-inflammatory drug
NSS ............................ Normal sheep serum
P ............................... Probability
PAF ............................. Platelet activating factor
PBS ............................ Phosphate buffered saline
PG ............................. Prostaglandin
pg ............................ Picogram
pH ............................. Hydrogen ion concentration (log)
PLA_2 ........................ Phospholipase A_2
PMN .......................... Polymorphonuclear neutrophil
RIA ............................. Radioimmunoassay
RNA ........................... Ribonucleic acid
ROM ............................ Reactive oxygen metabolites
rpm ............................ Revolutions per minute
sa ............................. Rabbits that received saline alone
sd ......................... Standard deviation
se .......................... Standard error
SM .......................... Submucosa
t₀ .......................... Time zero
TA .......................... Total activity
Tₜ .......................... Helper T-cells
TIMP ....................... Tissue inhibitor of metalloproteinase
TNB ........................ Trinitrobenzene sulphonic acid
tnb ........................ Rabbits that received trinitrobenzene sulphonic acid
TNF ......................... Tumour necrosis factor
Tx .......................... Thromboxane
UC .......................... Ulcerative colitis
w/v ........................ Weight/volume ratio
µl .......................... Microlitre
INTRODUCTION

Inflammatory bowel disease, namely Crohn's disease and ulcerative colitis, has an unknown aetiology, and is characterised by periods of relapse and remission. It is associated with high morbidity and prolonged non-specific medical treatment, and often requires surgical intervention in intractable cases. In addition to the human misery caused by these conditions, their management is costly (Hay & Hay, 1992) (Chapter 2).

A major limitation in understanding the pathogenic mechanisms responsible for inflammatory bowel disease has been the lack of an animal model that exhibits chronic and relapsing inflammation similar to that observed in the human disease. A model is necessary to allow more detailed study than can be achieved with human biopsy material, and has the advantage that different stages of disease activity can be investigated. While several naturally-occurring and experimentally-produced models of small intestinal or colonic inflammation have been described, most of these models have proved unsuitable for use as research tools, because they have exhibited only acute intestinal inflammation, required complicated or lengthy protocols, or are expensive (Chapter 5).

Perhaps the best model of IBD to date has been produced in the colon of the rat by the intrarectal administration of a hapten, trinitrobenzene sulphonic acid (TNB), in a barrier breaker, ethanol (Morris et al., 1989). This model overcomes many of the problems of previous ones: it does not require complex manipulations to produce the disease, it shares many of the histological and clinical features of inflammatory bowel disease, and it is reproducible. The model is based on the hypothesis that inflammatory bowel disease arises from an inability of the reticuloendothelial system to clear luminal antigens that have entered the mucosa.

There is, however, good justification for developing a rabbit model in addition to the rat model. For example, the response to inflammatory mediators displays more similarities when comparing rabbit and human colons than when comparing rat and human colons; moreover, these responses are mediated by similar mechanisms (Percy et al., 1990). The rabbit also has the advantage of a larger colon than that of the rat, allowing it to be inspected endoscopically for inflammatory changes. Furthermore, some potentially important antibodies for immunohistochemistry are only available to the rabbit and not to the rat. In this thesis, a new rabbit model of inflammatory bowel disease is proposed.
The experiments presented herein examine by means of macroscopic and microscopic assessments the dose response and time course of a new chronic colitis induced by TNB in the rabbit.

Connective-tissue degradation and subsequent remodelling is a significant feature of inflammatory bowel disease (Bailey, 1992). Connective-tissue degradation, both during normal-tissue remodelling and in pathological conditions, is achieved mainly by the action of the matrix metalloproteinases. (The structure and function of connective tissue and the biochemistry of the matrix metalloproteinases are considered in Chapter 4.)

Previous experiments have demonstrated the ability of colonic explants from patients with inflammatory bowel disease to produce matrix metalloproteinase enzymes \textit{in vitro} (Horowitz \textit{et al.}, 1987; Sturzaker & Hawley, 1975). However, such studies do no more than indicate the capacity of the cells and tissues examined to synthesise these enzymes under the experimental conditions used.

Specific antisera to rabbit matrix metalloproteinases and their inhibitor, tissue inhibitor of metalloproteinases (TIMP), are available (Drs J J Reynolds and R M Hembry, Strangeways Research Laboratory, Cambridge, UK). Immunolocalisation studies, using these antisera, have the potential to determine the distribution of the matrix metalloproteinases and TIMP in different parts of the bowel wall both on the matrix and within cells. Such studies allow examination of the potential \textit{in vivo} role of these enzymes. Thus, an investigation of the temporal and spatial distribution of the matrix metalloproteinases and TIMP in the new model was undertaken (Chapter 6).

Increased concentrations of many inflammatory mediators and abnormal inflammatory cell phenotypes have been demonstrated in tissue from patients with inflammatory bowel disease, although whether these are primary or secondary occurrences is unclear (Chapter 3). Therapy for inflammatory bowel disease employs a variety of anti-inflammatory agents, all of which appear to derive at least a part of their effectiveness from their ability to limit the synthesis of eicosanoid products and to inhibit neutrophil infiltration (Gaginella & Walsh, 1992). It has been suggested that the response of a clinically-relevant animal model of IBD to current therapeutic agents should be similar to that of the human disease (Kim & Berstad, 1992). The use of high-dose glucocorticosteroids remains an important therapeutic approach for patients with ulcerative colitis or Crohn's disease (Rosenberg \textit{et al.}, 1990). Hence, studies concerned with determining whether the TNB-induced rabbit model of colitis would respond to glucocorticosteroid therapy were designed and carried out (Chapter 7). The investigations included: occult blood detection, erythrocyte sedimentation rate measurements, computer-aided image analysis of the macroscopic appearance,
histological assessment, and assay of myeloperoxidase activity and of stimulated \textit{ex-vivo} release of leukotrienes and prostaglandins.

The mechanism whereby TNB induces colitis in experimental animals is not known. Inflammation and injury induced by TNB have been attributed to both its direct cytotoxic properties and to an as-yet-undefined immune-mediated mechanism (Yamada \textit{et al.}, 1992). Morris and co-workers have suggested that rats may become immunologically sensitised to the damaging effects of TNB (Morris \textit{et al.}, 1989), following their observation that re-exposure to TNB, several weeks after an initial challenge, produced an enhanced response. To investigate whether a type IV hypersensitivity reaction is the underlying immunological mechanism in the new model, experiments were performed that involved the re-initiation of colitis with a second dose of TNB six weeks after the initial dose (\textit{Chapter 8}).

In summary, the hypotheses explored in this thesis are as follows:

\begin{enumerate}
\item \textit{Trinitrobenzene sulphonic acid induces a chronic inflammatory colitis in the rabbit colon that resembles human inflammatory bowel disease.}

\item \textit{The matrix metalloproteinases and their inhibitor TIMP play a role in the pathogenesis of chronic colitis.}

\item \textit{The administration of a glucocorticosteroid to the new model decreases the severity of the disease.}

\item \textit{A type IV hypersensitivity reaction underlies trinitrobenzene sulphonic acid-induced colitis.}
\end{enumerate}
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INFLAMMATORY BOWEL DISEASE

2.1 Introduction

Non-specific inflammation of the colon, regarded by the physicians of the last century as a single disease entity, has now been separated into sub-groups, such as ulcerative colitis and Crohn’s disease of the colon, ischaemic colitis and the iatrogenic antibiotic-associated colitis. The term ‘inflammatory bowel disease’ usually denotes Crohn’s disease and ulcerative colitis, which are both chronic inflammatory conditions of unknown aetiology and have many features in common.

In recent years the incidence of inflammatory bowel diseases has risen dramatically, although some epidemiological studies suggest that the incidence of ulcerative colitis has reached a plateau (Srivastava et al., 1992). In the western world the annual incidence of ulcerative colitis ranges from 4 to 13 cases per 10^5 persons with a prevalence of 50 to 80 per 10^5 (Munkholm et al., 1992; Tysk & Jarnerot, 1992b). The incidence of Crohn’s disease is increasing: for example, in Wales the 1930s incidence level of 0.1 per 10^5 had increased to 8.3 per 10^5 in the 1980s (Rose et al., 1988). Ulcerative colitis and Crohn’s disease primarily affect young adults (ages 20 to 40), but they may present at any age from within the first year of life to the 80s. Many studies show a secondary peak of incidence of inflammatory bowel disease in elderly people (Calkins & Mendeloff, 1986). Ulcerative colitis may be more common in women than in men, although many of the more recent studies have failed to find a sex difference. Crohn’s disease, regardless of geographical location, shows relatively equal incidence in both sexes.

To date, there is still no curative medical treatment, let alone a preventative measure, for either Crohn’s disease or ulcerative colitis. Inflammatory bowel disease is associated with high morbidity, prolonged medical treatment, and often surgical intervention in intractable cases. In addition to the human misery caused by these diseases their management is costly. It has been estimated that the cost of inflammatory bowel disease to the US health care system in 1990 was between $1.8 billion and $2.6 billion (Hay & Hay, 1992). It is clear that research pursuing increased understanding of the nature of inflammatory bowel disease is essential.
2.2 Recognition of inflammatory bowel disease

The first description (c A.D. 300) of inflammatory bowel disease may have come from Aretaeus of Cappadocia (Aretaeus, 1856), however, as Clifford Hawkins points out in his historical review of inflammatory bowel disease (Hawkins, 1990) this may represent the 'bare bones of the past clothed with a writer's imagination'. Before the middle of the nineteenth century, abdominal operations were not carried out, and detailed pathological studies of diseased tissues were uncommon. Although there were rapid advances in surgical, histological and bacteriological techniques in the latter half of the nineteenth century, a better understanding of intra-peritoneal inflammation was hindered by the prevalence of abdominal tuberculosis. Intestinal lesions that were neither of a neoplastic nor of an acute inflammatory nature were commonly considered tuberculous.

2.2.3 Ulcerative colitis

Credit for the recognition of ulcerative colitis as a distinct disease entity has traditionally be given to Samuel Wilks. In 1859, he wrote to the Medical Times and Gazette describing the post-mortem examination that he had performed on a young woman, who had died during early pregnancy (Wilks, 1859b). In the report he described an idiopathic acute colitis, but his final opinion was that the woman had died as a result of a 'vegetable irritant' given to cause abortion. His conclusion almost resulted in the execution of a Dr Smethurst for what was most likely to have been an acute case of ulcerative colitis. Further cases of 'idiopathic dysentery' were reported by Wilks, and it was pointed out that although the disease was rare, mild cases that did not come to post-mortem might have been overlooked or misdiagnosed (Wilks, 1859a). In 1888, Sir William Hale-White also described cases of colonic ulceration with no obvious cause (Hale-White, 1888). Perhaps the first complete account of ulcerative colitis as a disease distinct from bacillary dysentry was given by Sir Arthur Hurst (Hurst, 1935), who also described the sigmoidoscopic appearances. However, Hurst considered the disease to be primarily an infective dysentry influenced by other factors, which led to a chronic disease process. He based this view on the identical sigmoidoscopic features of ulcerative colitis and dysentery, the isolation of dysenteric organisms from the faeces of some patients with ulcerative colitis, and the apparent effectiveness of polyvalent anti-dysenteric sera in the treatment of ulcerative colitis.

2.2.2 Crohn's Disease

Crohn's disease, an eponym for idiopathic regional enteritis, established itself in the medical literature as a separate disease entity following the classic report of Crohn,
Ginzburg and Oppenheimer in 1932. However, it may have been Giovanni B. Morgagni who documented the earliest case of Crohn's disease. In his great work ‘De Sedibus et Causis Morborum’, where he was the first to attach symptoms in life to the anatomical appearances in the organs after death, he describes a young man who had a clinical history very suggestive of Crohn's disease with perforation (Morgagni, 1761). The young man had been suffering from recurrent attacks of diarrhoea for most of his life, and at the age of 20 years was getting severe abdominal pain and rectal bleeding. His disease seems to have run a fluctuating course until he suddenly became severely ill and died. At autopsy, the terminal ileum and adjacent large bowel were diseased, with inflammation and ulceration of the mucosal surface, and there was marked mesenteric lymphadenopathy. Perforation had caused a fatal peritonitis. Thirty-seven years later Combe and Saunders described another case of a young man who 'had been for many years troubled with flatulence and complaints in the bowels' (Combe & Saunders, 1813). He then started to get pain after eating and eventually suffered from intestinal obstruction. He died in an emaciated state. At autopsy there was marked thickening and stenosis of the distal ileum, with three 'skip' areas in the colon. Combe and Saunders postulated that the loss of the absorptive function from the lower ileum caused his marked emaciation. There was, of course, no histological evidence to support the clinical and autopsy diagnosis of Crohn's disease.

At the beginning of this century, Lartigau, in a study of cases thought to be chronic hyperplastic tuberculosis of the intestine, noted that many of the tubercles were only aggregations of lymphoid cells, with one or more giant cells present (Lartigau, 1901). There was little tendency to necrotic change and the histological features of tubercle bacilli were often absent. Lartigau realised that there might be other types of granulomas affecting the intestine besides tuberculosis. In 1903, Koch described two young females with granulomatous lesions in the ileo-caecal region where tests for tuberculosis proved negative (Koch, 1903).

The clearest description prior to 1932 of both the clinical and pathological features of Crohn's disease was given by a surgeon, T. Kennedy Dalziel (Dalziel, 1913). Dalziel had operated on a doctor who, after having had diarrhoea for some time, had then developed subacute obstruction. At laparotomy there was widespread thickening of the small and large intestines and resection was not possible. During the following years Dalziel encountered seven more patients with similar chronic inflammation of the intestine that was not tuberculous. These patient complained of weight loss and passing some blood and mucus per rectum, but there was little elevation of temperature. The pathologist recognised the transmural nature of the inflammation, a characteristic feature of Crohn's disease. Johnne's disease caused by mycobacterium paratuberculosis in cattle had been
Figure 2.1: The first pages from Giovanni B. Morgagni's book “De sedibu et Causis Morborum”, in which he was the first to attach symptoms in life to the anatomical appearance of the organs after death. Among the cases reported, he described a young man who had a history very suggestive of Crohn’s disease.
recorded in England six years earlier (McFadyean, 1907), and Dalziel addressed the possible aetiological relationship that might exist between Johnne's disease and the chronic interstitial enteritis from which his patients suffered. However, no acid-fast bacilli in the human specimens were identified. Following Dalziel's report, nearly all further relevant publications came from German or American hospitals. In 1920, Tietze produced a review of all the non-tuberculous intestinal granulomas that had been recorded in the world's literature (Tietze, 1920). There is little doubt that some of the cases would today be classified as Crohn's disease, but Tietze's review did not define a clinical entity. However, it must have strengthened the belief that there could be diseases, other than the prevalent abdominal tuberculosis, that could cause thickening and narrowing of the intestine.

Crohn in 1930 persuaded Berg at Mount Sinai Hospital to operate on a 17-year-old boy (Crohn, 1967). The patient had suffered from diarrhoea, abdominal pain and a mass, and had intermittent fever. Chest X-ray and the skin tests for tuberculosis were negative. Resection of ileum was successful. During the next two years Berg and Crohn collected thirteen similar cases involving the terminal ileum; many had fistulas from earlier operations. However, when the work was published Berg declined to have his name in the list of authors and instead suggested that Ginzburg and Oppenheimer, who were also investigating intestinal granulomas, should join Crohn as co-authors (Crohn et al., 1932). They described both the clinical and the pathological details of the disease of the terminal ileum much more fully than any previous workers had done. While admitting that the aetiology of the process was unknown, they realised that the disease did not belong to any of the then recognised granulomatous or accepted inflammatory groups.

There was a strange reluctance for many years to accept that Crohn's disease could affect the colon. A right-sided localised form of colitis had been recorded by Bargen and Weber in 1930 (Bargen & Weber, 1930). Eight years later Crohn and Berg described similar cases (Crohn & Berg, 1938), but until the early 1950s gastroenterologists and surgeons tended to regard Crohn's disease as being exclusively a small intestinal granulomatous condition. In 1952 Wells clearly distinguished between ulcerative colitis and what he termed 'segmental colitis', which he believed was a variant of Crohn's disease (Wells, 1952). It was characterised by thickening and fibrosis of the colon with a tendency to 'skip'. In spite of this clear description, another eight years were to pass before Lockhart-Mummery and Morson (1960) finally brought general recognition of the colonic involvement (Lockhart-Mummery & Morson, 1960).
It was Crohn and his colleagues, Ginzburg and Oppenheimer, who were responsible for establishing idiopathic regional enteritis or Crohn’s disease (as it later became known) as a separate disease entity in 1932.
2.3 Pathology

2.3.1 General

The features of ulcerative colitis and Crohn’s disease reported in the early descriptions and the spectrum of clinical manifestations have remained largely stable. In general terms, the clinical, macroscopic and histological features usually permit a confident diagnostic distinction between ulcerative colitis and Crohn’s disease.

2.3.2 Ulcerative colitis

The principal symptoms of ulcerative colitis include diarrhoea, rectal bleeding, the passage of mucus, and abdominal pain. However, it should be recognised that a small number of patients with ulcerative colitis may be constipated, even when the disease is active. The severity of the clinical illness is generally proportional to the extent of bowel involvement and the intensity of inflammation. Although extensive disease, particularly pancolitis, is usually associated with severe inflammation, this association varies widely from patient to patient. It has been suggested that patients whose disease begins late in life have more extensive and more refractory disease than those affected by the disease earlier in life. Disease of moderate or severe activity may be associated with extra-intestinal manifestations; these include: iritis, erythema nodosum, pyoderma gangrenosum, sclerosing cholangitis and liver disease. The most important long-term complication of ulcerative colitis is the development of carcinoma of the colon. The risk is greatest in patients who have had total colitis for 10 years or more (Lennard et al., 1990).

In rare cases of ulcerative colitis the rectum may be spared; however, in most instances the inflammation extends in a continuous fashion from the rectum with variable proximal extension. Generally, it appears that in about 50 per cent of patients the disease is limited to the rectum and rectosigmoid, while the patients have disease that extends beyond the sigmoid (Haug et al., 1988; Morris & Rhodes, 1984). In mild inflammation the mucosa is hyperemic, oedematous and granular. In more severe disease mucosal granularity is replaced by broad areas of superficial ulceration covered with a mucopurulent exudate. Acute dilatation may eventually develop, with the bowel becoming thin, friable, and susceptible to perforation. In longstanding disease pseudopolyps (inflammatory polyps) may be present in the colon surrounded by areas of ulceration. In remission, the mucosa may appear normal, but in those patients that have frequent recurrence the mucosa becomes atrophied. In contrast to Crohn’s disease fibrosis is uncommon, although smooth strictures may be present in some patients with chronic disease.
Chapter 2

Ulcerative colitis displays a continuous distribution of disease contrasting with the patchy involvement seen in Crohn’s disease, when normal mucosa may be interspaced between abnormal areas. The inflammatory process in ulcerative colitis is usually confined to the mucosa and superficial submucosa of the large bowel. The histopathological features, although non-specific, are notable for the formation of crypt abscesses and the depletion of mucin from goblet cells. Normal crypt architecture is also distorted in active disease; branching and bifurcation are common and crypt atrophy is a characteristic of ulcerative colitis (Talbot & Price, 1987). Usually the lamina propria is densely infiltrated by neutrophils and lymphocytes as well as other acute and chronic inflammatory cells. However, the absence of inflammation in the deeper layers of the bowel is characteristic. As the intensity of the inflammatory infiltrate increases, extensive superficial mucosal ulceration develops. The histopathological features of ulcerative colitis can account for the bloody diarrhoea that is the cardinal symptom of the disease.

Recurrence of active disease after the presenting illness has been controlled is a characteristic feature; however, the frequency and severity of the relapses are highly variable. In a study conducted in 1985 of the clinical course of 783 patients with ulcerative colitis diagnosed between 1960 and 1978, 50 per cent of the patients were free of symptoms at any one time (Hendriksen et al., 1985). Almost all patients (97 per cent) had at least one relapse during a 10-year period.

2.3.3 Crohn’s Disease

Crohn’s disease is much more variable in its clinical manifestations than ulcerative colitis, reflecting a seemingly more complex inflammatory process. The propensity for involvement of the distal small intestine and full-thickness inflammation and scarring of the bowel often lead to an illness characterised by abdominal pain and diarrhoea. Several mechanisms may contribute to the diarrhoea observed in Crohn’s disease patients, including partial bowel obstruction, mucosal destruction and malabsorption, malabsorption of bile salts, bacterial overgrowth, rapid transit as a result of fistulas, and the secretory effects of inflammatory mediators such as the prostaglandins. Symptoms of obstruction are common and result from inflammation and oedema or from strictures due to fibrosis of the intestine wall. Obstruction occurs most frequently in the distal ileum. Systemic signs, including fever and weight loss, may figure prominently in the clinical illness and are somewhat more common than in ulcerative colitis. Nutritional deficiencies related to the specific segments of the gastrointestinal tract affected also contribute to the clinical picture. Anaemia may result from iron, folate, or vitamin B₁₂ deficiencies. A lactase deficiency in this disease is commonly associated with involvement of the small intestine. Often growth retardation and delayed maturation may be the only manifestations of the illness in children.
Although involvement of the terminal ileum, colon, or both is most common, any site within the alimentary canal can be affected by this segmental inflammatory process. Despite the substantial and steady increase in the prevalence of Crohn's disease, the relative distribution of affected sites has not changed dramatically. In approximately 30 per cent of patients only the large bowel is affected, and in an additional 30 per cent the large bowel and the small intestine are affected. Nearly 40 per cent have disease restricted to the small intestine (Farmer et al., 1975). In addition, the inflammatory process can extend beyond the gastrointestinal tract with the formation of fistulas. Initially ulceration may occur as small aphthoid-like ulcers overlying dense areas of lymphoid tissue. However, in contrast to the superficial ulceration seen in ulcerative colitis, deep linear ulcers are present in advanced disease. Extensive linear ulceration with relative preservation of intervening tissue give the mucosa a typical 'cobblestone' appearance. Although Crohn's disease confined to the large intestine may resemble ulcerative colitis or other forms of colitis, the involvement is typically segmental and frequently spares the rectum. Except for small rectovaginal fistulas which are sometimes seen in ulcerative colitis, major perianal lesions and fistulation into surrounding tissues and organs in inflammatory bowel disease are exclusively a feature of Crohn's disease.

Active disease is characterised by an infiltrate in which macrophages and lymphocytes predominate. Although the infiltrate present during acute and chronic inflammation in the mucosa can resemble that found in ulcerative colitis, extension of the infiltrate into the deeper layers of the bowel wall is a distinguishing feature. The aggregation of macrophages leads to the development of the important diagnostic non-caseating granulomas in biopsies of 25 to 30 per cent of cases, and in surgical specimens of colon in as many as 50 to 75 per cent of cases (Talbot & Price, 1987). Crohn and his colleagues on histological examination of many of their specimens had seen granulomas composed of epithelioid cells and sometimes giant cells, but they did not regard them as an essential feature. At present, the transmural nature of the inflammation is recognised as being as important as the type of infiltrating cells. Previously, some pathologists would not diagnose Crohn's disease unless granulomas were present. Another striking feature of Crohn's disease is collagen deposition contributing to the formation of strictures.

Crohn's disease is also a chronic recurring illness, although approximately 45 per cent of patients are free of symptoms at any one time. As with ulcerative colitis nearly all patients have a recurrence within 10 years of their initial episode of Crohn's disease (Binder et al., 1985).
Figure 2.3: Diseased ileum removed from a patient with Crohn’s disease. The ileum has been opened longitudinally to reveal a region of ulcerated mucosa. The wall of the ileum is thickened, forming a stricture (from the excessive deposition of collagen). At either end of the specimen the mucosa appears relatively normal, reflecting the focal nature of Crohn’s disease.
2.4 **Aetiology**

2.4.1 **General**

Despite the rising incidence of inflammatory bowel disease world-wide, the aetiology remains obscure. The search for a cause has encompassed epidemiological studies into possible genetic and environmental factors, microbiological investigation, and the search for defects in mucosal function and the immunological response. Increasingly, a multifactorial cause appears to be most likely. Some of the most frequently proposed hypotheses will be discussed.

2.4.2 **Epidemiological data**

2.4.2.1 **Geographical distribution**

While ulcerative colitis and Crohn's disease are a world-wide phenomenon, the precise incidence varies. Difficulties of diagnosis in regions where infective colitis, especially that arising from abdominal tuberculosis, is common may account for some of the variation in incidence between the developing and developed world (Mayberry & Mann, 1989). Better diagnostic facilities and increasing medical awareness have allowed the recognition of the diseases in most countries. However, high-incidence areas include the United Kingdom, the United States, and Northern Europe (Kildebo *et al.*, 1989; Srivastava *et al.*, 1992; Stowe *et al.*, 1990; Tysk & Jarnerot, 1992b). Low incidence areas include Southern and Eastern Europe, Asia, and Japan (Higashi *et al.*, 1988; Ruiz, 1989; Vucelic *et al.*, 1991a; Vucelic *et al.*, 1991b). Although it is difficult to obtain accurate figures, the incidence rates in the latter areas are probably about ten-fold less than in the high-incidence areas. Interestingly, a recent study found that geographic variations of Crohn's disease and ulcerative colitis were significantly correlated, suggesting that similar factors may be shaping the distribution of both diseases (Sonnenberg, 1990a).

2.4.2.2 **Variation between ethnic groups**

However, even within a high-incidence area considerable variation exists between ethnic groups. Epidemiologic studies have examined those genetically disparate groups that are exposed to similar environments, thereby evaluating the role of genetic factors in the pathogenesis of inflammatory bowel disease. In the United States, Jews are more prone to ulcerative colitis than are non-Jews: the incidence of ulcerative colitis in Jewish whites was 13 per $10^5$ persons compared with 3.8 per $10^5$ in non-Jewish whites (Mendeloff & Calkins, 1988). Low incidence rates for ulcerative colitis are found in blacks in the United States (1.4 per $10^5$) and in Cape Town (0.6 per $10^5$).
Jews also have a greater incidence of Crohn’s disease than do age- and gender-matched controls (Odes et al., 1989). Minor variations in incidence have been found between urban and rural populations (Brown et al., 1971), but these have not been consistent and there is probably no true difference. There may be a slightly higher incidence of ulcerative colitis in the higher salaried or better educated members of a population (Sonnenberg, 1990b; Sonnenberg, 1990c), but once again the differences are slight.

2.4.2.3 Familial component

A familial incidence of ulcerative colitis and Crohn’s disease has also been identified by epidemiological studies. For example, siblings of patients with Crohn’s disease are 17 to 35 times more likely to develop Crohn’s disease than members of the general population (Fielding, 1986). For ulcerative colitis, figures vary between studies, probably reflecting a referral bias among other factors, but between 10 per cent and 30 per cent of patients will have at least one other family member affected (Farmer & Michener, 1986; Paulley, 1950). New York and Cleveland data suggest a preponderance of parent-sibling combinations, but in the United Kingdom the disease was more commonly shared by siblings. Most familial association is within the first degree relatives. Furthermore, the other affected family members may have either ulcerative colitis or Crohn’s disease, although the majority will have ulcerative colitis.

A recent epidemiologic study in twins, using the Swedish twin registry, found that eight of 18 unselected monozygotic twin pairs were concordant for Crohn’s disease while for ulcerative colitis only one of 16 monozygotic twin pairs was concordant (Tysk et al., 1988). Crohn’s disease thus seems to show stronger genetic influences than does ulcerative colitis. However, the Swedish twin data is inconsistent with a simple autosomal recessive mode of inheritance. While family and marker studies clearly indicate genetic influences, the less than 100 per cent concordance rate between monozygotic twins described above and the less than 100 per cent penetrance of disease suggest that environmental factors must also play a role in the clinical expression of Crohn’s disease. The interplay of genetics and environment has been highlighted in a study of the incidence of inflammatory bowel disease in spouses and the identification of an extremely high risk for Crohn’s disease in offspring when both parents have acquired the disease (Bennett et al., 1991).

2.4.2.4 Environmental factors

As family studies fail to explain the observed incidence of inflammatory bowel disease the search for environmental risk factors has been a popular field of study in recent years. Migration studies have examined populations with the same gene pool living in two
different environments. A recent Israeli study (Fireman et al., 1989) measured prevalence of Crohn's disease in two Jewish immigrant groups, European/Americans and Asian/Africans. Prevalence of Crohn's disease in both groups rose significantly from 1970 to 1980, from 13 to 26 per 100,000 in the European/American population and from 1.7 to 12.4 per 100,000 in the Asian/African population. This rise in incidence among the immigrant groups is greater than the rise seen in their native countries and so must be due in part to the change in their environment.

In the search for potential environmental risk factors, attention has focused on smoking, oral contraceptives, and diet. Smoking has been associated with increased clinical relapse rates in Crohn's disease (Calkins, 1989), with the effect in women in one report appearing to be dose-related (Pinchbeck et al., 1988). Some of these same studies have linked non-smoking and especially termination of smoking to an increased incidence of ulcerative colitis (Motley et al., 1988). In the Swedish population there has been a gradual change in the gender distribution of ulcerative colitis between 1930 and 1990 (Tysk & Jarnerot, 1992a). A female predominance has given way to a male predominance of the condition among adults, but not in children. It has been suggested that this change may be due to changes in smoking habits since during this period Swedish males have tended to give up smoking, whereas women have taken up the habit.

A role for oral contraceptives has been postulated as a risk factor in Crohn's disease. The incidence of Crohn's disease has been shown in two studies to be 1.5 to 1.7 times as great in women who are using oral contraceptives as in those who are not (Logan & Kay, 1989; Sandler et al., 1992). No such effects were observed in patients with ulcerative colitis (Lashner et al., 1990). Other studies have found no association, leaving the case unproven (Lashner et al., 1989).

The constant exposure of the gut to ingested products has made diet a popular area of epidemiologic research. Beginning with dietary influences in the intrauterine environment, one study found that mothers of children with Crohn's disease had taken significantly less vitamin and mineral supplementation during pregnancy than had mothers with healthy children (Gilat et al., 1987). These data, however, were not corrected for the many confounding factors that may be linked to vitamin and mineral intake during pregnancy. Increased consumption of sugar is a well established risk factor in Crohn's disease (relative risk = 2.6) (Persson et al., 1992). There is evidence that the convenience food culture of the late 20th century may play a role in inflammatory bowel disease; the relative risk of Crohn's disease has been found to be increased for persons with a high intake of fast foods (relative risk = 3.4) (Persson et al., 1992). The only link with ulcerative colitis is possibly increased consumption of fast foods (relative risk = 3.9). Fast food outlets often recycle their cooking oil and this is suggested as a potential
explanation. In a small study on Asians with inflammatory bowel disease, patients with Crohn’s disease recycled cooking oil more than healthy controls and patients with ulcerative colitis (Chuah et al., 1992).

Clearly, there are many other environmental factors that may have a bearing on the pathogenesis of these diseases.

2.4.3 Infectious

2.4.3.1 Bacterial

Despite the recognition of Crohn’s disease as a separate disease entity after it was convincingly distinguished from intestinal tuberculosis in 1932, a bacterial origin for Crohn’s disease has remained a popular field of investigation owing to the similarity of the disease with Johnne’s disease in cattle and to tuberculosis (Dalzeil, 1913).

In the last few years there has been a surge of interest in the research community and the media (Brown, 1991), due largely to the isolation of a genetically identical pathogenic Mycobacterium paratuberculosis from several patients with Crohn’s disease (Sanderson et al., 1992). This pathogenic organism has been isolated from only a few patients and direct evidence for its involvement in the disease process is not clear. Using polymerase chain reaction amplification to detect a probe specific for Mycobacterium paratuberculosis, DNA was detected in 26 of 40 Crohn’s disease patients, but in only 1 of 23 ulcerative colitis patients, and in 5 of 40 control tissues (Sanderson et al., 1992). However, the authors selected only obviously diseased tissue from the specimens of Crohn’s disease and ulcerative colitis. This selection procedure begs the question as to whether the presence of this organism merely reflects opportunistic infection rather than being the primary cause. In a conflicting study (Tanaka et al., 1991), researchers used ELISA techniques to detect concentrations of serum IgG and IgM that reacted with protoplasmic and surface antigen of Mycobacterium paratuberculosis. They also used immunoblotting and immunohistochemical methods to detect Mycobacterium paratuberculosis on tissue sections from inflammatory bowel disease patients. No correlation was found between the presence of Mycobacterium paratuberculosis and Crohn’s disease.

Numerous attempts have been made to isolate bacteria specific to tissues from patients with Crohn’s disease and ulcerative colitis. The early investigations, which utilised standard techniques suitable for recovering bacteria with normal cell walls, found no differences compared to controls. In 1978, Parent and Mitchell reported the isolation of a cell wall defective bacteria from tissues of Crohn’s disease patients, but not from controls (Parent & Mitchell, 1978). Some of these variants reverted to normal bacteria
and were subsequently identified as *Pseudomonas maltophilia*. Initial nucleic acid hybridisation studies provided provocative, but inconclusive, evidence for the association of *Pseudomonas maltophilia* with inflammatory bowel disease. However, subsequent studies indicated that the earlier findings may have reflected the presence of bacteria in the lumen that were not involved in the disease process (Yoshimura *et al*., 1984a). Belsheim and co-workers reported the isolation of cell wall defective bacterial L-forms at significantly higher frequency from patients with inflammatory bowel disease than from controls (Belsheim *et al*., 1984). There have not been any confirming reports from other laboratories.

### 2.4.3.2 Viral involvement

In 1925, intranuclear inclusion bodies were described in the mucosa of a patient dying with ulcerative colitis, suggesting that a virus may have a role in the disease (Von Glahn & Pappenheimer, 1925). However, a number of other laboratories also sought the viral agents without success (Beeken, 1980; Hardin & Werder, 1975; Schneierson *et al*., 1962). In 1973 cytomeglovirus was cultured in a small study from one of four patients with Crohn's disease and three of six patients with ulcerative colitis. Later, Roche and Huang, using nucleic acid hybridisation techniques, failed to confirm the presence of cytomeglovirus DNA in tissues taken from patients with either disease (Roche & Huang, 1977). In 1975 a group in the USA reported the recovery of a filterable transmissible agent with the characteristics of a small RNA virus from tissues from patients with Crohn's disease. Although this group had performed appropriate controls, such as UV irradiation, subsequent studies showed that the cultures used were contaminated with mycoplasma (Kapikian *et al*., 1979).

Repeated failure to find a virus common to all patients essentially ended the active search for a viral cause of Crohn's disease until recently when transmission electron microscopy was used to examine the microvasculature of perfusion-fixed tissues from Crohn's disease and control patients. Paramyxovirus-like particles, and inclusions consisting of condensations of nucleocapsid, have been identified in giant cells and in endothelium at foci of vascular injury in all of the Crohn's disease patients examined. Tissues were also examined by *in situ* hybridisation for measles virus N-protein. Genomic RNA was positive in all cases of Crohn's disease, localising to foci of granulomatous vasculitis and lymphoid follicles. Hybridisation for measles virus RNA was also positive in a minority of control intestinal tissues (Wakefield *et al*., 1993). The authors suggest that Crohn's disease may be caused by a granulomatous vasculitis in response to this virus. These results have yet to be confirmed, but they do provide a compelling alternative to other more established aetiological hypotheses.
2.4.4 Mucosal abnormalities

2.4.4.1 Epithelial abnormalities

Several studies have suggested that the colonic epithelium in ulcerative colitis is abnormal and independent of the presence of mucosal inflammation. Morphological study of colonoscopic biopsy specimens from 15 patients with ulcerative colitis demonstrated ultrastructural features such as focal diminution and shortening of microvilli and vacuolisation of cytoplasmic organelles in epithelium from areas of inactive disease and of apparently uninvolved colon (Delpre et al., 1989). These findings indicate that these epithelial abnormalities are not the result of a hostile mucosal microenvironment secondary to inflammation, but occur before inflammation develops. However, the identification of factors, mucosal or luminal, responsible for these abnormalities is needed to establish support for this argument as it may be possible that the biopsy specimens came from previously diseased mucosa.

A previous functional study of colonic epithelial cells from patients with ulcerative colitis has shown that they are unable to oxidise fatty acids adequately (Roediger, 1980). Further evidence for their metabolic dysfunction has been found in the detoxification of phenols by sulphation (Ramakrishna et al., 1991). Failure to inactivate phenols may be of biological importance to the colonic epithelium because toxic phenols are generated from fermentation of proteins by colonic bacteria, which are found in the colonic lumen. In a recent study, the ability of isolated colonic epithelial cells to sulphate phenols was demonstrated in vitro. In vivo, using rectal dialysis bags, the sulphation of paracetamol was shown to be reduced and was often absent in patients with ulcerative colitis, active or quiescent, but was normal in Crohn’s disease patients (Ramakrishna et al., 1991).

2.4.4.2 Mucus defects

Intestinal and colonic mucus plays a role not only as a lubricant, but also as a protective factor; in the gastrointestinal tract reduced mucus production or abnormal mucus could result in the exposure of the mucosa to harmful contents of the lumen. Colonic mucus has previously been shown to be abnormal in patients with inflammatory bowel disease, and this abnormality is present in remission, suggesting that it may be a primary contributing factor in disease pathogenesis (Burton & Anderson, 1981; Raouf et al., 1992; Reid et al., 1984). Raouf and co-workers have measured the ability of rectal biopsies to incorporate radioactive sulphate into mucin, and demonstrated that patients with ulcerative colitis had decreased incorporation of radioactive sulphate into mucin, regardless of disease activity (Raouf et al., 1992). This observation suggests that abnormal sulphation of mucus may be important in ulcerative colitis. Sulphation of
mucus contributes to its resistance to degradation, and therefore this defect could represent a reduction of the host defence mechanisms. Whether this is a primary or secondary abnormality remains to be determined.

2.4.4.3 Mucosal permeability

Structural defects in the colonic epithelium or reduced resistance to injury may be responsible for the increased permeability that has been observed in some inflammatory bowel disease patients. There has been a great deal of interest in the hypothesis of increased intestinal permeability as a pathogenetic factor in Crohn's disease (Shorter et al., 1972). The intestinal epithelium is an external surface and comes into contact with a wide variety of potentially harmful compounds and organisms. These agents include bacteria and bacterially synthesised products, food antigens, and ingested chemicals and toxins. If the permeability characteristics of the intestine in patients with Crohn's disease are intrinsically abnormal, the uptake of antigenic compounds into the mucosa from the lumen might be the initiating factor leading to the subsequent chronic inflammatory response of inflammatory bowel disease.

Permeability studies of inflammatory bowel disease patients and their healthy relatives to investigate the possible connection between permeability abnormalities and genetic predisposition to inflammatory bowel disease have been conflicting. Excretion of the hydrophilic polyethylene glycol-400 in urine after oral ingestion has been adopted as a useful marker of intestinal permeability. A 2-fold increase in polyethylene glycol-400 permeation in Crohn's disease patients and their first degree relatives was found (Hollander et al., 1986). However, another study using polyethylene glycol-400 could not confirm these findings (Ruttenberg et al., 1992). Absorption of inert sugars such as lactulose, rhamnose, and mannitol has also been tested and permeability abnormalities could not be found in the relatives, but patients with inflammatory bowel disease had increased lactulose permeation compared to the control group (Katz et al., 1989). However, two other investigations did show an increased lactulose/mannitol ratio in first degree healthy relatives of Crohn's disease patients compared to controls (May et al., 1992; Pironi et al., 1992). The most extensive study to be published has examined the epithelial permeability in a larger group of patients with Crohn's disease and ulcerative colitis and in their healthy relatives and evaluates possible differences in absorption of the polyethylene glycol-400 and the three inert sugars lactulose, rhamnose, and mannitol (Munkholm et al., 1994). No differences between any of the groups were observed.

Despite conflicting results, the idea of a genetically transmitted increased permeability offers an attractive aetiological explanation for these diseases. An alternative hypothesis based on intestinal permeability suggests that sensitisation to a luminal antigen may arise...
soon after parturition when the epithelium is more leaky. This hypothesis does not require a genetically increased intestinal permeability.

2.4.4.4 Small vessel thrombosis

Another pathogenic mechanism that may be added to the list of factors favouring injury to the mucosa in inflammatory bowel disease as a contributing factor to the aetiology is the hypothesis that Crohn’s disease is caused by small vessel thrombosis. Evidence for micro-infarctions in the intestine, followed by granulomatous inflammation of vessel walls, has come from histological examination (Wakefield et al., 1991). Indeed healing by fibrinous stricture formation is a characteristic of ischaemic intestinal injury. Epidemiological evidence presented earlier in this chapter (section 2.4.2.4) suggests that the oral contraceptive pill and smoking increase the risk of Crohn’s disease. These two potentially thrombogenic agents may augment disease activity by exacerbating underlying vascular injury and tendency to focal thrombosis. The main protagonists of this hypothesis believe that a viral infection is the mechanism for the initiation of the thrombogenic injury (Wakefield et al., 1993). Imbalance of the mechanisms controlling thrombosis could also contribute to this process; accordingly, the concentration of the anticoagulant protein S was found to be below the normal range, suggesting that some patients might be at increased risk for thromboembolic complications (Aadland et al., 1992). The hypothesis is not, however, unchallenged in the literature: examination of 149 granulomas from 10 patients with Crohn’s disease revealed no evidence for a vasculitic origin of Crohn’s disease (Talbot et al., 1992). On the contrary, the evidence favoured a granulomatous lymphangitis.

2.4.5 Immunological factors

Most of the abnormal cellular and humoral immune mechanisms described in inflammatory bowel disease are likely to have secondary roles; a number of abnormalities have, however, been characterised that may have a primary aetiological role leading to the development of inflammatory bowel disease.

2.4.5.1 Autoantibodies

In a review of the large population of patients with inflammatory bowel disease in Oxford, Snook and co-workers found a significantly greater association of ulcerative colitis in patients with autoimmune disease than in the general population. A similar association was not found in Crohn’s disease. Autoimmune injury to colonic epithelium could be a primary event in the pathogenesis of ulcerative colitis, as numerous studies have confirmed that a heterogeneous group of circulating anticolonic antibodies are often found in the circulation of patients with ulcerative colitis. These have relative
disease specificity and their concentrations often relate to disease activity. The artificial stimulation of polyclonal antibodies by Epstein-Barr virus-transfected mucosal cells has been frequently found to produce anticolonic antibodies in ulcerative colitis. The relevance to \textit{in vivo} production or to pathogenesis is uncertain, especially because non-specific immune stimulation with interferon-\(\alpha\) is associated with the development of antibodies to simple epithelium of the gastrointestinal tract. The studies so far have not definitively shown that these antibodies in any way damage or modulate colonic epithelial cells, although cytotoxicity of a colonic cancer cell line has been observed by a minority of sera from ulcerative colitis patients. Thus, it appears likely that at least most anticolonic antibodies represent secondary pathogenic events or epiphenomena (Snook \textit{et al.}, 1989).

Recently attention has been focused on the presence of anti-neutrophil cytoplasm antibodies present in the majority of ulcerative colitis patients (Cambridge \textit{et al.}, 1992; Duerr \textit{et al.}, 1991; Peen \textit{et al.}, 1993). The inflammatory bowel disease-associated antineutrophil antibody has a perinuclear pattern but does not react with myeloperoxidase, being distinct from those found in Wegener’s granulomatosis and in vasculitis (Duerr \textit{et al.}, 1991). All studies to date have confirmed that anti-neutrophil cytoplasm antibodies are found with much higher frequency in patients with ulcerative colitis (32 per cent to 83 per cent) than in patients with Crohn’s disease (2 per cent to 25 per cent). Although many investigators have not found any relationship between the presence or titre of anti-neutrophil cytoplasm antibodies and disease extent or activity, Cambridge and co-workers found that anti-neutrophil cytoplasm antibodies were absent in patients with limited proctitis.

2.4.5.2 Macrophages

Mononuclear cell infiltration of colonic mucosa is a characteristic feature of idiopathic inflammatory bowel diseases. One of the problems in understanding the role of these cells has been the phenotypic heterogeneity of mucosal macrophages. There is some evidence that intestinal macrophages are involved in antigen presentation and in the modulation of immunoglobulin synthesis (Mahida, 1990). In active ulcerative colitis and Crohn’s disease, there is a population of cells that exhibit a low-affinity Fc\(\gamma\)R (3G8\(^{+}\)) and a population that expresses RFD9, a marker for epithelioid cells. Although 3G8\(^{+}\) macrophages are seen in intestinal infections, the presence of 3G8\(^{+}\) and RFD9\(^{+}\) populations appears to be unique for ulcerative colitis and Crohn’s disease. The pattern of RFD9\(^{+}\) cells is interesting, since they are clustered deep in the lamina propria (Allison \& Poulter, 1991). The origin and function of these cells are unknown. RFD9 is not simply an activation marker. Thus these cells seem to appear in response to specific local stimulus.
2.4.5.3 Lymphocytes

There has been a great deal of interest in many aspects of lymphocyte biology in inflammatory bowel disease. However, many discrepant results have been reported, especially regarding T-cell function. Most investigators appear to agree that the distribution of T-cell subsets (CD4⁺:CD8⁺) remains unchanged in inflammatory bowel disease patients (Selby et al., 1984). Although many reports have also found diminished T-cell suppressor activity, supporting the view that an uncontrolled inflammatory response underlies the chronicity of the diseases, most find a reduction in diseased states only (Hodgson et al., 1978b), suggesting that the observation is secondary to the inflammatory process.

T-cells may be activated by a number of different mechanisms. Specific activation by antigens is dependent on their triggering by antigen-specific T-cell receptors. There are two distinctly different classes of T-cell receptors, both of which are heterodimers that are the product of two genes: the more common, αβ heterodimer, and the much less common γδ heterodimer. T-cells expressing γδ heterodimers have been of considerable interest recently because they are much more frequent in the intestinal epithelium, particularly in the intraepithelial compartment, and molecular diversity of the antigen recognition sequences of these molecules is much more limited compared to αβ. However, quantification of the proportions of these two classes of T-cells in the intestinal mucosa of patients with inflammatory bowel disease indicates that the αβ heterodimer T-cell receptor-expressing population is increased both in the intraepithelial lymphocytes and lamina propria populations, whereas there is no increase in the γδ population (Cuvelier et al., 1992). This finding is in contrast to that in patients with coeliac disease, where the latter population is expanded. The relevance of this discovery is unknown.

2.4.5.4 Neuronal

Among the many factors that exert important immunomodulatory influences on the gut is the enteric nervous system. It seems probable that any defect in neuropeptide release may have consequences on mucosal immunoregulation in inflammatory bowel disease. There is considerable evidence that the enteric nervous system is altered in both Crohn's disease and in ulcerative colitis. A recent study has shown that the density of VIP-immunoreactive nerve fibres is decreased in the lamina propria and submucosa of ulcerative colitis and Crohn's disease patients (Yoshiro et al., 1992). The reduction in density in the lamina propria was also linked to the amount of inflammation. Although such studies do little more than highlight associations, it seems likely that the neuro-immune homeostasis in the bowel wall of these patient is upset. Therapeutically,
beneficial effects of administering lidocaine have been demonstrated in one hundred consecutive cases (Bjorck et al., 1992). Treatment of the mucosa with 2 per cent enemas gave symptomatic relief and eventual healing after 6 to 34 weeks in 83 per cent of the patients with proctosigmoiditis and in all patients with proctitis. This phenomenon is thought to be due to blockade of hyper-reactive autonomic nerves in the mucosa of ulcerative colitis patients.

2.5 Treatment

2.5.1 General

The approaches to the medical management of Crohn's disease and ulcerative colitis are substantially the same. However, the variability of the clinical presentation in these disorders requires that therapy be modified to account for the distribution and extent of disease as well as other factors in individual patients. In general, medical therapy is the approach of choice in these chronic diseases. However, surgical intervention may become necessary in intractable cases. Proctocolectomy, when necessary, is 'curative' for patients with ulcerative colitis which only affects the colon. In contrast, the high likelihood of recurrence in Crohn's disease makes surgery less appealing for patients, and surgery where necessary is mostly conservative.

2.5.2 Medical management

The aims of medical therapy for inflammatory bowel disease are (1) to provide symptomatic relief, (2) to ensure adequate nutritional status, (3) to reduce inflammation of the intestine, and (4) to maintain remission.

Glucocorticosteroids are the mainstay of therapy in patients with moderate to severe ulcerative colitis or Crohn's disease. Interestingly, for Crohn's disease, the comprehensive National Co-operative Crohn's Disease Study demonstrated efficacy of glucocorticosteroids only in patients with small-intestinal involvement, despite the accepted view that they may also be helpful in some patients with Crohn's colitis (Lennard-Jones, 1983). Oral prednisone or intravenous methylprednisolone are used most frequently when glucocorticosteroids are warranted (Podolsky, 1991). Methylprednisolone has been demonstrated to decrease disease recurrence in patients with ulcerative colitis or Crohn's disease in remission (Brignola et al., 1988). However, the use of glucocorticosteroids must always be tempered by the awareness that they carry a considerable risk of side effects and potential complications. There are two main categories of side effects seen with the use of glucocorticosteroids: those related to high dose treatments and those related to withdrawal. The primary complications seen with prolonged glucocorticosteroid therapy include hypertension, hyperglycaemia,
immunosuppression, osteoporosis, peptic ulcer, myopathy, growth retardation and Cushing's syndrome. The main problem associated with glucocorticosteroid withdrawal is iatrogenic acute adrenal insufficiency leading to hypotension and possibly death. Because oral glucocorticosteroids are associated with many side effects there have been attempts to produce rapidly metabolised glucocorticosteroids as a next generation therapy (Ford et al., 1992). Topical hydrocortisone is still the mainstay of therapy for distal proctocolitis, although its efficacy is limited by its relatively low potency. The use of high doses of topical glucocorticosteroids has been restrained by the appreciation that as much as 20 per cent of the drug may be absorbed, leading to systemic effects. Development of new topical preparations that are less likely to produce systemic effects, because of increased first-pass metabolism in the liver and enhanced potency, are proving effective in clinical trials (Bansky et al., 1987). These properties allow the safe delivery of higher, more effective doses of steroids to the affected areas and may enhance the usefulness of topical steroids in diseases affecting the distal colon.

Sulphasalazine, a medication initially used to treat rheumatoid arthritis in the 1940s (Svartz, 1942), is extensively used for the management of ulcerative colitis that is mild to moderately severe, as well as for that of large bowel involvement in Crohn's disease. Recent studies have proposed that sulphasalazine has several actions that attenuate inflammation in the bowel (Gaginella & Walsh, 1992). Sulphasalazine may be an inhibitor of cyclooxygenase and 5-lipoxygenase, resulting in a reduction of prostaglandin and leukotriene production. In addition, sulphasalazine appears to block the chemotactic activity of formylated bacterial peptides - compounds that recruit leukocytes to the bowel, perpetuating tissue damage. Sulphasalazine reduces both the frequency and the severity of recurrent ulcerative colitis. In contrast, no prophylactic benefit of this drug has been demonstrated after acute disease activity has been controlled in Crohn's disease (Van Hees et al., 1981). Approximately 20 per cent of patients are hypersensitive to sulphasalazine or display less specific intolerance (Taffat & Das, 1983). Hypersensitivity can be characterised by rash, arthritis, pericarditis, pancreatitis, and pleuritis. In men, there is a very high incidence of oligospermia with impaired fertility (Birnie, 1981). The hypersensitivity and intolerance induced seem to be largely a response to the sulphapyridine moiety to which 5-aminosalicylic acid is linked.

The 5-aminosalicylic acid moiety confers the majority of the anti-inflammatory properties of sulphasalazine (Azad Khan et al., 1977) and the presence of sulphapyridine is necessary for therapeutic efficiency because it prevents absorption of the drug in the proximal gastrointestinal tract, which would make the 5-aminosalicylic acid unavailable to the colonic mucosa. When sulphasalazine reaches the colon, 5-aminosalicylic acid is liberated by the action of bacterial azo reductase. These observations, together with the
associated side effects of sulphasalazine, led to development of other ways to deliver 5-
aminosalicylic acid to the colon. Topical 5-amino salicylic acid may be given as an enema
to control distal proctocolitis. A number of forms of orally active 5-amino salicylic acid
have been developed. Several use newer methods of drug delivery: for example, 5-
aminosalicylic acid is encapsulated in a pH-sensitive slow-release matrix (Dew et al.,
1983), or 5-aminosalicylic acid is conjugated to itself to form a dimer that is hydrolysed
by the colonic bacterial azo reductase (Lauritsen et al., 1984). These compounds have a
spectrum of pharmacological activity that is similar to that of sulphasalazine and offer
little additional benefit to patients who can tolerate sulphasalazine. However,
approximately 80 to 90 per cent of people who are intolerant of sulphasalazine are
tolerant of these other 5-amino salicylic acid derivatives.

Despite inconsistencies in the results of initial trials of azathioprine and its active
derivative, mercaptopurine, these two agents are considered to be efficacious in the
treatment of inflammatory bowel disease. The drugs are thought to act by interfering
with nucleotide conversions, thereby inhibiting DNA and RNA synthesis and
chromosomal replication. Typically, 6-mercaptopurine and azothioprine have been used
to treat patients with active Crohn's disease, with particular interest in their effect on
steroid sparing, fistula healing, perianal disease, and maintenance of remission
(Markowitz et al., 1990; Present et al., 1980). In contrast to sulphasalazine,
mercaptopurine appears to diminish the likelihood and severity of relapses in patients
with Crohn's disease. Although less data are available, mercaptopurine and azathioprine
also appear to be effective in minimising the requirement for glucocorticosteroids in
patients with refractory ulcerative colitis (Liang et al., 1992). With appropriate
monitoring, these agents appear to be safe and well tolerated. Problems, however,
include the very slow onset of action, bone marrow suppression, hepatotoxicity and
possible mutagenic potential (Present et al., 1989).

Two other immunosuppressive agents have recently emerged as potentially useful in this
patient population. In a double-blind placebo-controlled study, methotrexate at low
doses (5mg three times per week for 52 weeks) was reported to reduce the number of
disease relapses in 33 patients (46 per cent versus 80 per cent) with refractory Crohn's
disease, but there was no difference in the Crohn's disease activity index (Arora et al.,
1992). Cyclosporine has been evaluated more fully by a number of workers. A
controlled trial in 71 patients with Crohn's disease showed a somewhat higher rate of
response over a three-month period in those receiving 5 to 7.5mg/kg/day cyclosporine
than in those receiving a placebo, but fewer than half these patients had a sustained
response when the dosage of the drug was tapered (Brynskov, 1989). Cyclosporine binds
endogenous intracellular receptors, the immunophilins, and the resulting complex
targets the protein phosphatase, calcineurin, to exert an immunosuppressive effect. The small benefit afforded by cyclosporine needs to be balanced with its serious side effects during long-term therapy often required in patients with Crohn’s disease.

**Metronidazole** in high doses can be extremely effective in improving recalcitrant perineal fistulas (Brandt *et al.*, 1982). Dose-related toxicity often limits its use and fistulas frequently recur after the dosage is reduced or the drug is discontinued. The mechanism of action of metronidazole in this setting is unclear. This agent is also sometimes useful in the treatment of Crohn’s disease confined to the large bowel, although it is without effect in ulcerative colitis.

In the management of inflammatory bowel disease, the role of *dietary therapy and bowel rest* has long been debated. Bowel rest may alleviate some of the acute symptoms of ulcerative colitis and Crohn’s disease, and parenteral alimentation can ensure adequate nutritional intake. The availability of long-term total parenteral nutrition and repletion of specific nutritional deficiencies represent important advances in the care of patients with short-bowel syndrome or refractory disease (Whittaker, 1987). Total parenteral nutrition can be shown to restore the growth rate of paediatric patients with Crohn’s disease (Kleinman *et al.*, 1989). However, it has not been possible to demonstrate that total parenteral nutrition itself has a direct effect on inflammatory bowel disease and this approach requires a continuing commitment by the patient to a diet that many will find difficult to sustain.

Leukotrienes may contribute to the amplification of intestinal inflammation. This has stimulated interest in means of inhibiting their synthesis or physiological action. Conceptually, such leukotriene inhibitors might be especially effective and free of many of the toxic effects of other agents. A number of *5-lipoxygenase inhibitors* appear to have beneficial effects in animal models. A small-scale pilot study has recently validated the potential effectiveness of a 5-lipoxygenase inhibitor in the management of ulcerative colitis (Collawn *et al.*, 1992). There have also been attempts to treat patients with *fish oil*, which contains large amounts of eicosapentanoic acid. It has been shown that eicosapentanoic acid is metabolised by 5-lipoxygenase to form leukotriene B5 and prostaglandin E3, with a subsequent 53 per cent reduction in leukotriene B4. However, a one year placebo-controlled trial indicated that the treatment provided little benefit (Hawthorne *et al.*, 1992).

2.5.3 Surgical management

Most patients with ulcerative colitis will not require surgery, although as many as 25 per cent will eventually need an operation (Truelove, 1988). Conversely, the majority of
Crohn’s disease patients will require at least one surgical procedure for this condition within their lifetime (Sachar, 1990).

2.5.3.1 Ulcerative colitis

In recent years, colectomy with ileal pouch-anal anastomosis has become the treatment of choice for most patients undergoing elective surgery for ulcerative colitis. The commonly accepted indications for surgery in ulcerative colitis include exsanguinating haemorrhage, toxicity or perforation, significant dysplasia or suspected cancer, systemic complications and intractability. Conventional proctocolectomy and ileostomy is generally appropriate for elderly patients and for patients who are unsuited for a restorative anastomosis. Resections of smaller portions are generally inappropriate for patients with ulcerative colitis, since they are associated with a high likelihood of disease recurrence and a continued risk of colon cancer. Occasionally, partial colectomy is warranted in patients with segmental colitis. The functional outcome of ileoanal anastomosis has been improved by revised techniques for construction of a pouch reservoir from the terminal ileum, but it seldom matches the normal state. Before the techniques were revised, as many as 46 per cent of patients were incontinent after the operation (Beart, 1988). This complication is now largely avoided as a result of changes in the surgical techniques used to create the pouch, especially with the adoption of the J-pouch, W-pouch and S-pouch configurations. Patients average two to eight stools per day, where frequency is related most directly to the size of the pouch; a larger size reduces the frequency. As many as 20 per cent of patients have difficulty maintaining continence during the day as well; approximately 15 per cent require re-operation, and some ultimately require a conventional ileostomy. Poor postoperative continence is most closely related to the impairment of internal anal-sphincter pressure. Despite these problems, patients’ degree of satisfaction with the procedure on the whole is moderately high, perhaps reflecting the relative symptomatic improvement and the acceptable perceived body image (by avoiding a stoma). A troublesome and an unresolved complication is the development of pouchitis in the newly created ileal reservoir, which is reported to occur in between 10 per cent and 50 per cent of patients undergoing this procedure for ulcerative colitis (Pemberton, 1993). This inflammatory process resembles ulcerative colitis in many respects, but appears to differ from ulcerative colitis in that the inflammatory process frequently responds to treatment with antibiotics, such as metronidazole; however, its nature and methods of prevention are still unclear.

2.5.3.2 Crohn’s disease

A colectomy with ileal pouch-anal anastomosis is inappropriate for patients with underlying Crohn’s disease of the colon, because of the high risk of disease recurrence
and perineal complications. The goals of surgical treatment in Crohn’s disease are distinct from those in ulcerative colitis. The most common indication for surgery in Crohn’s disease is intestinal obstruction, which is caused by fibrosis and stricture. Approximately 30 per cent of patients with Crohn’s disease require surgical intervention within the first year of diagnosis. The remainder will require surgery at a rate of 5 per cent per year (Boer Visser et al., 1990; Wright et al., 1987). Cure is unlikely, and resection should be conservative so as to preserve as much bowel as possible. Careful analysis indicates that patients who have microscopical evidence of disease at the margin of resection have the same prognosis as those without such changes (Hamilton et al., 1985). Thus, resection should be limited to those areas most affected by gross disease. Subsequent recurrence is frequent and is most common immediately proximal to the site of a previous anastomosis or stoma (Cameron et al., 1992; Olaison et al., 1992). The disease probably recurs soon after surgery even if manifestations are delayed; one year after an initial operation, recurrent lesions were observed endoscopically in as many as 73 per cent of patients (Rutgeerts et al., 1990).

2.6 Summary

Crohn’s disease and ulcerative colitis are chronic idiopathic inflammatory diseases that affect the gastrointestinal tract, and for which the aetiology is unknown. Although the diseases have many similarities, differential diagnosis is possible in most cases. Crohn’s disease may affect any part of the gastrointestinal tract, but ulcerative colitis is by definition confined to the colon. Histologically, Crohn’s disease is characterised by patchy transmural inflammation with deep fissuring ulceration and frequently granulomas. Ulcerative colitis is, on the other hand, characterised by continuous inflammation that is usually confined to the mucosa. It seems likely that the cause of the idiopathic inflammatory bowel diseases is multifactorial, comprised of both genetic and environmental factors. Chronicity develops, accompanied by release of excessive inflammatory mediators, resulting in substantial tissue degradation and remodelling. None of the possible aetiologies attempts to explain the characteristic relapsing course of the diseases.

Our lack of understanding of the aetiology has resulted in the use of non-specific therapies that have remained unchanged for decades and are associated with a high incidence of side effects. Surgery can be ‘curative’ for ulcerative colitis patients, but not for those with Crohn’s disease. The development of new agents, better analogues of older agents, and improved modes of drug delivery is the target for future therapy. This will only be achieved through improved understanding of the mechanisms of gastrointestinal inflammation and the underlying primary aetiological factor(s).
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3 INFLAMMATION IN INFLAMMATORY BOWEL DISEASE

3.1 Introduction

The pathogenesis of intestinal inflammation in inflammatory bowel disease may be divided into two stages. The first stage is the initiating event which triggers an acute inflammatory response. The second stage is the amplification of the inflammatory response, which involves a number of inflammatory cells including lymphocytes, mast cells, macrophages and polymorphonuclear neutrophils. The amplification of the inflammatory response is controlled by soluble mediators including histamine, the kinins, products of the complement pathway, prostaglandins and leukotrienes, and the cytokines. The amplification of the inflammatory response is especially important in the pathogenesis of inflammatory bowel disease, as it is this stage that governs the tissue destruction and the histological and functional changes characteristic of inflammatory bowel disease. Moreover, so long as the initiating event remains unknown, it is likely that further advances in the medical therapy of inflammatory bowel disease will result from pharmacological modulation of inflammatory mediators.

In this chapter emphasis will be placed upon the role of the polymorphonuclear neutrophil in inflammation and upon the eicosanoids. The polymorphonuclear neutrophil has frequently been implicated as the most important cellular mediator of tissue destruction in inflammatory bowel disease (Weiss, 1989), and the eicosanoids are thought to be responsible for the sustained inflammatory response. In addition, the benefit of therapeutic agents currently employed in the treatment of inflammatory bowel disease is thought to be partly due to their inhibition of eicosanoid biosynthesis (Flower, 1988; Gaginella & Walsh, 1992).

3.2 Characteristics of the inflammatory response to injury

Inflammation is the reaction of vascularised living tissue to local injury (Robbins et al., 1989). Cornelius Celsus, a Roman writer of the first century AD, described the four cardinal signs of inflammation: rubor, tumor, calor, and dolor (redness, swelling, heat, and pain) (Celsus, 1831). A fifth clinical sign, loss of function, was later added by Virchow (Virchow, 1860). These features of the inflammatory response are induced by
changes in haemodynamics, changes in vascular permeability, and leukocyte exudation. These three reactions may overlap and some share common mediator mechanisms. It was Hunter who realised that inflammation was not a disease, but a non-specific response that has a restorative effect on its host (Hunter, 1794). With the development of the microscope, Cohnheim was the first to describe the changes in the vascular system occurring in the development of inflammation in the mesentery and tongue of the frog (Cohnheim, 1889). Dilatation, principally of the arteries, but also of the veins and capillaries, was observed and this appeared to be the first event, lasting for approximately half an hour. There was an increase in the number of white blood cells at the periphery of the veins and these cells emigrated through the vessel walls into the tissue. The tongue and mesentery became swollen due to exudation of fluid into the tissue. Cohnheim thought the most important event was a change in the vessel wall allowing a series of steps connected with the movement of blood during which leukocytes passively crossed the endothelium. On the basis of his observations Cohnheim explained that the cardinal signs of inflammation were due to an increased volume of blood in the inflamed area, with infiltration of fluid into the tissue contributing to the swelling. Heat was due to an increased blood flow from the internal organs and pain was associated with stimulation of the sensory nerves. His observations were remarkably accurate.

3.2.1 Acute inflammation

The term acute inflammation refers to a response that is abrupt in onset and of short duration. Acute also refers to a rather specific type of response involving an exudative reaction during which fluid, serum proteins, and leukocytes leave the blood stream and enter the area of injury. Acute inflammation may become chronic in the temporal sense if the injurious factor is persistent.

Following a very brief period of vasoconstriction immediately on injury, the arterioles vasodilate and the microvasculature at the site of injury becomes filled with blood. Vasodilatation results from a relaxation of the smooth muscle layer of arterioles and the sphincter of precapillaries. This opens previously inactive capillaries and may result in as much as a tenfold increase in blood flow in the injured area. Postcapillary venules dilate as more blood flows in through the capillaries. In addition, vessels become more leaky following contraction of the endothelial cells in small postcapillary venules. When the endothelial cells contract they draw away from each other, allowing fluid and plasma proteins to leave the vessels.

The movement of leukocytes, predominantly polymorphonuclear neutrophils, from the blood into the wound site is also an important event of the acute inflammatory reaction. However, it should be noted that the movement of leukocytes and the increase in
permeability occur independently of each other. The change in permeability is an immediate response, resulting in oedema, whereas movement of leukocytes occurs at a later stage. Both these phenomena can be inhibited separately (Hurley, 1964).

3.2.2 Chronic inflammation

There is no clear dividing line between acute and chronic inflammation; in fact chronic inflammation may often have many acute features. If the cause of the initial injury is not completely eliminated, acute inflammation will gradually progress to chronic inflammation, but there is no set time sequence. Chronic inflammation may develop in two ways depending on the nature of the inflammatory stimulus or stimuli involved. It may supersede an acute inflammatory response that is not completely resolved, or it may develop in the absence of an antecedent acute response. Characteristically, chronic inflammation is usually associated with irreversible destruction of normal connective tissue. Probably the most acceptable definition of chronic inflammation is to describe it as a state of arrested healing. Complete healing is prevented by the continued presence of the sustaining factors.

The histological hallmarks are infiltration by mononuclear cells, proliferation of fibroblasts, increased fibrosis and connective tissue destruction. Although polymorphonuclear neutrophils are usually considered a characteristic of acute inflammation, many forms of chronic inflammation, lasting for months, continue to show large numbers of neutrophils and suppuration. Conversely, the presence of lymphocytes does not always mean that inflammation has been present for long periods. This is especially true of viral infections. In acute hepatitis or viral myocarditis, for example, lymphocytes predominate even in the first few days of the acute disease.

3.2.3 Hypersensitivity reactions

When an adaptive immune response occurs in an exaggerated and inappropriate form causing tissue damage and inflammation, the term hypersensitivity is applied. Coombs and Gell described four types of hypersensitivity, in each of which the tissue injury was attributable to different facets of the immune response (Coombs & Gell, 1975). Hypersensitivity may be classified into types I, II, III, and IV. Types I to III are immediate responses that are mediated by antibody, while type IV, or delayed-type hypersensitivity, is a delayed response that is mediated by T-cells where antibody plays a subordinate role. In some cases, for example rheumatoid arthritis, systemic lupus erythematosus, and allergic contact dermatitis, hypersensitivity may be the cause of chronic lesions. In other conditions, hypersensitivity develops as a secondary response.
Both immediate and delayed types of hypersensitivity may develop into chronic inflammation.

A hypersensitivity reaction may be an important feature of the pathogenesis of inflammatory bowel disease. Both humoral models, types II and III, and cell-mediated models of delayed-type hypersensitivity have been proposed as the underlying cause of inflammatory bowel disease (Beck et al., 1988; Brkic et al., 1992; Callahan et al., 1963; Hodgson et al., 1978a; Hotta et al., 1986).

3.3 Cellular involvement

The accumulation of leukocytes, principally neutrophils and monocytes, is the most important feature of an inflammatory reaction. Leukocytes engulf and degrade bacteria, immune complexes, and the debris of necrotic cells. However, it is conceivable that these defensive reactions may themselves prolong inflammation and increase tissue damage by the release of proteolytic enzymes, chemical mediators, and toxic oxygen radicals (Weiss, 1989). The accumulation of leukocytes in inflamed tissue can be divided into five phases: margination, adhesion, emigration and chemotaxis, phagocytosis, and the release of leukocyte products (Robbins et al., 1989).

A reduction in blood flow in an inflamed organ may follow the initial increase. In this situation leukocytes assume positions in contact with the endothelium (margination). The cells first tumble slowly along the walls of the capillaries and venules, before finally coming to rest at some point (adhesion). Following margination, a number of factors may influence adhesion. Evidence suggests that increased leukocyte adhesion in inflammation involves specific interactions between complementary adhesion molecules present on the leukocyte and endothelial surfaces. The surface expression of these adhesion molecules is either induced, or enhanced, or altered by inflammatory agents and chemical mediators, resulting in increased adhesiveness (Harlan & Liu, 1992).

Emigration is the process by which motile leukocytes escape from the blood vessels to the perivascular tissues. Neutrophils, eosinophils, basophils, monocytes and lymphocytes are all thought to migrate by similar mechanisms, although less is known about lymphocyte emigration. Following adhesion, the leukocytes move along the endothelial surface and insert pseudopodia into junctions between the endothelial cells. They then move through widened interendothelial junctions, eventually assuming a position between the endothelial cell and the basement membrane. Traversing the basement membrane to enter the extravascular space is accomplished through the release by the cells themselves of proteases, particularly the matrix metalloproteinases, that are able to
degrade basement membrane. Once in the extravascular space the leukocytes move along chemical gradients (chemotaxis) toward targets to be phagocytosed.

3.3.1 Mast cells

Mast cells are not leukocytes and do not phagocytose; however, mast cells are important in any inflammatory event as they contain histamine, an important inflammatory mediator released immediately following injury, inducing vessel dilatation. They are a normal constituent of nearly every organ, but their apparent number varies significantly between different species and, within a single species, between different organs and tissues. There are at least two distinct mast cell populations (Irani et al., 1986). Connective tissues are found to contain mast cells with granules rich in heparin, whereas the lamina propria of the gastrointestinal tract contains mucosal mast cells of a smaller size with granules that react for biogenic amines and for chondroitin sulphate proteoglycan but not for heparin. Connective tissue mast cells are dispersed selectively around small arteries and their nearby nerve bundles. Mucosal mast cells are particularly common in the terminal ileum at the site most frequently affected by Crohn's disease (Fox et al., 1993).

Mast cells readily degranulate in response to agents that interact with their surfaces, enhancing calcium ion influx and inhibiting membrane-bound adenylcyclase. Mast cells bind the Fc portion of the IgE antibody and complement which induces degranulation. Following a stimulus causing degranulation, the granules move to the cell periphery; adjacent granules merge, forming granule complexes. A shallow invagination of the plasma membrane leads to fusion with the granule surface and release of its contents.

Mucosal and submucosal mast cell hyperplasia is a feature of the chronic inflammatory bowel diseases. Degranulated mast cells are often observed in areas of active disease, suggesting that the inflammatory mediators released from these cells contribute to the pathophysiology of these disorders. A recent hypothesis suggests that epithelial cell-derived proteins interact with the mast cells of patients with inflammatory bowel disease to trigger the local release of mast cell mediators (Fox et al., 1993). Histamine release was found to be increased by more than 10 per cent in a third of chronic inflammatory bowel disease specimens in response to epithelial cell-derived protein preparations derived from small bowel epithelium. Normal controls did not respond to the preparations, suggesting that epithelial protein-induced release of intestinal mast cell mediators may contribute to the inflammation in these chronic gastrointestinal disorders. However, the specificity of the response was not tested and the study did not include tissue from patients with non-active disease.
3.3.2 The role of neutrophils in inflammatory bowel disease

The phagocytic cells form an important arm of the non-specific defences against pathogens. Two types of phagocytic cell are found in the body: the polymorphonuclear leukocyte (neutrophils, eosinophils and basophils) and the monocyte. The infiltration of an organ or tissue by polymorphonuclear neutrophils is a hallmark of acute inflammation. Polymorphonuclear neutrophils, with potential to kill cells via generation of toxic oxygen metabolites, have the primary role of defending the body against invading pathogens. However, the polymorphonuclear neutrophil is being increasingly implicated as a mediator of tissue-destructive events (Weiss, 1989). This phenomenon may be a consequence of the inability of the neutrophil to distinguish between foreign and host antigens. When normal host tissue is inappropriately identified as foreign or damaged structures, the receptors on the plasma membrane of the polymorphonuclear neutrophil are triggered, eliciting the cell’s destructive potential.

In response to assorted stimuli polymorphonuclear neutrophils become activated and pass through the capillary walls and move along chemotactic gradients, such as leukotriene B4, towards foreign material (LeDuc & Nast, 1990). This material is then phagocytosed and degraded intracellularly by a combination of oxidants and hydrolytic enzymes. The generation of toxic oxygen metabolites assumes a critical role in host defence against bacterial invasion, intravascular coagulation, and possibly tissue injury. The role of phagocytic cells in host immunity was discovered by the Russian scientist Metchnikoff, in the late 1880s. He emphasised that cellular ingestion of foreign matter was a manifestation of host defence and an attempt by cells to ingest and destroy the invading pathogenic entity. His observations led to the theory of phagocytosis, which became fundamental to the concept of cellular immunity. The polymorphonuclear neutrophils are the first phagocytes to arrive at sites of acute inflammation and, indeed, the presence of these cells serves as a histological indicator of an inflammatory response (Robbins et al., 1989). Resting phagocytes consume little oxygen since they rely mainly on glycolysis for ATP production and are rich in stored glycogen. The professional phagocytic cells – neutrophils, monocytes, and macrophages, and eosinophils – all display an enhanced rate of oxygen consumption when engulfing their prey (Segal & Abo, 1993). At the onset of phagocytosis these cell types show a marked increase in oxygen uptake that is not prevented by cyanide, and is thus unrelated to mitochondrial electron transport. This respiratory burst can be ten or twenty times the resting respiratory rate in neutrophils (Morel et al., 1992). At the same time, there is an increased consumption of glucose by the cells, which is fed into the pentose phosphate pathway. The killing of many bacterial strains by neutrophils is greatly decreased under anaerobic conditions. Indeed, patients suffering from chronic granulomatous disease, in which phagocytosis is
normal but the respiratory burst is absent, show persistent and multiple infections (Curnutte et al., 1974). The classic type is inherited as an X-linked recessive disorder and typically presents in the first two months of life as severe skin sepsis caused by Staphylococcus aureus.

Phagocytes employ as antimicrobial agents a number of compounds generated by the partial reduction of oxygen or reactive oxygen metabolites (ROMs) (Halliwell & Gutteridge, 1985). For the production of these agents oxygen is initially reduced to superoxide (O$_2^-$) using NADPH. A large part of the O$_2^-$ produced by this enzyme is converted to H$_2$O$_2$ in a subsequent dismutation reaction. Much of this H$_2$O$_2$ is delivered into phagosomes, or into the surrounding medium, but a portion diffuses into the cytoplasm, where it is detoxified by the glutathione-peroxidase-glutathione-reductase system. Both the O$_2^-$-forming enzyme and glutathione reductase generate NADP$^+$ in the course of their activity; this NADP$^+$ is converted back to NADPH by the hexose monophosphate shunt. The nature of NADPH oxidase, the respiratory burst enzyme, is still conjectural, although a flavoprotein and a type b cytochrome have been proposed as possible components (Morel & Vignais, 1984). Even less clear is the mechanism of NADPH oxidase activation. Since phorbol 12-myristate 13-acetate and 1-oleoyl-2-acetyl-glycerol elicit a vigorous respiratory burst, protein kinase C is generally thought to be involved in the activation process (Baggiolini et al., 1988). Receptor agonists like fMLP, however, induce superoxide formation, H$_2$O$_2$ production and oxygen consumption at a much faster rate than protein kinase C ligands, suggesting that NADPH oxidase activation does not depend solely on protein kinase C.

Formation of superoxide or hydrogen peroxide may be directly injurious to tissue; however, it is thought more likely that the mediators of tissue damage are the secondarily-derived oxidants such as hydroxyl radical and hypohalous acid. In fact, few studies have successfully demonstrated that human neutrophils use either O$_2^-$ or H$_2$O$_2$ alone to produce a toxic effect (Weiss, 1989). Myeloperoxide (MPO) is the enzyme responsible for the production of hypochlorous acid (HOCl), which is the most powerful oxidant generated in large quantities by the neutrophil, making up 5 per cent of a neutrophil's dry mass. Once discharged from the neutrophil MPO alone exerts little if any effect. However, in combination with H$_2$O$_2$ purified MPO can oxidize the halides Cl$, Br^-$, or I$^-$ to their corresponding hypohalous acids. Because the plasma concentration of Cl is more than 10$^3$ times that of the other halides, the H$_2$O$_2$-MPO system probably use Cl$^-$ at most sites in vivo.

The gastrointestinal tract is particularly well endowed with the enzymatic machinery necessary to form large amounts of ROMs. In addition to granulocytes, other sources of ROMs in the gastrointestinal tract include mucosal oxidases such as xanthine oxidase,
amine oxidase, and aldehyde oxidase. Recent work from many laboratories (Grisham & Granger, 1988) suggests that neutrophils may play a role in the development of tissue injury in a variety of disease states in the gastrointestinal tract, including: gastritis, necrotizing enterocolitis, ischaemia reperfusion injuries, Crohn's disease and ulcerative colitis. In these disease states, a popular hypothesis suggests that transient ischaemic episodes may initiate a cascade of self-perpetuating cycles of ROM formation, inflammation and, ultimately, mucosal injury (Grisham & Granger, 1988). *In vitro* studies suggest that 5-aminosalicylic acid is a potent scavenger of free radicals, and this has been proposed as its mechanism of action (Miyachi *et al.*, 1987). However, 4-aminosalicylic acid, which appears to have similar therapeutic activity, only has weak scavenging activity (Nielsen & Ahnfelt, 1988).

Clinically, evidence from almost 80 years ago in a large patient population also challenges the hypothesis that ROMs are the primary mediators of the damage observed in some inflammatory conditions. At the start of World War I, an alarming increase in the number of deaths due to infectious complications in wounded soldiers spurred a search for more effective disinfectants. Screening of more than 200 compounds for bactericidal activity *in vitro* led investigators to conclude that the most promising compound was HOCl (Dakin, 1915). HOCl was chosen without any knowledge of neutrophil metabolism and was used in large quantities at 0.5 per cent to perfuse a variety of wounds, ranging from deep flesh wounds, compound fractures, and suppurating joints to life-threatening gangrenous infections. *In vitro*, 0.5 per cent HOCl can solubilise strips of tissue, but *in vivo* it exhibits microbicidal activity without causing tissue damage or interfering with wound healing (Smith *et al.*, 1915). The high reactivity of HOCl seems to ensure that its toxicity is rapidly dissipated within a short distance of its site of generation. Given this information, it seems clear that ROMs alone do not appear to account for the observed damage.

Neutrophils also synthesise matrix-degrading metalloproteinase enzymes and neutrophil elastase. An alternative role for oxygen metabolites may be as the activators of proteolytic destruction by the polymorphonuclear neutrophil proteases (Weiss, 1989). HOCl seems to selectively inactivate antiproteases by the oxidation of methionyl residues on the antiprotease molecule to the sulphoxide with the loss of biological activity. Oxygen-derived free radicals may also activate the latent human neutrophil metalloproteinases, collagenase and gelatinase.

### 3.3.3 Eosinophils and basophils

Eosinophils are similar to neutrophils in that they contain conspicuous granules and have lobulated nuclei; as the name eosinophil implies, their granules have an affinity for
eosin. Eosinophils respond to chemotactic factors released from degranulating mast cells including histamine and eosinophil chemotactic factor (Goetzl & Austen, 1975). These mediators cause eosinophils to accumulate in the areas of IgE-mediated hypersensitivity reactions. Eosinophils play an important role in degrading certain mediators of inflammation, such as histamine, and probably play a key role in the control of immediate hypersensitivity reactions. Blood eosinophilia appears to be more closely related to atopic diseases associated with increased levels of IgE than to immunologic diseases mediated by other immunoglobulins. Eosinophils are particularly abundant in parasitic infections where they bind to IgE coated parasites and degranulate, releasing their toxic content causing damage to the parasites. Eosinophils are frequently attracted to chronic inflammatory lesions where they are thought to phagocytose immune complexes and thus limit the inflammatory response.

Basophils are also members of the granulocyte family, but are the least numerous. These cells share many characteristics with the tissue mast cell; their granules contain histamine and heparin. Like mast cells they can also produce slow reacting substance of anaphylaxis and contain eosinophil chemotactic factor and platelet activating factor. Basophils degranulate in response to the same stimuli that cause mast cells to degranulate, including anaphylatoxin, IgE binding, and certain immune complexes.

3.3.4 Macrophages

In inflammatory bowel disease there is an increase in the mucosal macrophage population (Thyberg et al., 1981). There is also an increase in monocyte turnover and activation from which the mucosal macrophage population is likely to be derived (Meuret et al., 1978). In the circulation, monocytes are relatively immature cells. When they leave the bloodstream and enter the tissues they differentiate into various types of macrophage, depending on the tissue. Like neutrophils, macrophages are motile phagocytic cells that defend the body against pathogens. Unlike neutrophils, however, they may live for several months and can divide in areas of inflammation. Macrophages respond to chemotactic stimuli such as cytokines and anaphylatoxins. Whereas neutrophils are the first leukocytes to enter an area of injury, macrophages are often the most numerous cells in the later stages of inflammation. The principal functions of macrophages in inflamed tissue are the removal of dead and dying cells, debridement, and acting as the primary line of defence against certain micro-organisms (for example, *Mycobacterium tuberculosis* and *Mycobacterium leprae*). The macrophage also secretes a large number of products that are proinflammatory, including: lysosomal enzymes, complement proteins, interferon, reactive oxygen metabolites, arachidonic acid metabolites, platelet activating factor, interleukin-1, plasminogen activator, and specific matrix metalloproteinases. Unlike neutrophils, macrophages lack myeloperoxidase and
lactoferrin but do utilise a hydrogen peroxide killing system. As with neutrophils, phagocytosis triggers a respiratory burst (Johnston, 1988).

3.3.5 Platelets

Platelets are small fragments of cytoplasm derived from megakaryocytes which reside in haematopoietic marrow. They also have the potential to be phagocytic and can ingest immune complexes, virus particles, and bacteria. In contrast to the other leukocytes, which migrate through intact blood vessels, platelets do not interact with normal vasculature; instead platelets interact with damaged blood vessels, adhering to sites of injury or disease (Roth, 1992). Normal blood vessels are lined by a continuous, non-thrombogenic, layer of endothelium. Disruption of this lining exposes subendothelial elements, such as extracellular collagen, to the circulating platelets in the blood. Platelets are able to distinguish between endothelium and subendothelium, avoiding the former and adhering avidly to the latter. This phenomenon keeps the surface of normal vessels free of platelets, while encouraging platelet deposition on exposed subendothelium. Depending on the vascular insult, attached platelets may initiate either normal, haemostatic events, or abnormal, thrombotic events. For example, platelet plugs at sites of vascular injury limit blood loss, while platelet masses on atherosclerotic vessels impair blood flow and contribute to coronary and cerebral ischaemic events (Packham & Mustard, 1986). Pathological thrombotic events may have a place in the aetiological events associated with Crohn's disease (Wakefield et al., 1991).

3.3.6 Lymphocytes in inflammation

The development of chronic inflammation gives rise to the presence of cells of the specific immune system, namely the lymphocytes. These cells are normally found throughout the body - in lymph nodes, the spleen, the bloodstream, lymphatics, Peyer's patches, tissues and secretions. There are three classes of lymphocytes. Two classes, the B-cells and T-cells, are programmed to respond to specific antigens. The third class of lymphocytes, killer cells and natural killer cells, do not recognise antigen.

B-cell precursors come from bone marrow and constitute 10 to 20 per cent of the lymphocytes in circulating blood. They give rise to antibody-producing plasma cells. Five distinct classes of antibody can be produced: IgM, IgG, IgA, IgD, and IgE, depending on the nature of the challenge. Antibody binding to specific antigens results in the neutralisation of bacterial toxins, virus inactivation, and phagocytosis of bacteria following opsonisation and complement activation. B-cells also have proinflammatory effects following binding to phagocytes, promoting degranulation. Inappropriate
antibody production is often associated with many inflammatory diseases, especially hypersensitivity reactions.

Most of the circulating lymphocytes are T-cells, which are present in the blood as well as the lymph. After the precursor cells leave the bone marrow they undergo further maturation in the thymus gland. Thus, they are named thymus-dependent lymphocytes or T-cells. T-cells have a potential life-span of a few months and recirculate continuously from the blood stream to the lymphatics. T-cells are the lymphocytes involved in cellular immune reactions. However, any given antigen usually activates both T-cells and B-cells, and in most cases they are functional partners in producing full immunological reactivity. A specific subpopulation of T-cells act as ‘helpers’ in antibody formation by providing cofactors for stimulating the proliferation and differentiation of B-cells. These have been designated T4-helper lymphocytes. Other T-cells, T8-suppressor lymphocytes, have the ability to suppress B-cell proliferation and antibody formation and thus can serve in a regulatory capacity controlling the immune process. T8-cytotoxic cells are primarily involved in killing virally infected cells. The T4 and T8 refer to surface markers on the T-cells. These surface markers have been designated CD4 and CD8.

The third class of lymphocytes include killer cells and natural killer cells. These lack the CD4 and CD8 surface antigens and are unable to recognise antigen. Killer cells possess receptors by which they can bind to antibodies and can recognise and attack cells coated with antibody. The important aspect of this is that these cells do not have to recognise specific antigens; once contact is made they are capable of recognising and attacking IgG-coated tumour cells and virus-infected cells non-specifically. This process is termed antibody-dependent cellular cytotoxicity. Natural killer cells are capable of lysing some tumor cells and virally infected cells in vitro, but their role in vivo is unclear. They differ from killer cells in that the Fc receptor of natural killer cells is not involved in cell lysis. They may represent a natural defence that is equivalent to acquired immunity, but is relatively non-specific. Natural killer cells do not require prior antigenic stimulation, but their activity is enhanced significantly by viral infection. Interferon and interleukin-2 also enhance the activity of natural killer cells.

Impaired natural killer cytotoxicity has been found in patients with inflammatory bowel disease in peripheral blood (Manzano et al., 1992). The natural killer cells in the intestinal mucosa differ from those in peripheral blood by the expression of different cell markers (Van Tol et al., 1992). Interestingly, high levels of spontaneous cytotoxicity have been found in the normal mucosa in Crohn’s disease patients, suggesting that immunologic abnormalities may be present even in histologically normal tissue.
An important immunological abnormality that has received considerable attention in the study of inflammatory bowel disease over the years concerns abnormal expression of immunoglobulin subclasses in the mucosa. Normally, few IgG-producing cells are found in the intestinal mucosa. In inflammatory bowel disease, there is a marked increase - by up to 30 times - in the number of IgG-producing cells. Furthermore, ulcerative colitis and Crohn's disease differ in that the former is characterised by an increase in IgG\textsubscript{1} and the latter by an increase in the expression of IgG\textsubscript{2} (Helgeland et al., 1992). The significance of these changes is unknown.

3.4 Mediators of Inflammation

Not only are the aetiologies of ulcerative colitis and Crohn's disease unknown, but the soluble mediators that amplify and modulate the inflammatory response have also not been fully explored (table 3.1). Soluble mediators of inflammation share certain biological effects. Several of these compounds increase vascular permeability, and some, including C5a and several arachidonate metabolites, are neutrophil chemotactic agents. A combination of these mediators is involved in most inflammatory processes, making it difficult to assign responsibility for any portion of the inflammatory process to a particular mediator. The large number of potentially important mediators complicates inflammatory bowel disease treatment, in that pharmacological agents directed against one mediator may have no effect on others.

<table>
<thead>
<tr>
<th>Chemical mediators of inflammation</th>
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<tbody>
<tr>
<td><strong>Cells</strong></td>
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<tr>
<td>Stored</td>
</tr>
<tr>
<td>Histamine</td>
</tr>
<tr>
<td>5-hydroxytryptamine (Serotonin)</td>
</tr>
<tr>
<td><strong>Synthesised on demand</strong></td>
</tr>
<tr>
<td>Eicosanoids and PAF</td>
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<tr>
<td>Cytokines</td>
</tr>
<tr>
<td>Reactive oxygen metabolites</td>
</tr>
<tr>
<td><strong>Plasma</strong></td>
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<tr>
<td>Kinins</td>
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<tr>
<td>Complement</td>
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*Table 3.1: The chemical mediators of inflammation.*
3.4.1 Chemical mediators of the vascular response

The participation of the kinins in the initiation and maintenance of the inflammatory process has been confirmed by many reports. Despite their rapid inactivation by a variety of peptidases, local generation of kinins may be maintained during the acute and chronic phases of inflammation (Wedmore & Williams, 1981). Indeed the kinins may be the only endogenous system which is continuously present and active at the site of inflammation. Bradykinin is formed from a precursor, high-molecular-weight kininogen, through the action of the plasma enzyme kallikrein (Rocha e Silva et al., 1949). Plasmin also has the capacity to generate bradykinin from the precursor molecule. A second kinin, kallidin can be generated from another precursor, low-molecular-weight kininogen, under the influence of tissue kallikreins. Both kinins produce qualitatively similar effects by their ability to enhance the vascular permeability of post-capillary venules. This increase in permeability may be the result of endothelial cell contraction and a widening of intercellular junctions, changes which can be prevented by β-adrenergic agonists. The kinins are also some of the most potent naturally occurring pain-producing species (Steranka et al., 1988).

Histamine and 5-hydroxytryptamine are vasoactive amines that also mediate the increase in vascular permeability of acute inflammation and their release is stimulated by, among other peptides, the kinins. The actions of histamine on the microcirculation are of particular importance. After an inflammatory insult or injury, mast cells accumulate and degranulate near the arteriolar bed. Vasodilatation occurs. There is an increased escape of fluid from the post-capillary venules because of endothelial cell contraction. Vasodilatation is mediated by the activation of histamine H1- and H2-receptor types (Black et al., 1972). By contrast, the increase in vascular permeability is a response to histamine H1-receptor activation alone. Many of the vascular effects of histamine are enhanced by the prostaglandins (Williams & Morley, 1973). Histamine can inhibit its own release by negative feedback and may reduce basophil and eosinophil granulocyte and polymorph chemotaxis and enhance chemokinesis. The role of histamine in inflammation is complex and its analysis difficult. It is likely that histamine, like other inflammatory mediators, has pro-inflammatory and anti-inflammatory properties.

Histamine may be involved in the pathogenesis of ulcerative colitis, since an increased number of mast cells are present in the colon, and some patients benefit from treatment with the anti-allergic drug, disodiumcromoglycate (Heatley et al., 1975). To investigate the possible role of histamine in ulcerative colitis, the release of histamine from the wall of the rectum has been measured using an in vivo rectal dialysis technique to collect the exudate (Rampton et al., 1980). Increased levels of histamine were found in patients with proctocolitis in all disease states. The highest levels were in those patients with
active disease, but these were not significantly different from levels in remission, and
some patients with active disease had normal levels of histamine. The effect of treatment
with disodiumcromoglycate on histamine release, eosinophil count and general disease
activity has been determined in patients with active ulcerative colitis (Rampton et al.,
1982). Patients were given an oral dose of the drug for two weeks. Histamine release,
stool consistency, urgency of defecation, rectal bleeding, mucosal appearance on
sigmoidoscopy and mucosal function shown by rectal potential difference and electrolyte
transport were all unchanged by the drug treatment. Disodiumcromoglycate did reduce
the eosinophil content, which is elevated during active ulcerative colitis, but overall the
use of disodiumcromoglycate was not of benefit, suggesting that histamine is not a
primary factor.

3.4.2 Complement

In inflammation the complement products of greatest interest are the anaphylatoxins
C3a, C4a and C5a. Their main effects include mediating increased vascular permeability
and chemotaxis. The initial vascular changes are, in part, a consequence of their
stimulating effect on histamine release and other mediators from mast cells and basophils
(Johnson et al., 1975). A prolonged increase in permeability may be caused by the release
of permeability factors from neutrophils following C5a stimulation. C5a is the most
potent anaphylatoxin in terms of chemotaxis and vascular permeability (Williams &
Jose, 1981). The anaphylatoxins are susceptible to degradation by carboxypeptidase N to
form the des-Arg derivatives. When injected into the skin they trigger an inflammatory
weal-and-flare reaction, macroscopically and microscopically indistinguishable from the
reaction caused by the native anaphylatoxin (Swerlick et al., 1988). However, it is now
accepted that the des-Arg derivatives of all the anaphylatoxins do not stimulate
production of histamine from mast cells (Damerau et al., 1980). This evidence lends
further support to the view that histamine may not have a role to play in anaphylatoxin-
induced inflammatory reactions in vivo. Two of the anaphylatoxins, C3a and C5a, may
have immunoregulatory properties (Folkman et al., 1985): C3a suppresses, and C5a
enhances, antibody responses. C3a is, however, unable to suppress cellular proliferative
responses such as the T-cell response to phytohaemagglutinin or T-cell and B-cell-
proliferative responses to pokeweed mitogen. C5a enhances antigen-induced immune
cell proliferation but has no effect on non-specific proliferation. Unconfirmed reports
suggest that in inflammatory bowel disease there may be defects in C5a generation
(Elmgreen et al., 1983), but the specificity of this observation and the pathogenic
relevance remain to be established.
3.4.3 Cytokines

There is increasing evidence that the pathogenesis of chronic inflammatory bowel disease is associated with persistent lymphocyte and macrophage activation (Brynskov et al., 1992). The activated leukocytes release proteins that exert profound, often remote, effects on other leukocytes as well as on non-leukocyte cells such as fibroblasts. The principal role of the majority of these mediators, known as cytokines, is to influence the cells of the immune system in the activation of T-cells, B-cells and macrophages. Investigations characterising the release of cytokines have been a popular area of inflammatory bowel disease research, both to improve our understanding of their involvement in the disease process and to identify new markers of disease activity.

In acute inflammation interleukin-1 is produced, mainly by activated macrophages. It has a broad range of proinflammatory effects which may amplify and promote continued inflammation in the gut. In experimental animal models of colitis as well as in patients with inflammatory bowel disease, the production of interleukin-1 by peripheral blood mononuclear cells and lamina propria mononuclear cells is increased (Gionchetti et al., 1992; Rachmilewitz et al., 1989). Tissue samples obtained from areas of active inflammation have also been shown to possess increased levels of interleukin-1, which correlate with degree of inflammation, as well as with clinical disease activity (Nakamura et al., 1992); however, there is little evidence to suggest a direct pathogenic effect of interleukin-1.

Interleukin-1 receptor antagonist is a glycoprotein that competitively binds to interleukin-1 receptors located on T-cells, B-cells, mesenchymal cells, macrophages, and neutrophils. It competitively inhibits binding of interleukin-1 to its receptor and suppresses the proinflammatory effects of interleukin-1. In a rat acetic acid-induced colitis model, pretreatment with interleukin-1 receptor antagonist was associated with decreased inflammation and necrosis (Thomas et al., 1991). This result suggests a potential therapeutic benefit for an interleukin-1 receptor antagonist in human inflammatory bowel disease.

Interleukin-2 is produced mainly by activated lymphocytes, it stimulates the proliferation and differentiation of T-cells and increases the activity of natural killer cells. The mechanisms of action of interleukin-2, and its involvement in the intricate cascade of immunological events, have not been clearly defined. Many studies using peripheral blood mononuclear cells, lamina propria mononuclear cells, serum levels, and tissue levels of interleukin-2 have been performed, in attempts to characterise its role in inflammatory bowel disease (Kusugami et al., 1991; Mullin et al., 1992; Nakamura et al., 1992). Conflicting results have been obtained, possibly because observations using
isolated lamina propria mononuclear cells may not truly represent events happening in vivo or because these cells have been isolated from surgical specimens, which may have been exposed to non-physiological environments during the isolation and culture procedure. Similar criticisms have been levelled against the use of peripheral blood mononuclear cells to study physiological interleukin-2 production; these cells may not truly represent the mononuclear cells present in the inflamed gut tissue. Finally, conflicting observations may also arise from differences in the current therapeutic approaches being used in individual patients.

Interleukin-2 receptor is a protein expressed on the surface of activated T-cells. The precise role of interleukin-2 receptor is not well characterised. Soluble interleukin-2 receptor has been detected in serum from patients with inflammatory bowel disease as well as in serum from patients with other inflammatory disorders such as rheumatoid arthritis, infectious colitis, and some viral infections (Crabtree et al., 1990). In studies examining fresh biopsy specimens, there was a trend towards increased interleukin-2 receptor tissue levels in patients with inflammatory bowel disease, correlating with areas of gross inflammation. However, no clear correlation between tissue levels of interleukin-2 receptor and clinical activity has been observed (Matsuura et al., 1992).

Recent research has focused on interleukin-6 and interleukin-8 as markers of disease activity and possible markers of disease specificity. Interleukin-6 is a cytokine that activates other immune cells and stimulates the production of hepatic acute-phase reactants. Serum levels of interleukin-6 have been shown to be increased in active Crohn's disease, but not in active ulcerative colitis (Mahida et al., 1991a). However, these levels did not correlate with disease activity or levels of acute-phase reactants. In another study, tissue levels of interleukin-6 were found to be elevated in both active Crohn's disease and ulcerative colitis (Mitsuyama et al., 1991). These levels correlated well with areas of gross inflammation as well as with disease activity. Similarly, tissue interleukin-6 messenger RNA expression has been found to be increased both in active ulcerative colitis and in active Crohn's disease, but correlated with mucosal disease activity only in ulcerative colitis (Isaacs et al., 1992).

Interleukin-8 is a proinflammatory cytokine that has been shown to be a potent chemoattractant agent. Tissue levels of interleukin-8 have been shown to increase in active ulcerative colitis but not in active Crohn's disease (Mahida et al., 1992). As with interleukin-6, tissue expression of interleukin-8 messenger RNA was increased in active ulcerative colitis and Crohn's disease, but was correlated with mucosal disease activity only in ulcerative colitis (Isaacs et al., 1992). Contrary to these findings, serum levels of interleukin-8 were not found to be elevated in either ulcerative colitis or Crohn's disease (Mahida et al., 1992).
Tumour necrosis factors α and β induce fever, increased acute phase protein synthesis, and endothelial cell activation. Most studies examining the role of tumour necrosis factor α and β in inflammatory bowel disease have failed to demonstrate a difference between the serum and tissue levels in patients with the disease and the corresponding levels in control groups (Hyams et al., 1991; Murch et al., 1991). Tumour necrosis factor α is a less potent inflammatory agent than interleukin-1, although it can dramatically enhance leukocyte infiltration in response to interleukin-1 after intra-articular injection (Henderson & Pettipher, 1989).

Interferons produced by leukocytes (IFN-α), mononuclear cells (IFN-γ), and fibroblasts (IFN-β) are responsible for immune and epithelial cell differentiation and expression of class II surface antigens. These functions are important for effective cell-mediated cytotoxicity and in the inflammatory process; therefore, the role of interferon as a mediator of these events and its role in the pathophysiology of inflammatory bowel disease have been widely investigated. Studies using isolated peripheral blood mononuclear cells, and lamina propria mononuclear cells, to measure interferon production in patients with inflammatory bowel disease have been conflicting. Earlier work demonstrated that cultured lamina propria mononuclear cells, but not peripheral blood mononuclear cells spontaneously-produced increased levels of interferon in patients with Crohn's disease (Fais et al., 1991). However, others report that spontaneous production of interferon from isolated peripheral blood mononuclear cells and lamina propria mononuclear cells was not significantly different in patients with ulcerative colitis or Crohn's disease from corresponding control groups (Nakamura et al., 1992).

Although all the cytokines discussed here are potentially important mediators in the inflammatory process, the precise roles of each in the pathogenesis and pathophysiology of inflammatory bowel disease are unclear. The conflicting results in the cytokine studies discussed here probably reflect the wide variety of techniques used to isolate and measure cytokines, and the variety of sources (for example, blood or tissue).

3.4.4 The eicosanoids

At present there is a variety of anti-inflammatory medications, ranging from sulphasalazine and its derivatives through non-steroidal anti-inflammatory drugs (NSAIDs) to glucocorticosteroids, all of which appear to derive at least a part of their effectiveness from their ability to limit the body's synthesis of arachidonic acid derivatives. In ulcerative colitis and Crohn's disease, the effectiveness of available medications is limited. In addition, side effects remain a problem, even with the newer medications.
The elucidation of the arachidonic acid metabolic pathways is one of the most important developments in inflammation research over the past three decades (figure 3.1). Prostaglandins, thromboxanes, leukotrienes and the lipoxins are all metabolites of eicosatetraenoic acid (arachidonic acid); hence the collective term eicosanoids is used to describe them. This unsaturated fatty acid is derived mainly from cell membrane phospholipids, and release of arachidonic acid is the rate-limiting step in eicosanoid production. Eicosanoids are not stored in the body, and so an increase in levels in response to a stimulus occurs through their rapid biosynthesis. Arachidonic acid is usually located on the second carbon atom of phospholipids, and release depends on the activation of specific phospholipases, such as phospholipase A$_2$ (Flower & Blackwell, 1976). There are several ways in which this release may be stimulated (for example, through mechanical stress or receptor-linked release), but since phospholipase A$_2$ activity is dependent on the level of intracellular calcium, it is likely that the main stimuli all elevate the level of intracellular calcium. The phospholipase A$_2$ enzymes can generally be divided into two major groups, soluble phospholipase A$_2$ and cytosolic phospholipase A$_2$ (Clark et al., 1991b; Sharp et al., 1991). There are clear functional differences in vitro between the two forms, but a specific inhibitor of cytosolic phospholipase A$_2$ or soluble phospholipase A$_2$ is required to help discern the relative contributions of the different forms of phospholipase A$_2$ to physiological release of arachidonic acid. Calcium ionophores, such as A23187, are thought to stimulate eicosanoid production at this stage in the pathway by increasing intracellular calcium and activating phospholipase A$_2$.

One branch of the arachidonic acid cascade pathway leads via cyclooxygenase to production of prostaglandins, the biological activity of which was first recognised in the 1930s (Goldblatt, 1934; Von Euler, 1934), as well as to production of thromboxanes (Hamberg et al., 1975) and prostacyclins (Moncada, 1976). Extensive research pursued throughout the 1970s led to recognition of the varied roles these substances play in normal homeostatic regulation as well as in inflammation.

Cyclooxygenase is a bifunctional enzyme with cyclooxygenase activity catalysing conversion of arachidonic acid to PGG$_2$ by the incorporation of oxygen, and peroxidase activity converting PGG$_2$ to PGH$_2$. Iron(III)-protoporphyrin IX is required for both activities. In the presence of PGE and PGD isomerases, PGE$_2$ and PGD$_2$ are formed respectively. PGF-reductase acts on endoperoxide to produce PGF$_{2\alpha}$. All these end products are relatively stable and biologically active. A less stable biologically active product is prostacyclin (PGI$_2$), formed by the enzyme prostacyclin synthetase (Moncada, 1976). This has a half-life of only 3 minutes and is hydrolysed to the inert product 6-keto PGF$_{1\alpha}$, which may be assayed. Thromboxane A$_2$ is formed in the presence of thromboxane A synthetase. This has an oxane ring instead of a cyclopentane structure.
and is hydrolysed very rapidly to thromboxane B\textsubscript{2}, which is inert and stable. The lung and spleen are able to synthesise the whole range of cyclooxygenase products, but other tissues are more selective; for example platelets synthesise mainly thromboxane A\textsubscript{2}, and blood vessel walls synthesise primarily prostacyclin. The inactivation of prostaglandins occurs to a large extent enzymatically in the lung. One passage through the pulmonary circulation removes 80 to 90 percent of the prostaglandins (Samuelson \textit{et al.}, 1979), although enzymes that catalyse degradation are widely distributed in the body. In 1971 three publications demonstrated that low concentrations of aspirin could inhibit prostaglandin production (Ferreira \textit{et al.}, 1971; Smith & Willis, 1971; Vane, 1971). In fact, all ‘aspirin-like’ drugs or non steroidal anti-inflammatory drugs (NSAIDs), are able to suppress prostaglandin production, by inhibition of cyclooxygenase. However, the suggestion that inhibition of colonic prostanoid synthesis is an important mechanism to explain the therapeutic efficacy of these drugs is challenged by the observation that in inflammatory bowel disease non-steroidal anti-inflammatory drugs are of no therapeutic benefit and even induce clinical deterioration.

Two homologous cyclooxygenase enzymes - cyclooxygenase 1 and cyclooxygenase 2 - are now known to exist, although the details of their functions have not been established. Cyclooxygenase-1 is constitutively expressed and cyclooxygenase-2 is inducible (O’Neill & Ford-Hutchinson, 1993). Both cyclooxygenase-1 and cyclooxygenase-2 expression are inhibited by glucocorticosteroids. The selective inhibition of cyclooxygenase-2 may give rise to new anti-inflammatory drugs with a therapeutic action in inflammatory bowel disease, while maintaining the homeostatic role of low-level prostaglandin release.

Prostaglandins appear to be associated with the acute phase of inflammation in which acute inflammatory cells infiltrate an injured site. The maximum increase in vascular permeability occurs simultaneously with the onset of prostaglandin release (DiRosa, Giroud, & Willoughby, 1971).
MEMBRANE PHOSPHOLIPID

\[ \text{Phospholipases (A}_2\text{, C)} \]

\[ \text{ARACHIDONIC ACID} \]

\[ \begin{align*}
   &\text{15-HPETE} \rightarrow 15\text{-HETE} \\
   &\text{12-HPETE} \rightarrow 12\text{-HETE} \\
   &\text{5-HPETE} \\
   &\text{PG I}_2, \text{PG D}_2, \text{PG F}_{\alpha\alpha}, \text{TxA}_2 \\
   &\text{PGE}_2, \text{6-KETO PG F}_{1\alpha}, \text{TxB}_2, \text{LTA}_4, \text{LTC}_4, \text{LTD}_4, \text{LTI} \\
\end{align*} \]

\[ \begin{align*}
   &\text{COOH} \\
   &\text{OH} \\
   &\text{CH}_3 \\
\end{align*} \]

**Figure 3.1: The biosynthetic pathways of eicosanoid metabolism leading to formation of prostaglandins (PGs), prostacyclin (PGI\textsubscript{2}), thromboxanes (Txs) and leukotrienes (LTs).**

Prostaglandin E\textsubscript{2} and PGI\textsubscript{2} cause dilatation of the microcirculation; effects are observed at doses of 0.1 to 10ng. When injected into human skin, both PGE\textsubscript{2} and PGI\textsubscript{2} induce
erythema (Higgs et al., 1979; Solomon et al., 1968). Prostaglandin D2 also induces erythema in human skin, although it is less potent than PGE2. Blood flow changes induced by PGs in rabbit skin have been measured directly using 133Xenon clearance; it was found that PGE2 and PGI2 were potent vasodilators, whereas PGF2α and PGD2 were effective only at high doses (Williams, 1979). The relative contributions of different PGs to hyperaemia may depend on the species, the tissues involved, and the nature of the inflammatory reaction.

Prostaglandins cause oedema by increasing the vascular permeability; however, they are not as effective as histamine, bradykinin or 5HT. Moreover, prostaglandins enhance the increased permeability produced by other mediators (Williams & Peck, 1977). This ability to potentiate reactions is their principal role in inflammation, and they also enhance pain produced by histamine and bradykinin and the itching caused by histamine (Ferreira, 1972). The hyperalgesic effect of PGE2 is cumulative and long-lasting, whereas PGI2, which is a more potent hyperalgesic agent, has a short-lived action (Ferreira et al., 1978).

In vitro, prostaglandins have been shown to be chemotactants for leukocytes, but only at much higher concentrations than occur in an inflammatory reaction. Inhibitors of prostaglandins have no effect on the movement of the polymorphonuclear leukocytes and only a small effect on monocyte migration (Salmon et al., 1983); therefore, the effect of prostaglandins on cell motility seems unlikely to be of significance in an inflammatory reaction.

Surprisingly, prostaglandins of the E series and PGI2, given systemically, can suppress acute and chronic inflammatory reactions. Early experiments indicated that high systemic doses of PGE1 or PGE2 could suppress adjuvant arthritis in rats. Although these effects could be due to a non-specific reduction in blood pressure, other evidence indicates that leukocyte-dependent oedema in the rabbit can be suppressed by 15-methyl PGE1 (a synthetic analogue of PGE1) or by PGI2 at doses which are not hypotensive (Rampart & Williams, 1986). The latter data are consistent with the finding that PGEs and PGI2 suppress neutrophil responses to a range of stimuli in vitro. However, much lower doses of PGEs are required to inhibit the proliferation of lymphocytes and the production of lymphokines than are required to suppress neutrophil function. Therefore, inhibition of lymphocyte function by PGEs in vivo may be a more likely explanation for the effect of these prostanoids on adjuvant arthritis. Prostaglandin E2 also suppresses other autoimmune diseases such as murine nephritis, and this correlates with inhibition of T-cell function rather than inhibition of immune complex formation (Kelly et al., 1987). Prostaglandins E1, E2 and PGI2 decrease macrophage activation. PGE2 inhibits interleukin-1 mediated inflammation (Otterness et al., 1988) and the production of
mature TNF-α protein in macrophages (Kunkel et al., 1987). Clearly, the findings that prostaglandins can suppress lymphocyte and macrophage function may have implications for NSAID therapy.

Prostaglandins, in addition to their role in inflammatory disease, have other biological effects on the gastrointestinal system. Prostaglandins E₂ can inhibit gastric secretion when administered intravenously, possibly by preventing the reactions of the parietal cells, and can exert a cytoprotective effect on the epithelium of the stomach, the small intestine, and possibly the large intestine, which prevents ulcer formation (Hawkey & Rampton, 1985).

In patients with active ulcerative colitis and Crohn’s disease intestinal prostaglandin generation is increased, as shown by measurements of their levels in fresh tissue, organ culture of biopsies, rectal dialysis and quantitation in stool (Donowitz, 1985). The enhanced prostanoid production in active inflammatory bowel disease was shown to be due mainly to the increased number of stimulated mononuclear cells in the inflamed bowel. In remission, colonic prostaglandin generation is similar to that in normal subjects. Activity of adenylate cyclase, the enzyme responsible for the synthesis of cyclic-AMP, is stimulated in active ulcerative colitis, and this stimulation is mediated by the increased prostaglandin generation. The enhanced colonic prostaglandin generation in active ulcerative colitis may also contribute to the pathogenesis of the diarrhoea by inhibiting activity of Na-K-ATPase, the enzyme responsible for the absorption of sodium (Rachmilewitz, 1980).

The lipoxygenase pathway, the other major pathway of the arachidonic acid cascade, was identified by Borgeat and Samuelsson in 1979 (Borgeat & Samuelsson, 1979). The lipoxygenase pathway is further divided into three pathways, each of which is initiated by the oxygenation of arachidonic acid by 5-, 12-, and 15-lipoxygenases (figure 3.1).

The leukotrienes generated from the 5-lipoxygenase branch of the arachidonic acid cascade fall into two distinct series: a 5, 12-dihydroxy compound with the conjugated double bonds at the 6, 8, and 10 positions; and another consisting of 5-sulphidopeptide (cysteinyl) compounds with double bonds at the 7, 9, and 11 positions (figure 3.1).

The 5-lipoxygenase pathway begins with insertion of a dioxygen group at the 5 position by the enzyme 5-lipoxygenase, creating 5-hydroperoxyeicosa-6,8,11,14-tetraenoic acid (5-HPETE); 5-HPETE in turn either is reduced to the side-product 5-hydroxyeicosa-6,8,10,14-tetraenoic acid (5-HETE) or rearranges to 5,6-epoxyeicosa-7,9,11,14-tetraenoic acid (leukotriene A₄). It is at this point that the main pathway branches: allylic hydrolysis of LTA₄ by LTA hydrolase produces the dihydroxy compound LTB₄,
while action of LTC$_4$ synthetase (glutathione transferase) replaces the oxygen with a glutathione moiety to produce the first of the leukotriene sulphidopeptides, LTC$_4$. Successive removal of the γ-glutamyl and glycyl residues then produces LTD$_4$ and LTE$_4$, respectively.

The 5-lipoxygenase enzyme is found in the cytosolic fraction, although its substrate, arachidonic acid, is associated with membrane phospholipids. Recent investigations have defined a 5-lipoxygenase-activating protein and suggested that the binding of 5-lipoxygenase to 5-lipoxygenase-activating protein on the membrane is essential for cellular leukotriene synthesis (Rouzer et al., 1990). The development of a 5-lipoxygenase-activating protein-specific inhibitor (MK-886) represents a significant improvement over previous 5-lipoxygenase inhibitors; this development allows for the assessment of the effectiveness of a specific inhibitor of leukotriene synthesis that does not act via redox-related inhibition of 5-lipoxygenase and does not inhibit super-oxide anion generation by neutrophils. MK-886, through its interaction with 5-lipoxygenase-activating protein, prevents 5-lipoxygenase translocation from the cytosol to the membrane and thereby inhibits leukotriene synthesis.

Leukotrienes of the two series typically exert different effects. LTB$_4$ tends to exert classically proinflammatory effects, whereas sulphidopeptide leukotrienes act primarily as smooth muscle constrictors.

LTB$_4$ is probably the most potent leukotriene. Among the effects of LTB$_4$ on cells of the immune system are stimulation of chemokinesis and chemotaxis of polymorphonuclear neutrophil movement towards the site of leukotriene release (Ford-Hutchinson et al., 1984). LTB$_4$ enhances polymorphonuclear neutrophil adherence to vascular epithelium, especially in areas of tissue injury (Grimbourne et al., 1984). This adherence presumably plays a role in LTB$_4$-induced vascular permeability, since the resulting oedema does not occur when polymorphonuclear neutrophils are depleted beforehand. Adherence is the first step in the migration of polymorphonuclear neutrophils into extravascular tissue. On arriving in regions of high LTB$_4$ concentration, these cells are stimulated to release lysosomal enzymes and to produce oxidants such as superoxide (see below). LTB$_4$ also stimulates chemotaxis and oxidant production by eosinophils.

Interactions of LTB$_4$ with other parts of the immune and inflammatory systems are complex. For example, LTB$_4$ promotes clonal expansion of both CD4+ (helper) and CD8+ (suppressor/killer) T-lymphocytes. It then stimulates interferon-γ production by helper cells, but suppresses production of this substance by suppressor/killer cells (Rola-Pleszcynski et al., 1987). LTB$_4$ also stimulates the production of both interleukin-1 and
interleukin-2 which have further proinflammatory effects (Rola-Pleszczyński & Chavaillaz, 1986).

The contractile effects of the leukotrienes are varied in gastrointestinal tissue. LTC₄, LTD₄ and LTE₄ cause contraction of the longitudinal muscle of isolated guinea-pig ileum (Piper & Samhoun, 1983), whereas neither LTC₄ nor LTD₄ cause contraction of the rat duodenum or ileum (Goldenberg & Subers, 1982). In a recent study the relative mechanical properties of the longitudinal and circular muscle layers of the human and rabbit colon were found to be similar. Furthermore, with respect to a range of inflammatory mediators including the sulphidopeptide leukotrienes, the pharmacological profile of the rabbit distal colonic muscularis propria in vitro is similar to that in humans (Percy et al., 1990). In the rat model of inflammatory bowel disease induced by the administration of TNB, colonic transit disturbances were blocked by two different LTD₄ receptor antagonists (Pons et al., 1992).

Like LTB₄, the sulphidopeptide leukotrienes exhibit complex interactions with other components of the inflammatory process. For example, these compounds stimulate production of platelet activating factor (PAF) by the vascular endothelium (McIntyre et al., 1986), while PAF in turn stimulates leukotriene production by lung tissue (Sirois, 1987).

The leukotrienes exert their activities through specific cell-surface receptors. Polymorphonuclear neutrophils possess two distinct LTB₄ receptors: a high-affinity, low-density receptor which is believed to function in chemotaxis and adherence; and a low-affinity, high-density receptor which mediates the release of oxidants and lysosomal enzymes (Bomalaski & Mong, 1987). This dual mechanism allows leukotrienes to attract polymorphonuclear neutrophils originally at considerable distances from the inflammatory site, without stimulating release of their inflammation-related products until locally high leukotriene levels occur. Eosinophils, however, appear to have only a single LTB₄ receptor. The number of receptors for the sulphidopeptide leukotrienes remains unknown.

Many groups have demonstrated that lipoxygenase products are present in the mucosa of patients with ulcerative colitis or Crohn's disease (Boughton-Smith et al., 1983; Peskar et al., 1987; Sharon & Stenson, 1984). However, their role in the inflammatory response of inflammatory bowel disease is less clear. Therapies currently available for ulcerative colitis and Crohn's disease, such as sulphasalazine and glucocorticosteroids, have some 5-lipoxygenase inhibiting activity. In all cases, however, the 5-lipoxygenase inhibition is weak and is coupled to other biochemical and pharmacological activities. The value of 5-lipoxygenase inhibition has yet to be demonstrated in human clinical trials, but
promising results have been obtained with a variety of compounds in various animal models (Laursen et al., 1990; Morteau et al., 1993; Wallace & Keenan, 1990; Wallace et al., 1989).

3.4.5 Neurogenic factors

For many years, inflammatory processes have been thought of purely in terms of interactions involving cells of the immune system and the chemical mediators that they release. Increasingly, however, neural elements have been shown to play an important part in the inflammatory process. Inflammation is often associated with pain and hyperalgesia, and it is clear that some of the agents produced during inflammation can activate nociceptive sensory neurones, either directly or indirectly. Thus, there is a complex interaction between nerves and other cell types. The role of neuronal mediated inflammation in inflammatory bowel disease is becoming an increasingly important subject for investigation (Bjorck et al., 1992; Yoshiro et al., 1992).

3.5 Regeneration and repair

Wound healing is an important component of inflammation. Although often thought of as a distinct reaction, it commences soon after injury while acute injury is still evident, but healing cannot be completed until the injurious agent has been neutralised or destroyed (Robbins et al., 1989). The essential features of regeneration and repair are the same for all soft tissues. Since many of the histological events in the healing process have been determined from the study of skin wounds, the general features of skin healing are described below, followed by an account of the way in which intestinal healing differs.

Primary wound healing, or healing by first intention, occurs in surgical wounds when the edges are correctly apposed. This is the desirable result in surgical practice. The events can be split into phases, although each phase merges smoothly into the next. In the first phase of healing, angiogenesis occurs as new blood vessels bud from capillaries in the wound edges. Lymphatic channels develop more slowly, and drainage of the wound does not take place in the early stages of healing. Undifferentiated mesenchymal cells from the perivascular connective tissue appear in the wound towards the end of the first phase and differentiate into fibroblasts.

The second phase of healing is characterised by fibroplasia and begins by the fifth day. Macrophages become less predominant and give way to fibroblasts. The fibroblasts synthesise proteoglycans and new collagen, recognised histologically as reticulin, while the new vessels become canalised and some develop into arterioles and venules. Collagen production increases, although the fibres are still randomly arranged. One to two weeks after a surgical wound, the second phase gives way to the third phase of healing. Cell
numbers diminish and much of the new vasculature disappears, leaving a dense network of collagen fibres in ground substance that together constitute the relatively avascular scar tissue. A slow process of organisation and remodelling occurs, removing the excess collagen and, in skin, leaving those fibres which are correctly oriented along lines of tension. The remaining few inflammatory cells work to remove any residual wound debris. This phase is on-going, gradually merging with normal turnover and remodelling processes.

In wounds where the extent of tissue loss prevents the apposition of the edges, healing takes place by secondary intent. Granulation tissue fills the defect and the inflammatory reaction is intense. A process of contraction to reduce the size of the wound takes place, brought about by the presence of myofibroblasts, but fibrous tissue formation is also greater and the resulting scar is correspondingly more pronounced.

In skin wounds epithelial repair accompanies the processes described and normally within 24 hours the basal epidermal cells at the edge of the wound begin to migrate over the wounded dermal surface, enlarging and multiplying to produce a thickened advancing edge. The edges meet and bridge the defect and the thickened epidermis fills the surface of the wound as keratinisation begins.

Intestinal healing following a wounding procedure follows essentially the same sequence of events as in skin wounds. Cellular infiltration takes place over the first two to three days, creating a reparative framework of macrophages and fibroblasts with new vessel formation. The surface columnar epithelial cells do not migrate but mitosis in the glandular crypts provides cells for the restoration of epithelial continuity.

Healing is tightly controlled by chemical mediators. Cytokines, notably interleukin-1, have far-reaching effects on connective tissue formation, encouraging proliferation of fibroblasts and increasing collagen and fibronectin synthesis. Interleukin-1 also promotes collagenase production, increasing connective tissue destruction (Dayer & Seckinger, 1988; Pasternak et al., 1986). The situation is clearly complex since the same mediators contribute to both sides of the equation and intricate feedback mechanisms are necessary (Krane et al., 1988).

3.6 Summary

The inflammatory reaction is a complex process encompassing humoral, cellular, and neural responses. The initial reaction to injury is a transient vasoconstriction, but it is followed rapidly by vasodilatation of the arterioles and venules. Dilated vessels leak increased amounts of fluid and plasma proteins into the connective tissues. Movement of the leukocytes from the vessels into the injured tissue follows, and is directed by the
release of chemotactic factors in the tissues. The polymorphonuclear neutrophils and the newly arrived or resident macrophages engulf microbial agents, damaged cells, and extracellular materials, and may release products that further amplify the inflammatory reaction. In varying degrees mast cells, eosinophils and basophils, lymphocytes, and platelets also play important roles, depending on the nature of the inflammatory response. Vascular and cellular phases of inflammation are co-ordinated by the release of a large number of pharmacologically active inflammatory mediators, such as the eicosanoids, from both resident and immigrant cells. However, it is difficult to ascribe any of the features of inflammation to the release of a single mediator. Increased concentrations of many of these mediators have been shown in inflammatory bowel disease, although whether these are primary or secondary occurrences is unclear.

Repair begins while inflammation is still present and is on-going, gradually merging with normal turnover and remodelling processes. Thus, tissue injury is followed by a set of interrelated cellular and humoral reactions that remove or neutralise injurious agents and eliminate damaged tissue in order to promote healing.
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4 COMPOSITION AND TURNOVER OF CONNECTIVE TISSUE

4.1 General

Connective tissue provides much of the structural framework for the body. Its degradation and subsequent remodelling is a significant feature of the inflammatory bowel diseases (Bailey, 1992). The matrix metalloproteinases are a family of enzymes that, together, are able to degrade all the components of the extracellular matrix. In this chapter an overview of the structure and function of connective tissue will be presented with emphasis on the components of the extracellular matrix and the ability of the matrix metalloproteinases to degrade them.

4.2 Connective tissue structure and function

Connective tissue is composed of cells and extracellular materials (fibres and ground substance) that provide the supporting and connecting framework for all the other tissues of the body. Thus, epithelia, endothelia, mesothelia, nerves, and muscle either rest on or are surrounded by connective tissue. The extracellular fibres are assembled mainly from collagen and elastin. The ground substance, which fills the spaces between cells and fibres, contains soluble precursors of the fibrous proteins, proteoglycans, glycoproteins, other molecules secreted from the cells, and molecules passed by the vascular filters. The cellular composition of adult connective tissue includes, depending on site, the fibroblast, chondroblast, osteoblast, odontoblast, reticular cell, and adipocyte. Macrophages and mast cells are also classified as indigenous cells of connective tissues, although they originate from bone marrow precursors. The materials that form the extracellular matrix are synthesised by the resident cells. The concept of the extracellular matrix as an inert supporting structure is known to be inaccurate (Hay, 1991). Interactions between the cells and the extracellular matrix components influence cell metabolism, shape and differentiation. The proportion of cells to fibres and ground substance, and the amounts, kinds, and organisation of the extracellular materials vary greatly from tissue to tissue, reflecting the function of the tissue.
4.2.1 Collagen

Collagen is a protein that forms the majority of the extracellular matrix in practically every tissue of the body. Depending on the structural and functional requirements of a particular tissue, the fibres may be abundant, as in dense connective tissue, or sparse, as in loose connective tissue. In tendons and ligaments, the collagen fibres are thick and long and are grouped into large parallel bundles to provide the required tensile strength. By contrast, collagen fibres tend to be wrapped helically around the long axes of tubular expansile structures such as blood vessels, intestine, and glandular ducts. In the intestine, where it constitutes 15 per cent of the dry weight (Gottrup, 1981), collagen forms a flexible supporting layer which allows expansion of the lumen and movement of the contents without loss of the integrity or elasticity of the wall. Collagen molecules are also components of basement membranes, structures that characteristically lie beneath epithelia, endothelia, and mesothelia. Using light microscopy, basement membranes appear as a single layer of variable thickness, but with the electron microscope the components can be resolved. Just beneath the epithelium is an unstained layer containing proteoglycans and glycoproteins known as the lamina rara, followed by a distinct and constant layer, the basal lamina, composed of a stained mesh of non-banded collagenous fibrils. Beneath the basal lamina another layer contains thin fibres of banded collagen (reticulin). The basal lamina and reticulin are formed from different molecular types of collagen.

Analysis of the molecular structure of collagen has shown the prototypic collagen molecule to consist of three polypeptide chains (α-chains) of equal length, each with a molecular weight of approximately 95kDa (Ramachandran, 1988). The aminoterminal of the three chains are at the same end of the molecule. The individual chains form a left-handed helix, but the three chains are coiled around the central axis to form a right-handed major helix. Stability of the triple helical structure is conferred by the repeating triplet glycine-X-Y, where X and Y can be any amino acid but are often proline and 4-hydroxy proline, respectively. The latter two imino acids direct the helical conformation by their rotational restrictions, and hydroxyproline also helps to stabilise the triple helix by contributing to interchain hydrogen bonds. The amino acid 5-hydroxylysine is unique for collagen, but it is much less abundant than hydroxyproline. Galactose, or the disaccharide galactose-glucose, is covalently linked to the hydroxyl group of some of the hydroxylysine residues, but the function of these sugars is unknown. At both the amino- and carboxy-termini of each α chain 16 to 25 residues form non-helical regions, because of their low content of glycine and imino acids, which are called telopeptides. Lysine and hydroxylysine residues in the telopeptides participate in covalent cross-linking of collagen molecules after the molecules aggregate to form fibres (Eyre et al., 1984). The
Telopeptides are remnants of much larger globular extensions originally present in procollagen, the precursor form of collagen.

There are at least 15 kinds of assembled collagen molecules in tissues, which are products of at least 24 different structural genes (Anderson, 1992). These molecules all contain the collagen triple helical structure as defined above, but the constituent \( \alpha \)-chains in the respective genetic types have different amino acid sequences. The collagen molecules can differ with respect to the length, number and location of their triple helical domains. Type I, the most abundant of the collagens, was the first to be isolated and extensively analysed, and so is considered to be prototypic. Type I molecules contain two \( \alpha \)-chain classes, each determined by a different gene and hence differing in primary structure. Type I molecules typically assemble to form the very thick, distinctly cross-banded collagen fibres of connective tissues. Collagen types II and III each contain only one chain class and tend to form thin cross-banded fibres. Type IV collagen is mostly recovered as a heterotrimeric molecule, but the existence of homotrimeric forms has not been excluded. The fibrillar non-banded basal lamina layer of basement membranes contains the type IV collagen. Knowledge of the molecular forms and modes of fibrillar assembly is incomplete for collagen types IV to XV.

The biosynthesis of collagen is complex, for it involves the synthesis of pro-\( \alpha \) chain classes, post-translational hydroxylation and glycosylation of specific residues, and extracellular modifications of the precursor so that the final molecule can be incorporated into a type of fibre appropriate to the tissue. Controls clearly must operate at the level of genomic transcription so that the proper kinds and amounts of messenger RNA are released to the cytoplasm (Ramirez & DiLiberto, 1990). Translational controls may further modulate the balanced synthesis of different chain classes. Presumably the hydroxylation, glycosylation, assembly, and extracellular processing of the precursors are critically determined by the primary structure of the pro-chains.

Non-covalent interactions between collagen molecules are mainly responsible for the assembly of the collagen fibre; once formed, however, the fibre becomes stabilised by covalent intermolecular cross-links. These cross-links are initiated when a specific copper-dependent amine oxidase secreted by the fibroblast converts certain lysyl and hydroxylysyl-amino groups to aldehydes. Most of these converted residues are at the ends of the \( \alpha \) chains. Intra- and inter-molecular cross-links between chains then form.

Several diseases result from defects in collagen metabolism, for example: osteogenesis imperfecta, Ehlers-Danlos syndromes, and scurvy (Hay, 1991). Osteogenesis imperfecta is a heritable disorder that is characterised by multiple bone fractures, blue sclerae, and thin skin. In each variant of the disease, mutations of genes encoding the synthesis of
pro-α1(I) or the pro-α2(I) chain may result in insufficient synthesis of normal type I collagen molecules, in the formation of molecules that are unstable, or in the formation of collagen molecules that cannot form helices. Ehlers-Danlos syndromes are a clinically and genetically heterogeneous group of disorders arising for abnormal collagen metabolism. Perhaps the best characterised of these is Ehlers-Danlos type IV which results from reduced secretion of type III collagen (Aumailley et al., 1988). In scurvy, collagen fibres are not formed and consequently bone growth and dentition are abnormal, wounds and fractures do not heal, and capillaries are susceptible to rupture. This disease is related to the requirement for vitamin C in the enzymatic hydroxylation of the prolyl and lysyl residues of collagen. A procollagen molecule lacking hydroxyproline residues has an unstable triple helix and is prone, therefore, to denaturation and proteolytic turnover. Diseases with an inflammatory component, whether of infectious, metabolic, or autoimmune origin, inevitably affect collagenous structures, and new collagen fibres are deposited as part of the reparative response to tissue damage. The resulting collagen scars can further limit function and contribute to the morbidity of the disease.

4.2.2 Elastic fibres

With the development of higher organisms came the need for tissue flexibility and the necessity to withstand stretch. As their name suggests, elastic fibres stretch easily and return to their original length when the deforming force is removed. They are found in tissues normally subject to stretching and expansile forces. The thickness, length, and disposition of the fibres differ in different tissues. In arteries, elastic fibres form two thick concentric lamellae, the elastica interna and externa, and elastic fibres are also dispersed through the media of the artery as a concentric, highly fenestrated network. In mesentery, fasciae, and skin, the thin and scattered fibres form networks between bundles of collagen. Under electron microscopy most of an elastic fibre appears to be composed of an amorphous material the structure of which is neither fibrillar nor periodic. However, at the periphery of the amorphous component microfibrils of approximately 110Å diameter occur. Elastin, the insoluble protein that remains after connective tissues are digested with dilute alkali (Lansing et al., 1951), contains about 33 per cent glycine residues and about 11 per cent proline. Unlike collagen, elastin is composed mostly of nonpolar hydrophobic amino acids such as valine, and it has little hydroxyproline and no hydroxylysine. Uniquely, elastin contains desmosine and isodesmosine derivatives of lysine, which function as covalent cross-links in and between the polypeptide chains. The microfibril portion contains the protein fibrillin that contains much less glycine, and is enriched in hydrophilic amino acids. Fibrillin is glycosylated and contains cysteine residues that form disulphide cross-links which
stabilise the microfibril. Thus, when elastic fibres are treated with chemical agents that break disulphide bonds, the microfibrils are solubilised and elastin is left as an insoluble residue. Fibrillin is also a major structural component of fibrillar bundles that are not associated with elastin, and such fibrils may have a wide tissue distribution.

Fibroblasts and smooth muscle cells synthesise the molecules that form the elastic fibre. The microfibrillar protein and elastin are synthesised on the rough endoplasmic reticulum. Little is known about the detailed structure of the microfibrillar protein and how its synthesis, secretion, and extracellular assembly are co-ordinated with elastin biosynthesis. Elastin is synthesised and secreted as a single, soluble polypeptide chain of about 70kDa (Indik et al., 1987). Assembly and cross-linking of individual chains to form the amorphous component of the elastic fibre occur in the extracellular space. The desmosine and isodesmosine cross-links of elastin are initiated by an enzymatic reaction like that described for the cross-linking of collagen. However, the desmosines have the potential of cross-linking four proelastin chains.

The precise physicochemical properties that account for elastin's rubber-like characteristics have not been fully characterised, although they may involve the exposure of hydrophobic regions when the fibre is stretched (Gosline, 1978). Recoil then occurs when the nonpolar groups re-aggregate and expel water after the distending force has been removed.

4.2.3 The ground substance

The cellular and fibrous components of connective tissues are surrounded by materials collectively called the ground substance. Proteoglycans and glycoproteins form most of the interstitial ground substance.

4.2.4 Proteoglycan

The proteoglycans are secreted products of the resident cells of the connective tissues and are composed of polysaccharide chains (glycosaminoglycans) attached to a protein core. The protein cores are difficult to characterise, appear to be diverse in nature and are not genetically related (Bourdon et al., 1985; Krusius & Ruoslahti, 1986; Neame et al., 1987). The proteoglycans are thus classified according to the nature of their repeating disaccharide units, but considerable molecular heterogeneity can exist within each group. For example, keratan sulphate from the cornea is not the same molecule as keratan sulphate from cartilage, and there may be a unique heparan sulphate-containing proteoglycan in basement membranes. Even when a given proteoglycan is carefully isolated from a single tissue or cell type, the molecules are often different in size. This
heterogeneity can be due to differences in lengths of the protein cores or to variations in
the number, size, or degree of sulphation of the glycosaminoglycan chains.

Hyaluronic acid has the simplest glycosaminoglycan structure and is widely distributed
in loose connective tissues, where it is able to trap a large amount of solvent, giving rise
to highly viscous solutions. It is a major component of synovial fluid and the vitreous
body of the eye, and it has been extracted in significant amounts from cartilage, blood
vessels, skin, and umbilical cord (Toole, 1991). Hyaluronate fragments of between three
and 25 disaccharide units stimulate capillary angiogenesis (West et al., 1985). However,
hyaluronic acid may not be a true proteoglycan because there is no firm evidence that its
polysaccharide chains are covalently linked to protein. Its carbohydrate chains are
formed from repeating units of N-acetylglucosamine and D-glucuronic acid, and it is
estimated that about 2,500 such units form chains of approximately $10^6$ Daltons. In
contrast to the other glycosaminoglycans, hyaluronic acid is not sulphated.

In chondroitin sulphate the repeating unit is N-acetylgalactosamine and D-glucuronic
acid, and the hexosamine is variously sulphated. Approximately 250 repeating units
contribute to an estimated molecular weight of between 100kDa and 300kDa (Yada et
al., 1990). This molecule predominates in cartilage, bone, and blood vessels, but it has
also been identified in skin, cornea, and other connective tissues.

The repeating unit of dermatan sulphate is N-acetyl-galactosamine-4-sulphate and L-
iduronic acid. This is a stereo isomer of chondroitin 4-sulphate in which L-iduronic acid
has generally replaced D-glucuronic acid, although a few of the latter groups may still
occur in each polysaccharide chain (Poole, 1986). Dermatan sulphate is found mostly in
the skin, but it has also been demonstrated in blood vessels, heart valves, tendons, and
the connective tissues of the lung.

Polymers of N-acetyl-glucosamine-6-sulphate and galactose yield keratan sulphate
(Poole, 1986). In this structure the degree of sulphation of the hexosamine group can
vary considerably. Moreover, the galactose residues may be sulphated in position 6, and
galactosamine may also be present. Two keratan sulphate types have been identified:
Type I, located exclusively in the cornea, and type II, found in association with
chondroitin sulphate in skeletal tissues such as cartilage and the nucleus pulposus.
Aggrecan, the best known cartilage protein, bears 100 to 140 chondroitin sulphate
chains and 30 to 60 keratan sulphate chains. The keratan sulphates have characteristics
of both proteoglycans and glycoproteins. Type I keratan sulphate is unique among the
proteoglycans for having an N-glycosidic bond between an N-acetyl-glucosaminyl
residue and the amido nitrogen of an asparaginyl residue in the polypeptide chain. This
type of linkage group is commonly found in glycoproteins.
The heparan sulphate basic unit is N-acetyl-glucosamine and D-glucuronic acid, but some iduronic acid residues are also present (Gallagher et al., 1986). Sulphate esters are formed with the nitrogen group of deacetylated glucosamine and with oxygens of the hexosamine and iduronic acid residues. Proteoheparan sulphates are peripheral and integral membrane proteins in many cell types, and this class of molecule is localised in basement membranes. Heparin, the anticoagulant and antilipemic agent, is related structurally to heparan sulphate (Gallagher et al., 1986). Compared to the latter, however, it has a higher content of N-sulphated glucosamine and O-sulphated iduronic acid residues. Heparin is characteristically synthesised and stored within mast cells.

Translation of the protein moiety of these molecules is restricted to the rough endoplasmic reticulum, and the addition of the carbohydrate residues probably begins before the polypeptide is released from the ribosome (Hascall et al., 1991). The polysaccharide chains are assembled by catalysed stepwise transfers of monosaccharides from nucleotide sugar donors. The polysaccharide chains are subject to such modifications as trimming by glycosidases, addition of new terminal sugars, deacetylation, introduction of sulphate groups, and epimerisation of D-glucuronyl residues to L-iduronyl residues. Assembly of proteoglycan subunits into larger aggregates probably occurs after secretion from the cell. The polysaccharide chains of proteoglycans are thought to be generally unbranched. The sulphate and carboxyl groups in the repeating disaccharide units of the chains provide a high density of anionic charges that promote an elongated chain configuration. The proteoglycans, therefore, extend through a larger volume of solution than do uncharged, highly folded molecules with similar molecular weights. The elongated proteoglycans tend to become entangled and form three-dimensional nets with themselves and other polymeric molecules in the connective tissue spaces. In vivo, the proteoglycans presumably interact electrostatically with a variety of cationic molecules and, through such binding, play a significant role in the transport of electrolytes and water. Proteoglycans may also interact with positively-charged collagen molecules to affect collagen fibre formation. The permeability, transport, and osmotic functions of interstitial fluid can also be modified by the entangled proteoglycans acting as molecular sieves to exclude or entrap molecules of different sizes. Such networks increase the viscosity of interstitial fluids and can thus contribute to their lubricative and mechanical functions. The viscous or gel-like nature of tissue fluids may also help to limit the spread of bacteria.

4.2.5 Interactive glycoproteins

Both collagen and elastin are glycosylated and are therefore, strictly speaking, glycoproteins. However, several new glycoproteins have been characterised that merit the description 'interactive glycoproteins'. These proteins all tend to contain distinct,
functionally active polypeptide domains specialised for binding to cell surface receptors as well as to other molecules of the extracellular matrix. The molecules also form polymers by both covalent and non-covalent bonding.

Fibronectin is a major surface glycoprotein of the fibroblast, but it is also synthesised by other mesenchymally-derived cells, by epithelia and endothelia, and by some marrow-derived cell types (Mosher, 1989). The protein is synthesised as a 220kDa monomer with complex oligosaccharide chains linked to asparaginyl residues. Fibronectin appears at the cell surface as a disulphide-linked dimer; however, disulphide-linked fibrillar multimers are subsequently formed. Fibronectin is readily shed into the extracellular space and appears in the plasma; in this plasma form, fibronectin is also called cold insoluble globulin. Fibronectin mediates the attachment of fibroblasts to collagen gels \textit{in vitro} and immunofluorescence staining demonstrates fibronectin in most connective tissues, where it is associated with the interstitial collagens. Collagen and fibronectin form an integrated fibrillary network \textit{in vivo} that influences the adhesion, motility, growth and differentiation of cells (McDonald, 1988). Fibronectin has also been shown to bind to proteoglycans, fibrinogen, and fibrin, to help mediate platelet-collagen interactions, and to function as a chemotactic agent and opsonin (Morel \textit{et al.}, 1992).

Laminin is a major constituent of basement membranes. The molecule is composed of at least three large polypeptides joined by disulphide bonds (Von der Mark & Kühn, 1895). The molecule has the shape of an asymmetric cross with three short arms and one long arm. \textit{In vitro}, binding assays show that laminin self-associates, and binds to proteoheparan sulphates, to type IV collagen, and to cell-surface receptors. It is presumed that these associations are exploited in the assembly of basement membranes \textit{in vivo}. Another glycoprotein constituent of basement membrane is enactin (158kDa), that binds to laminin in a 1:1 ratio to form a stable non-covalent complex (Aumailley \textit{et al.}, 1989), and most studies reported in the literature actually relate to the laminin-enactin complex.

Thrombospondin is a large multifunctional glycoprotein released from platelets and synthesised and secreted by endothelial cells, fibroblasts, and smooth muscle cells (Mosher, 1990). Immunofluorescence demonstrates thrombospondin in the fibrillar extracellular meshwork of the cultured cells, and the molecule has been shown to interact with fibronectin, laminin, type V collagen, and fibrinogen. Thrombospondin may also regulate growth. Antibodies raised against thrombosporin inhibit smooth muscle cell proliferation by arresting the cells in the G1 phase of the cell cycle (Majack \textit{et al.}, 1988).

Vitronectin, otherwise known as S protein is a plasma glycoprotein that promotes cell adhesion and spreading \textit{in vitro}. It was first identified as a cell attachment factor with
high avidity for glass surfaces. The molecule binds to native collagens and to proteoglycans, and immunofluorescence demonstrates vitronectin on cell surfaces and on extracellular fibrils. Vitronectin inhibits the action of a terminal complex formed in the course of complement activation, and it also potentiates blood clotting (Tomasini & Mosher, 1991). Thus, vitronectin may be considered as one of the mediators of the inflammatory and repair reactions that occur at sites of tissue injury.

Thus, most molecules that form connective tissue matrices appear to have distinct domains specialised for binding to self, to other matrix molecules and to cell surfaces. In this manner, a complex structure is assembled that provides structural support and influences the attachment and movement of cells.

4.3 The matrix metalloproteinases

4.3.1 Background

The degradation of connective tissue matrix is a normal event in the physiological remodelling associated with morphogenesis and growth, as well as in processes such as angiogenesis, cell migration, cervical softening, uterine involution, and wound healing (Murphy & Reynolds, 1993). Although many proteases can cleave extracellular matrix components, the matrix metalloproteinases are believed to be the normal, physiologically relevant mediators of matrix degradation (Murphy & Reynolds, 1993). There are several reasons for this. The matrix metalloproteinases are secreted proteins, placing them in the proper location for matrix degradation, and their enzymatic activities are most potent at pH values close to neutrality.

The matrix metalloproteinases are endopeptidases secreted by mesenchymal and haemopoietic cells that contain Zn$^{2+}$ at the active site and are Ca$^{2+}$-dependent. Together these enzymes have evolved to degrade all the components of connective tissues (Murphy & Reynolds, 1993). The degradation of the extracellular matrix by the matrix metalloproteinases is strictly regulated throughout the processes of synthesis, secretion, and activation. The active forms of metalloproteinases may be inactivated by forming a complex with α2-macroglobulin (Sottrup & Birkedal, 1989) or tissue inhibitor of metalloproteinases (TIMP) (Cawston et al., 1981; Ward et al., 1991).

All inflammatory conditions are associated with lysis of connective tissue to a greater or lesser extent. In pathological conditions, such as inflammatory bowel disease, a local imbalance of the metalloproteinase regulation system may occur, and consequently the degradation pathway would be dominant over the inhibitory one, often reflected in the formation of ulcers. Conversely, excessive deposition of collagen could lead to the stricture formation observed in Crohn’s disease.
It is useful to subdivide the matrix metalloproteinases into three subclasses based on substrate specificity (table 4.2). These are the collagenases, gelatinases and stromelysins. However, the distinctions are arbitrary and show a tendency to become less clear as more is known about the enzymatic activity of the purified enzymes and substrates. In many cells there is co-ordinated production of these metalloproteinases, often paralleled by TIMP synthesis.

4.3.2 Synthesis

Matrix metalloproteinases are produced by a number of cell types including polymorphonuclear leukocytes (Murphy et al., 1989), chondrocytes and endothelial cells (Murphy et al., 1989), fibroblasts (Hipps et al., 1991) and macrophages (Hibbs et al., 1987), as well as tumour cells (Gavrilovic et al., 1985). Whether or not these cells actually synthesise matrix metalloproteinases may depend on their ability to respond to specific stimuli within their microenvironment. Cytokines and growth factors known to stimulate synthesis of metalloproteinases by connective tissue cells include interleukins, tumour necrosis factor, epidermal growth factor, fibroblast growth factor and interferons. While many of these are produced by mononuclear cells, undoubtedly some are also synthesised by connective tissue cells themselves, demonstrating important autocrine regulatory mechanisms (reviewed by Matrisian, 1992). Control is exerted at the transcriptional level; exposure of fibroblasts to epidermal growth factor, fibroblast growth factor or embryonal-carcinoma-derived growth factor leads to increased collagenase, stromelysin and TIMP mRNAs (Edwards et al., 1987). As well as stimulating the synthesis of collag enolytic enzymes, growth factors can also diminish their production (Girard et al., 1991). Transforming growth factor β can reduce the expression of procollagenase by human fibroblasts and additionally stimulate these cells to increase their output of TIMP (Overall et al., 1989). Glucocorticosteroids have been demonstrated in vitro to be strong inhibitors of metalloproteinase synthesis (Bauer et al., 1985; Cambray et al., 1981b). Dexamethasone also diminishes the level of stromelysin and collagenase gene induction caused by interleukin-1 (DiBattista et al., 1991), epidermal growth factor, phorbol esters and cAMP. These actions may be significant in the anti-inflammatory action of glucocorticosteroids.

The promoter regions for human and rat stromelysins 1 and 2 and rabbit interstitial collagenase have been shown to have common features. The genes all contain a TATA box and have AP-1 sites and PEA-3 sites within the most proximal 210 base pairs. Mutational analysis has shown that AP-1 alone, or in combination with PEA-3, regulates the basal levels of these genes (Angel et al., 1987; Auble & Brinckerhoff, 1991). Transcriptional factors that recognise and transactivate through these elements are the protooncogenes c-fos and c-jun, which transactivate through the AP-1 element, and
c-ets, which recognises the PEA-3 element (Wasylyk et al., 1990). Gelatinase B does not contain the same promotor regions as stromelysin and collagenase, and although gelatinase A does contain a TATA box and an AP-1 site, it is not clear if they are acting as true transcriptional elements (Huhtala et al., 1991; Templeton & Stetler, 1991). This may explain some of the observed differences in expression of these enzymes. AP-1 and PEA-3 are also transcriptional elements of the urokinase plasminogen activator gene (Nerlov et al., 1991). Urokinase converts plasminogen to plasmin, which in turn is thought to be responsible for the activation of collagenase and stromelysin.

4.3.3 Secretion of proenzymes

The synthesis of matrix metalloproteinase enzymes is usually followed by their secretion. Studies of collagenase synthesis in human fibroblasts have demonstrated that intracellular enzyme appears 15 minutes after stimulus and that after a further 15 minutes it is present extracellularly (Valle & Bauer, 1979). The interval between synthesis and secretion suggests that little intracellular storage of the enzyme takes place, a feature which contributes to the difficulty in identifying the cells responsible for metalloproteinase synthesis. Polymorphonuclear leukocytes are an exception to this, and appear to store the enzyme in secretory granules. Secretion of the matrix metalloproteinases may be accompanied by the cleavage of a signal sequence of twenty or so amino acids from the N-terminal of the enzyme (Docherty & Murphy, 1990).

4.3.4 Extracellular activation

The metalloproteinases are secreted in an inactive form. There is a decrease in molecular weight of about 10kDa on activation of metalloproteinases, but further proteolysis can occur by autolysis or the action of proteinases in a complex process yielding several active forms (Murphy et al., 1987). The process of activation is controlled by the relative concentrations of plasmin, plasminogen activator, and plasminogen activator inhibitor. A requirement for additional proteinases, such as plasmin, allows for another level of control to be placed on the degradative activity of the metalloproteinases. Plasminogen activator activity in cell cultures is raised by sex steroids, vasopressin, prolactin, parathyroid hormone and thyrotropin.

Activation of the latent proenzymes can be effected in vitro by proteinases such as trypsin and by organomercurials (Grant et al., 1987). The process in vivo is probably initiated by plasmin, although kallikrein (Nagase et al., 1990) and cathepsin G (Capodici et al., 1989), which has some activity at neutral pH, have also been implicated. For collagenase and stromelysin a sequence of reactions involving the initial generation of plasmin from plasminogen by plasminogen activator, and culminating in the activation of the latent
form of the enzyme has been envisaged (He et al., 1989). Other enzymes, such as cathepsin B and L, have also been implicated in the activation of collagenase and stromelysin. However, although plasmin can convert both procollagenase and prostromelysin to their active forms it is relatively ineffective against progelatinase, as indeed is stromelysin (Collier et al., 1988). Quite how progelatinase is activated remains unclear, but the process may be autocatalytic, involving self-degradation to the active species (Hipps et al., 1991).

4.3.5 Extracellular control

The activity of matrix metalloproteinases can be further controlled by both non-specific and specific inhibitors. Of the non-specific inhibitors, α₂-macroglobulin is the most important and in human plasma accounts for over 95 per cent of metalloproteinase inhibitory activity (Cawston & Mercer, 1986). Although α₂-macroglobulin may have a role in limiting metalloproteinase activity, its large size (725kDa) is likely to confine its action to an area in and around the vasculature (Cawston & Mercer, 1986). There is therefore a requirement for smaller molecules to control metalloproteinase activity within the tissue stroma, and the existence of specific matrix metalloproteinase inhibitors, tissue inhibitors of metalloproteinases (TIMP) provides an appropriate system. The TIMP family consists of at least two members, TIMP-1 and TIMP-2 (Ward et al., 1991). There is no precursor form of TIMP-1, which is a glycoprotein of molecular weight 28kDa. Synthesis of TIMP is increased in cells stimulated by interleukin-1, phorbol esters or glucocorticosteroids.

TIMP-1, the major form, was originally purified from rabbit bones in tissue culture (Cawston et al., 1981) and has since been identified in a variety of human fluids including plasma, amniotic fluid and saliva (Clark et al., 1991a). TIMPs act specifically against the matrix metalloproteinases by combining with them on a one-to-one basis to form irreversible complexes (Cawston & Mercer, 1986). TIMP-1 appears to be synthesised constitutively by most connective tissue cells, and its presence within the extracellular matrix may provide a final fail-safe mechanism for the control of matrix metalloproteinase activity. Net matrix proteolysis is only likely to occur when the level of activated enzyme exceeds that of the inhibitor. Thus, chondrocytes stimulated to produce collagenase fail to degrade collagen films unless the action of TIMP is blocked by the addition of an anti-TIMP antibody (Gavrilovic et al., 1987). As well as combining with the active enzyme, TIMP-1 has also been shown to form a complex with the latent pro form of 95kDa gelatinase B (Wilhelm et al., 1989). The in vivo function of this complex is uncertain, but it may provide a way in which the inhibitor can be sequestered in the tissues. When required, TIMP-1 may dissociate from the relatively
weaker TIMP-1 progelatinase complex and combine with the higher-affinity binding sites of the active enzymes.

Unlike TIMP-1, TIMP-2 is not glycosylated, a fact reflected in its lower molecular weight of 21kDa. However, it does share considerable similarity to TIMP-1 at the amino acid sequence level (Stetler et al., 1990). TIMP-2 is able to form a complex with both the latent pro form of 72kDa gelatinase and with the active enzyme. A further metalloproteinase inhibitor has been identified with an apparent molecular weight of 76kDa. In view of its larger size compared to TIMP it is referred to as large inhibitor of metalloproteinases, or LIMP, and has been shown to inhibit collagenase, stromelysin and gelatinase (Cawston et al., 1990).

4.3.6 Substrate specificity

The triple helical structure of tropocollagen is uniquely cleaved by interstitial collagenase at a locus three-quarters of the way between the amino- and carboxy-termini of the molecule. This occurs at a Gly-Ile bond in the $\alpha_1(I)$ chains and at a Gly-Leu bond in the $\alpha_2(I)$ chain. The resultant degradation products are designated $\frac{2}{4}(TC_A)$ and $\frac{1}{4}(TC_B)$ (Harris & Krane, 1974). These fragments are unstable and spontaneously unravel to allow further degradation by gelatinase and other proteases. However, although interstitial collagenase can degrade individual tropocollagen molecules it is less able to do so when these are combined to form intact collagen fibrils. This has led to the theory that for interstitial collagenase to act in vivo other non-specific proteases, such as cysteine and serine proteases, may be required (Harris & Krane, 1974). These proteases would act to break the cross-links which exist between the telopeptide, or non-helical regions, of the tropocollagen molecules. Once released, the collagen molecules would then be open to attack by interstitial collagenase. As well as extracellular degradation, collagen fibrils may also be phagocytosed and digested by lysosomal enzymes such as the cathepsins. Types IV and V collagen, which are present in basement membranes, can also be denatured by two mechanisms, one specific and involving gelatinase (type IV collagenase) (Murphy et al., 1985), and the other less specific and due to the action of proteases with a broader substrate specificity such as plasmin and the cathepsins. In vivo there may be a requirement for both if the insoluble basement membrane is to be successfully degraded. Gelatinase lyses type IV collagen to produce fragments resembling the $\frac{3}{4}$ and $\frac{1}{4}$ cleavage products produced by interstitial collagenase (Murphy et al., 1989), while plasmin is likely to attack the non-helical regions of the molecule. The cross-linked regions of type IV collagen are also susceptible to stromelysin, although this enzyme appears generally less potent against this substrate than gelatinase. In addition to their action against type IV collagen, the enzymes - gelatinase A, gelatinase B and stromelysin - all show some activity against elastin, although in the case of stromelysin this is slight
Matrilysin is the most effective matrix metalloproteinase against elastin. However, the serine proteinase elastase secreted by polymorphonuclear neutrophils is a powerful general proteinase and degrades elastin moderately well by non-specific electrostatic binding and by attacking alanine-rich regions.

The core proteins of proteoglycans have been shown to be vulnerable to attack by gelatinase and collagenase. However, this action is not confined to the matrix metalloproteinases alone. The serine proteases elastin and cathepsin G have also been shown to have activity. The majority of the glycoproteins are proteinase-sensitive and are susceptible to the action of many serine proteinases, such as plasminogen activator and neutrophil elastase. Stromelysin has also been shown to have activity against these molecules.

4.3.7 Expression in disease

Some of the first evidence that tissue destruction might result from an imbalance of the matrix metalloproteinases over TIMP came from studies with an experimental model of arthritis. The production of the matrix metalloproteinases and TIMP by synovial explants in culture, taken from rabbits during the course of a model arthritis, was measured, and showed that the induction of the lesion correlated with a reduction in the synthesis of TIMP and an increase in matrix metalloproteinase synthesis (Cambray et al., 1981a). Studies with human joint tissues from normal controls and arthritic patients indicated close similarities to the rabbit model and supported the idea that resorption is closely linked with the absence of sufficient TIMP to counteract increases in matrix metalloproteinase production (McGuire et al., 1981; Murphy et al., 1981). Extracts of cartilage from patients with osteoarthritis (Dean et al., 1989) also have considerably elevated levels of matrix metalloproteinases and only moderately increased levels of TIMP, compared to extracts of cartilage from controls. These workers have concluded from their detailed studies that there is a small excess of inhibitor over enzymes in normal human cartilage. In osteoarthritis, TIMP does not increase to the same extent as the matrix metalloproteinases, and this imbalance may contribute significantly to tissue breakdown.

The matrix metalloproteinases are also implicated in non-inflammatory pathological processes, the most significant being tumour invasion and metastasis. For a tumour cell to metastasise, it must break away from its neighbours, force its way through the surrounding stroma, and penetrate the basement membrane in order to enter the circulation. When it arrives at its destination, these steps must be repeated in reverse order. These steps require extensive degradation of the extracellular matrix components including interstitial collagens, basement membrane collagen, fibronectin, laminin, and
various proteoglycans. The ability of the matrix metalloproteinases to degrade most of the components of connective tissue makes the enzymes ideal candidates to mediate tumour invasion and metastasis. Most research has been centred on gelatinase because of its ability to degrade basement membrane collagen type IV. Gelatinase is frequently elevated in human and mouse tumours, and the levels of the enzyme correlate with the metastatic potential of a series of mouse melanoma cells (Goldfarb & Liotta, 1986). Stromelysin expression has also been shown to correlate with tumour progression. Prostromelysin mRNA is frequently detected in mouse skin carcinomas, but rarely found in benign papillomas (Matrisian, 1986). Stromelysin levels are particularly high in mouse skin tumours with a high probability of metastasis (Ostrowski et al., 1988). Prostromelysin, pro-stromelysin-2 and pro-PUMP mRNAs have been isolated from human tumours as well (Muller et al., 1988). Additional evidence of a role for matrix metalloproteinases in tumour invasion has come from studies with TIMP. An inverse correlation has been observed between TIMP levels and the invasive potential of mouse fibrosarcoma cells (Hicks et al., 1984). Denhardt and his colleagues have demonstrated the importance of TIMP in suppressing tumour invasion and metastasis by regulating TIMP protein levels through the use of antisense mRNA (Khokha et al., 1989). Cells that are not normally invasive by the human amnion invasion assay become invasive when they no longer produce TIMP as a consequence of the expression of antisense TIMP mRNA. The antisense TIMP cells also produce metastatic tumours in nude mice. These results suggest that expression of active metalloproteinases is required for invasion and metastasis, since inhibition of metalloproteinase activity suppresses the invasive phenotype. The stimulus for the elevated levels of the matrix metalloproteinases may come from activated oncogenes like Ha-ras, which is associated with a variety of human and animal tumours, and can induce matrix metalloproteinase expression (Wynford-Thomas, 1991). A level of complexity has been added recently by the discovery that an important source of the metalloproteinase production in epithelial tumours may be the adjacent stroma (Basset et al., 1990; Gallegos, 1992; Muller et al., 1993).

An interesting feature of inflammatory bowel disease pathology is that these diseases are associated with other connective tissue disorders in which errant matrix metalloproteinase expression has been implicated. For instance, sterile sero-negative arthritis is associated with ulcerative colitis more often than can be ascribed to chance (Hochberg et al., 1982). Ten to 15 per cent of patients with ulcerative colitis develop joint disease; the distinction from rheumatoid arthritis is emphasised by the frequency of cases where arthritis coincides with exacerbations of the intestinal disorder, the patients' rheumatoid factor titres are not raised, and antinuclear factors are not found. The clinical signs of the peripheral arthritis that may complicate Crohn's disease are indistinguishable from those associated with ulcerative colitis; the frequency of the
peripheral arthritis in the former is, however, somewhat greater than in the latter (Frahyha et al., 1975). In Crohn's-associated arthritis the synovial response may be granulomatous, resembling the intestinal lesions. Although peripheral joint disease is common and begins approximately six years after the onset of colitis, the anatomical sites affected often extend to those of the spine. In ulcerative colitis and in Crohn's disease the frequency with which ankylosing spondylitis occurs is greatly increased, so that either ulcerative colitis or Crohn's disease coexist with ankylosing spondylitics in approximately five to seven per cent of instances. Both HLA-B27-positive and HLA-B27-negative ankylosing spondylitis are 100 to 300 times more prevalent in patients with inflammatory bowel disease than could be expected by chance (Gardner, 1992).

4.4 Summary

Connective tissue is composed of both resident cells and extracellular matrix that provides the supporting and connecting framework for all the other tissues of the body. The extracellular matrix is assembled mainly from collagen and elastin, but also includes a complex network of glycoproteins and proteoglycans. In the normal course of events a low level of turnover of the components exists in all tissues. The principal protease enzymes responsible for this turnover are thought to be the matrix metalloproteinases; gelatinase, collagenase and stromelysin are together able to degrade all the components of the extracellular matrix. These proteolytic enzymes are tightly controlled at all stages of synthesis.

All inflammatory conditions are associated with lysis of connective tissue to a greater or lesser extent. In inflammatory bowel disease a local imbalance of the metalloproteinase regulation system may occur, and as a consequence of which the degradation pathway would be dominant over the inhibitory one.
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THE DEVELOPMENT OF AN ANIMAL MODEL OF INFLAMMATORY BOWEL DISEASE

5.1 Introduction

Advances in inflammatory bowel disease research have been hampered by the lack of a simple, yet clinically relevant, animal model. Although animal models of colitis cannot be used to identify the initiating or aetiological factors leading to colitis in human beings, they can be used to evaluate the mechanisms of tissue injury, the role of inflammatory mediators, and the efficacy of potential therapeutic agents. To produce an experimental animal model two approaches have been adopted: (1) the identification of a naturally occurring disease in another species that resembles the human disease, and (2) the search for an exogenous agent that can produce lesions macroscopically and microscopically similar to chronic inflammatory bowel disease by simple means in a convenient experimental animal. This introduction will focus on some of the most popular spontaneous and experimentally-induced models of small intestinal or colonic inflammation. The objective is to evaluate each model in terms of its clinical and histopathological features, aetiological mechanisms, and responses to certain drug therapies known to be effective in the treatment of human inflammatory bowel disease.

5.1.1 Inflammatory bowel disease in species other than man

Ideally, using an animal with a naturally-occurring inflammatory bowel disease would be the best way of studying the disease; however, there are few animals which have a sufficiently high incidence of naturally-occurring idiopathic colitis. This approach also invites criticism, since the intrinsic susceptibility of an animal species may not reflect the same physiological mechanism in man.

Table 5.1: Models of inflammatory bowel disease (next page).
<table>
<thead>
<tr>
<th>Model type</th>
<th>Species</th>
<th>Method</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naturally occurring</td>
<td>Dog, Horse, Pig</td>
<td>Spontaneous</td>
<td>Pathology consistent with IBD. However, low incidence and expensive. Could be infective. Histologically resembles UC and responds to sulphasalazine. Expensive, endangered species.</td>
</tr>
<tr>
<td></td>
<td>Cotton Top Tamarin, Macaque</td>
<td>Spontaneous</td>
<td></td>
</tr>
<tr>
<td>Transfection</td>
<td>Mouse</td>
<td>Injection of lymph-node homogenate from CD patients, M. paratuberculosis from CD patients.</td>
<td>Produced granulomatous inflammation. However, likely to be artefact. Segmental disease similar to Johne's disease in Cattle.</td>
</tr>
<tr>
<td></td>
<td>Goat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemotactic Peptides</td>
<td>Rabbit, Rat</td>
<td>Intracolonic administration of fMLP.</td>
<td>Acute inflammatory infiltrate with increase in mucosa permeability. However, no ulceration and not persistent.</td>
</tr>
<tr>
<td>Chemical irritation</td>
<td>Rat</td>
<td>Intra-colonic instillation of acetic acid.</td>
<td>Similar histology to UC. Mucin depletion and crypt abscesses. Healing by one week. However, cheap, reproducible and simple.</td>
</tr>
<tr>
<td>Sulphated polysaccharides</td>
<td>Guinea-pig, Mouse, Rat, Rabbit, Monkey, Hamster</td>
<td>Oral administration over an extended period in drinking water.</td>
<td>Histopathological similarities to IBD and an increased risk of cancer. Main problem is the length of time required to establish the disease and the biological variability.</td>
</tr>
<tr>
<td>Free radical</td>
<td>Rat</td>
<td>Administration of free radical initiator.</td>
<td>Acute mucosal inflammation, but no ulceration.</td>
</tr>
<tr>
<td>Immunological</td>
<td>Guinea-pig, Rabbit</td>
<td>Type II, III, IV hypersensitivity reactions by sensitisation to colonic extracts, formalin and exogenous antigen, or hapten (DNCB).</td>
<td>Variable response to colonic extracts. Formalin enemas with immune complexes or LPS produces acute UC like damage. Presensitisation with the hapten DNCB produces mucosal inflammation and ulceration that persists for more than a week. All require lengthy protocols.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intra-colonic instillation of trinitrobenzene sulphonic acid in an ethanol solution.</td>
<td>Simple induction. Persistent inflammation and ulceration that resembles CD. But nonphysiological and no periods of remission.</td>
</tr>
</tbody>
</table>
In some varieties of dog, such as Boxers, cases of inflammatory bowel disease have been described that resemble ulcerative colitis and Crohn's disease (Strandle et al., 1954; Van Kruinigen, 1972). The dogs may experience periods of remission and relapse of the disease. Following episodes of chronic diarrhoea the dogs display anorexia, fever, tenesmus, malaise and weight loss. The characteristic gross lesions of hyperaemia, oedema, and haemorrhage, are confined to the colon and rectum. Extensive ulceration is present in some dogs, whereas in others there is a diffuse or patchy mucosal thickening and minimal ulceration. The early lesions are characterised by an abrupt transition from normal to inflamed mucosa - a characteristic of Crohn's disease. Initially, the lamina propria is infiltrated with lymphocytes, plasma cells, mast cells, and some neutrophils. In severe cases, macrophages dominate the lesions. The wall of the colon becomes thickened, reducing the lumen diameter, and there is a loss of elasticity and motility of the colon. Large, ulcerated, denuded segments of colon are observed as the disease progresses. Most of these features are consistent with human chronic inflammatory bowel disease, suggesting that idiopathic colitis in dogs may be a good experimental model. However, the present incidence appears to be extremely low, making this model impractical. Other limitations of this model include the cost of experimental research with dogs and the difficulties associated with obtaining approval for work on dogs.

In horses, a rare but dramatic equine colitis has been described (Rooney et al., 1963). This is an acute, sporadic illness, characterised by the sudden onset of severe but non-bloody diarrhoea, that is often fatal. The histology displays oedema, hyperaemia and petechial haemorrhage, and there may be frank infarction. However, in some cases Clostridium difficile has been implicated. Pigs are also susceptible to diarrhoeal illness and histologically there is an acute inflammatory response, but there is a good response to anti-microbials. In both horses and pigs, as for the colitis described in dogs, the incidence is not high enough for these animal illnesses to be of practical use as an experimental model, and the cost would be prohibitive.

The best naturally-occurring animal models found so far are the idiopathic inflammatory bowel diseases in other primates. A fatal acute dysentery in four Siamang gibbons was identified in which no bacterial pathogen could be detected (Stout & Snyder, 1969). This colitis, however, was ascribed to the stress induced by 'socio-environmental upheaval'. A similar colitis was reported in orang-utans (Scot & Keymer, 1974). These episodes of colitis occurred sporadically and unpredictably. The cotton-top tamarin (figure 5.1) a New World primate species (Saguinus oedipus oedipus) has a high prevalence of spontaneous colitis and has a 25 to 40 per cent risk for developing colonic cancer after two to five years in captivity (Moore et al., 1988). As many as 50 per cent of colony-maintained cotton-top tamarins develop active colitis not associated with
Figure 5.1: The cotton-top tamarin. This New World primate species (Saguinus oedipus oedipus) has a high prevalence of spontaneous colitis and has a 25 to 40 per cent risk for developing colonic cancer after 2 to 5 years in captivity.
Chapter 3

identifiable pathogens and, as in humans, the activity of the disease process spontaneously enters periods of relapse and remission (Madara et al., 1985; Winter et al., 1989). Histologically, the colonic inflammation is characterised by haemorrhage, crypt abscesses, mucin depletion and an increase in the density of lymphocytes and plasma cells in the lamina propria (Madara et al., 1985). Biochemically, there is a reduction in colonic mucin glycoprotein analogous to that in human ulcerative colitis. In addition, significant histological improvement occurs when cotton-top tamarins are treated with sulphasalazine (Madara et al., 1985). These striking similarities between cotton-top tamarin colitis and human ulcerative colitis present an opportunity to study the aetiology of spontaneously occurring chronic colitis. Unfortunately, this expensive endangered species is not widely available, thus limiting its usefulness as a research tool.

Another spontaneous chronic colitis has been identified in juvenile rhesus macaques (Hendrickx et al., 1990), which may have recurrent episodes of persistent non-bloody liquid stool, weight loss, and growth retardation. Post mortem examination revealed uniform, continuous inflammation of the caecum, the colon, and the rectum. Histologically, there was chronic inflammation with crypt abscesses, goblet cell depletion, and surface cell alterations. Unfortunately, the same problems arise with the use of this species as a research tool as were noted above for the cotton-top tamarin.

5.1.2 Experimentally-induced models of inflammatory bowel disease

While naturally-occurring models suggest that idiopathic inflammatory bowel diseases are not specific to humans, these models are of limited use either because of their low incidence, or because of the ethical considerations, or because of the expense that would be incurred if the animals concerned were to be used as a research tool. For these reasons, many attempts have been made to induce in common laboratory animals colitis with similar pathology to human inflammatory bowel disease. A number of agents have been employed to induce disease, with varying degrees of success. Those most commonly used are discussed below.

5.1.3 Transfection models

The possibility that an infectious agent was responsible for the aetiology of Crohn's disease was recognised by Crohn, Oppenheimer and Ginzburg in their initial description of regional ileitis (Crohn et al., 1932). Noting the histological similarities between Crohn's disease and tuberculosis, the investigators carried out microscopic examinations of tissue sections and smears for mycobacteria, and attempted to culture mycobacteria from involved tissues. Mycobacteria were not recovered, and failure to isolate mycobacteria became a diagnostic criterion for differentiating Crohn's disease from
intestinal tuberculosis. Nevertheless, similarities between Crohn’s disease and other infectious diseases continued to provoke attempts to establish an infectious aetiology for Crohn’s disease. In 1970 Mitchell and Rees reported that granulomas were produced six to twenty months after injection of intestinal or lymph node homogenates from patients with Crohn’s disease into mouse footpads (Mitchell & Rees, 1970). Control tissue homogenates failed to produce inflammation. Preparation of some homogenates included passage through 0.2μm filters (Cave et al., 1975). Because conventional bacteria are removed by 0.2μm filters, the putative transmissible agent was thought to be a virus or cell-wall-defective bacteria (L-form). However, when coded slides were reviewed, some previously-reported granulomas were found to be associated with foreign bodies such as bone fragments or hair, and some were neoplasms rather than granulomas (Cave et al., 1979; Thayer, 1979).

Although a few other investigators reported positive results in animal transmission studies (Donnelly et al., 1977; Simonowitz et al., 1977), most attempts to reproduce these studies yielded either negative results or non-specific results (Yoshimura et al., 1984b). Following inconsistent results obtained in studies from several laboratories, a consensus developed among investigators that the initial observations had resulted from experimental artefacts, caused by foreign body contamination.

5.1.4 Mycobacterial infection

Despite frequent negative reports, the search for an infectious agent continues. Speculation that Crohn’s disease might be due to a mycobacterial infection has led to a number of attempts to produce Crohn’s disease-like lesions in animals. Mycobacterium paratuberculosis has recently been isolated from the Crohn’s disease tissue of some patients and has been given to apparently healthy goats which subsequently developed segmental granulomatous disease of the intestine (Chiodini et al., 1984). Mycobacterium paratuberculosis is now known to cause Johne’s disease in cattle; however, there are striking differences between this disease and human inflammatory bowel disease, where 10^6 times fewer mycobacteria are present (Sanderson et al., 1992). In another experimental, model guinea pigs inoculated with BCG (Bacillus Calmette-Guerin) vaccine in the serosa of the antimesenteric bowel wall produced a granulomatous infiltration in the bowel wall (Mitchell & Turk, 1989). A small amount of ulceration was observed in association with Peyer’s patches; however, fissuring and the ‘cobblestoning’ typical of Crohn’s disease were not observed. While mycobacterial infection remains a popular hypothesis for the aetiology of Crohn’s disease, the failure to transmit the disease to animals not typically prone to Mycobacterium paratuberculosis diseases, such as common laboratory animals, hampers its acceptance as a primary factor.
5.1.5 Induction of acute colitis by chemotactic peptides

Chemotactic peptides from endogenous aerobic and anaerobic bacteria in the intestinal lumen could potentially induce inflammation if they reached the mucosa. An experimental model of colitis reflecting this scenario as a possible cause of inflammatory bowel disease in humans has been developed in the rabbit (LeDuc & Nast, 1990). Intracolonic administration of n-formyl-methionyl-leucyl-phenylalanine, a potent polymorphonuclear neutrophil chemotactic peptide, produced a dose-dependent colitis within four days as assessed grossly and histologically (LeDuc & Nast, 1990). The colitis was characterised by an infiltration of polymorphonuclear neutrophils and eosinophils and an increase in mucosal permeability that was abolished by treatment with antineutrophil serum (Von Ritter et al., 1988). The colitis, however, was non-ulcerating and resolved within three days following treatment with few chronic inflammatory cells remaining. Furthermore, an increase in macrophage numbers, which is a typical feature of human inflammatory bowel disease, was not observed in any portion of the colons of the experimental animals. The model is, therefore, unsatisfactory for use as a model of inflammatory bowel disease.

5.1.6 Vascular impairment models for Crohn’s disease

Mesenteric ischaemia produces damage that is easily distinguished from ulcerative colitis or Crohn’s disease. However, in 1963 Boley postulated that vascular occlusion “may be the aetiologic factor for many cases of so-called non-specific ulcers”, following the production of segmental colitis in dogs by ligating major vessels supplying the colon (Boley et al., 1963). The pathological appearances were dependent on the degree of ischaemia inflicted and varied from slight mucosal congestion to extensive necrotic ulceration (Marston et al., 1969). Occasionally fibrous stricture formation was observed. However, the lesions did not persist, since a collateral supply was rapidly established. Other workers have also suggested that Crohn’s disease may have a vascular component, perhaps at a microvascular level, and have attempted to develop animal models. In dogs ceramic and glass microspheres were injected into the mesenteric circulation to induce colitis (Boley et al., 1965). This produced the macroscopic appearances of minimal mucosal oedema, longitudinal red or blue streaks in the mucosa, and in some cases total intestinal dissolution, depending on the size and number of spheres injected. Recently, interest in this type of model has been revived following observation of multifocal granulomatous microvascular lesions in the submucosa of tissue from patients with Crohn’s disease (Wakefield et al., 1991). Experiments similar to those performed in dogs were set up in ferrets (Hudson et al., 1992). Interruption of the mucosal collateral plexus by intra-arterial injection of a combination of 27μm and 90μm styrene microspheres resulted in focal mucosal inflammation, necrosis, and superficial ulceration. The same
group also suggested that the combination of anastomosis and microvascular injury can produce a pattern of intestinal inflammation similar to that seen in anastomotic recurrence in Crohn's disease (Osborne et al., 1993). In each of these models of microvascular injury, the lesions were resolved by two weeks and the microscopic damage observed was not typical of the chronic pathology observed in Crohn's disease, which limits the models' usefulness. However, it is possible that repeated focal ischaemia may produce histopathological features more consistent with Crohn's disease.

5.1.7 Chemical irritation

A transient mucosal ischaemia may be responsible for an ulcerative colitis-like disease induced in rats (Leung & Koo, 1991). Intrarectal instillation of acetic acid in concentrations ranging from four per cent to 33 per cent and in different volumes produces a colitis with many similarities to human ulcerative colitis. Recent work has attempted to establish an optimal dose and period of exposure (Fabia et al., 1992). Four per cent acetic acid for 15 seconds produced a moderate, superficial colitis on the first day after instillation; thereafter a uniform colitis evolved in all rats by the fourth day. However, intraluminal introduction of dilute HCl (pH 2.3) does not produce injury or inflammation (Yamada et al., 1992; Zeitlin & Norris, 1984), suggesting that penetration of the epithelium by the lipid-soluble undissociated form of acetic acid followed by dissociation and acidification in the mucosa probably accounts for the injury. The colitis that developed showed morphological similarities to human ulcerative colitis. Histologically, the model displayed uniform superficial colitis with crypt atrophy, mucin depletion, crypt abscesses and accumulation of inflammatory cells including granulocytes, lymphocytes and plasma cells. However, signs of healing and regeneration of the mucosa were seen at one week, and the mucosa was almost normal at two weeks following administration. The acetic acid model is frequently employed despite the transient nature of the damage produced. The ease of induction, the availability of the materials and the low cost of rats make this an attractive model.

5.1.8 Sulphated polysaccharide models of ulcerative colitis

There are several sulphated polysaccharides which when administered orally to animals will induce colitis having similarities to human inflammatory bowel disease and especially ulcerative colitis.

The oral administration to guinea pigs of an aqueous solution of carrageenan derived from the red seaweed, *Eucheuma spinosum*, produces colonic lesions which are similar to those of human ulcerative colitis (Marcus & Watt, 1969). Two types of ulcerative disease can be produced following a month of carrageenan administration: ulceration
localised mainly to the caecum by using one per cent undegraded carrageenan in the
drinking fluid; and extensive ulceration involving the caecum, colon, and rectum by
using five per cent degraded carrageenan in the drinking fluid (Watt & Marcus, 1975).
Recent refinement of the technique for inducing colitis (by using degraded carrageenan
of a uniform molecular weight and by decreasing the concentration of NaCl) has
improved the consistency of the damage produced (Kitsukawa et al., 1992).
Microscopically, the similarities include focal mucosal haemorrhages and cellular
infiltrates, oedema, crypt abscesses, irregular dilatation of the crypts with a loss of mucin-
secreting cells and degeneration of the epithelium, as well as ulceration involving the
mucosa (Watt & Marcus, 1971). Carrageenan has also been shown to induce lesions in
various other laboratory animals, including mice, rats, rabbits, and monkeys (Benitz et
al., 1973; Kitano et al., 1986; Marcus & Watt, 1971). There are no lesions in the small
intestine and, in contrast to the pattern of human inflammatory bowel disease, no
remission-exacerbation cycles of the colitis occur. The lesions tend to begin at the
caecum and progress distally. This feature, apart from the length of time required to
establish the model, is one of its principal problems, since in human ulcerative colitis
there is a tendency for the disease to affect the distal rather than the proximal colon. The
model is also unresponsive to sulphasalazine (Oestreicher et al., 1991).

A mechanism for the inflammation induced by carrageenan may begin with phagocytosis
by intestinal macrophages, followed by leakage of lysosomal enzymes into the mucosal
tissue and consequent tissue destruction and inflammation (Abraham et al., 1974).
Alternatively, it has been suggested that the acute inflammation observed with five per
cent degraded carrageenan may be simply an osmotic phenomenon, since the damage
can also be produced by giving the animals other osmotic cathartics such as 1 per cent
sodium sulphate in the drinking water (Sharratt et al., 1971). Several investigators have
tried to modify carrageenan induced inflammation with antibiotics. Pretreatment with
anti-microbials directed against coliforms failed to attenuate the disease process
(Onderdonk et al., 1978). On the other hand, pretreatment with metronidazole, an
antimicrobial primarily active against anaerobic bacteria, prevented carrageenan-induced
colitis in most animals (Onderdonk et al., 1978). However, metronidazole treatment of
established colitis showed no benefit. The results suggest that anaerobic bacteria play a
role in the initial events of carrageenan-induced colitis in the guinea pig model but do
not alter the course of the disease once the lesions have formed. Oral administration of
carrageenan also changes the intestinal microflora, particularly increasing gram-negative
anaerobes, and Bacteroides vulgatus has been incriminated in the pathogenesis of
carrageenan-induced colitis in the guinea pig (Breeling et al., 1988). Despite certain
shortcomings of this model an important additional phenomenon has also been noted.
High-grade dysplasia (nonpolypoid) involving the mucosal epithelium was induced in
three of five rabbits treated for 28 months with carrageenan in the drinking water (Kitano et al., 1986), providing further support for this colitis as a comparable model of human ulcerative colitis.

The discovery that sulphated polysaccharides of the carrageenan type could readily induce ulceration of the colon in a range of species prompted investigation of other related high molecular weight sulphonated products. Ulceration of the colon develops in rabbits (Marcus & Watt, 1971) and guinea pigs when they are fed sulphated amylopectin, even in concentrations as low as 0.1 per cent, in the drinking water over two to six weeks. The deleterious effect appears similar to that of degraded carrageenan; this is consistent with the similarities in structure and polyanionic behaviour between sulphated amylopectin and degraded carrageenan. Neither carrageenan nor sulphated amylopectin appear to have any adverse effects in humans.

An ulcerative colitis-like disease can be induced in hamsters, mice and rats by giving dextran sulphate sodium. The first evidence of colitis occurs at seven days following administration of 5 per cent dextran sulphate sodium in the drinking water, sooner than in carrageenan-fed animals (Cooper et al., 1993). As has been reported in the case of carrageenan-induced colitis, dextran sulphate sodium was found to cause a change in the intestinal microflora, and particularly an increase in the number of gram-negative anaerobes, including Bacteroidaceae (Okayasu et al., 1990). The macroscopic and microscopic changes included multiple erosions and inflammatory changes, including crypt abscesses, on the left side of the large intestine. Mice developed chronic colitis (including dysplasia), shortening of the large intestine, and prominent lymphoid follicles after three months of treatment with intermittent five per cent dextran sulphate sodium in the drinking water. The frequency of dysplasia or dysplasia with carcinoma in situ in the caecum and ascending colon was about 25 per cent after three months and 90 per cent after six months of dextran sulphate feeding (Tamaru et al., 1993). Morphological observations showed swollen macrophages in the inflamed colonic wall, a finding consistent with carrageenan-induced or amylopectin sulphate-induced colitis. The initial lesion of a non-inflammatory cell-mediated crypt loss may reflect faulty cell turnover or replication (Cooper et al., 1993).

Although these models display many of the histopathological features of inflammatory bowel disease, they suffer from the length of time required to induce the disease, which increases costs. None of the sulphated polysaccharides appear to have any adverse effects in humans, which detracts from their relevance as models.
5.1.9 Free radical mediated colitis

Recently, colitis has been induced in the rat using a free radical initiator, 2-2’-azobis(2-amidinopropane) dihydrochloride, and ethanol. The treatment evoked colonic mucosal erythema, oedema, and neutrophil infiltration with a corresponding rise in myeloperoxidase activity, but no ulceration. The development of colitis was prevented by sulphasalazine (Tamai et al., 1992). Free radical damage may also have a significant role in a new model of acute colonic inflammation induced by a single systemic injection of mitomycin C (Keshavarzian et al., 1992). Histological examination revealed a transient diffuse, colonic inflammation and injury that, like human ulcerative colitis, was limited to the mucosal layer. The rest of the gastrointestinal tract was spared. Chemiluminescence, a means of estimating levels of reactive oxygen species, was greater in the intact, inflamed colon of mitomycin C-treated rats than in bypassed segments. Furthermore, the reactive oxygen species scavengers allopurinol, catalase, and WR-2721 decreased inflammation severity (Keshavarzian et al., 1992). While this approach displays some promise as a model for ulcerative colitis, the lack of ulceration makes the model less than ideal.

5.1.10 Immunological methods

Another approach that has attracted researchers utilises immunological methods, since many feel that inflammatory bowel disease may represent an allergic response to some intraluminal component, either of dietary or microbiological origin. The inflammatory reactions induced fall into two categories: the humoral models, and cell-mediated immune reactions. The humoral models have been based on the premise that inflammatory bowel disease may reflect either immediate auto-antibody type II hypersensitivity or immune complex mediated type III hypersensitivity. Cell-mediated models of delayed type IV hypersensitivity involve presentation of antigen to T-cells where antibody plays a subordinate role.

The main distinction between type II and type III hypersensitivity is that type II reactions involve antibodies directed to antigens on the surface of specific cells or tissues, whereas type III reactions are due to antibodies against widely distributed antigens. Thus, damage caused by type II reactions is localised to a particular tissue or cell type, while damage caused by type III reactions affects those organs where the antigen-antibody complexes are deposited.

Several groups have examined the possibility that colitis might result from a cross reactivity between colonic self-antigens and endogenous bacteria. Colitis can be induced in mice by sensitising them to colonic extracts (Callahan et al., 1963) and in dogs by
intravenous injection of anti-colon antiserum prepared in ducks and rabbits (Leeven et al., 1961). Most other workers have, however, found that even if colon-auto-antibodies are generated as a result of immunisation with E. coli or colonic extracts (Cooke et al., 1968), histological colitis does not result.

Several models have been based on variants of the Arthus reaction in which administration of an antigen to a previously sensitised animal results in local immune complex formation. Kirsner showed that a haemorrhagic necrotising colitis could be produced as a result of the Arthus phenomenon in immunised rabbits (Kirsner, 1961) but that a more realistic model of human ulcerative colitis could be achieved using the Auer modification (Auer, 1920) of this reaction (Kraft et al., 1963). In this model, a massive inflammation is produced in the colon at the site of a mild non-specific inflammatory reaction elicited by rectal installation of dilute formalin and by the parenteral administration of egg albumin to immunised albino hybrid rats. In 1978 a similar experimental colitis in rabbits was described by Hodgson, following the intravenous injection of preformed immune complexes of human serum albumin and anti-human serum albumin into non-sensitised rabbits (Hodgson et al., 1978a). Once again tissue damage was localised to the colon by the Auer technique of rectal instillation of dilute formalin. In rabbits given intravenous immune complexes, formed in antigen-excess, a severe colitis was initiated with histological features including mucosal ulceration, mixed inflammatory cell infiltration in the lamina propria, and crypt abscess formation. However, the colitis induced by this technique was acute and chronic lesions typical of human ulcerative colitis did not develop. When the ratio of antigen to antibody is high, augmentation of the immune response occurs by encouraging fixation of the antigen on certain antigen presenting cells and subsequent up-regulation of B-cell activation. When the ratio is reversed, preformed immune complexes often suppress B-cell activation. In a later set of experiments the same group investigated prior sensitisation to colonic bacterial antigen of Kunin followed by local administration of formalin in the colon (Mee et al., 1979). Abnormalities, including increased numbers of plasma cells and lymphocytes, were observed up to six months following proctoscopy and rectal biopsy. The results imply that once the mucosal defences are weakened the inflammation may be maintained, as a result of allergic reactions to intraluminal antigens that are normally denied access to the mucosa. Despite the persistence observed in the model, ulceration was not a feature.

More recently, another rabbit model of human ulcerative colitis using lipopolysaccharide has been induced. Skin-sensitisation with lipopolysaccharide, and challenge with intrarectal instillation of lipopolysaccharide after one per cent formalin enema, produced petechiae by eight hours and ulcers and bleeding on the third day. Mild macroscopic
changes continued for about two weeks and tissue fibrinolysis of the colon increased significantly as the mucosal damage appeared. When the procedure was repeated after the initial treatment, the colitis was maintained for over a month (Hotta et al., 1986).

Haptens have been used by a number of researchers to stimulate delayed-type IV hypersensitivity reactions in guinea pigs, mice and rabbits. Delayed type hypersensitivity reactions are T-cell dependent, and are characterised by oedema and mononuclear cell infiltration one to two days after contact with antigen in sensitised individuals. The ongoing inflammation observed in inflammatory bowel disease has many of the appearances of delayed-type hypersensitivity reactions. When small haptens, that would not be antigenic by themselves, penetrate the epidermis they become conjugated, either covalently or non-covalently, to normal body proteins and may become allergenic. 2,4-dinitrochlorobenzene, a hapten, will sensitisate nearly all individuals. Eighty-five per cent of epicutaneously applied 2,4-dinitrochlorobenzene binds to the epidermal cell proteins, by their lysine-NH$_2$ residues, and the conjugates formed serve to sensitisate the animal. T-cell recognition of the conjugate is specific for the hapten-carrier conjugate and is not dependent on the separate recognition of hapten and carrier, which occurs in antibody formation.

In rabbits (Rabin & Rogers, 1978) and guinea pigs (Bicks & Rosenburg, 1964) animals were skin-sensitised to 2,4-dinitrochlorobenzene and challenged two weeks later with an intrarectal instillation of 2,4-dinitrochlorobenzene. The histology of the colon revealed mucosal ulceration, crypt abscesses and epithelial basophilia with increased mitotic figures. An increased inflammatory infiltrate in the lamina propria contained polymorphonuclear neutrophils as well as mononuclear cells. These lesions changed to those consistent with the healing phase of ulcerative colitis at two weeks, but at five weeks only a mild crypt disorganisation remained, with some submucosal fibrosis. Dinitrofluorobenzene has also been used to elicit this delayed type IV hypersensitivity in mice with similar results (Brkic et al., 1992). In keeping with type IV responses no circulation antibodies are necessary for the reaction, and the passive transfer of serum does not induce sensitivity. However, lymph node homogenates, buffy coat, and sterile peritoneal exudate do transfer the hypersensitivity (Bicks & Rosenburg, 1964).

Although some of these immunologically-produced models have many pathological features in common with human inflammatory bowel disease, all of them require lengthy and involved protocols, which limits their practical application.
Although the mechanism of action is not fully understood, the administration of another hapten, 2,4,6-trinitrobenzenesulphonic acid (TNB), in a barrier breaker, ethanol, produces colonic inflammation and ulceration that persists for up to eight weeks (Morris et al., 1989). The rationale for the model is based on the hypothesis by Ward that inflammatory bowel disease arises from an inability of the reticuloendothelial system to degrade a variety of normal gut luminal constituents (Ward, 1972). The necessary exposure to the defective cell results from an increase in intestinal mucosal permeability. Administration of 30mg of TNB in 0.25 cm$^3$ of 50 per cent ethanol can produce an inflammatory response, including mucosal and submucosal infiltration by polymorphonuclear leukocytes, macrophages, lymphocytes, connective tissue mast cells, and fibroblasts. Granulomas were observed in 57 per cent of the rats killed three weeks after induction of inflammation. Langhan's-type giant cells were also present. Segmental ulceration and inflammation, similar to the skip lesions in Crohn's disease, were common. Interestingly, the induction of colitis is associated with a significantly increased colonic Gram-negative aerobic bacilli count, and a positive correlation between the severity of colonic inflammation and the extent of bacterial translocation in colitic animals (Gardiner et al., 1993). The model has also been shown to respond to various therapies, including dietary manipulation (Guarner et al., 1992), epidermal growth factor (Luck & Bass, 1993), and leukotriene inhibitors (Wallace & Keenan, 1990; Wallace et al., 1989). The principal disadvantages of the model lie in the non-physiological method of induction and the fact that despite the long duration the model does eventually heal with no periods of remission and relapse.

5.1.12 Summary

Experimental models of colitis may be induced in a variety of ways. Irrespective of the initial perturbation, the mechanism underlying gastrointestinal inflammation ultimately involves a response to stimuli in the mucosal environment, so that pathological events are inevitably channelled into an inflammatory pathway, resulting in an inflammatory bowel disease-like pathology. With this latter consideration in mind, the TNB induced colitis in the rat has several advantages over other models. The inflammation is induced by a single intraluminal instillation, and the severity and duration appear to be reproducible. The inflammation results in many of the histopathological features which are associated with inflammatory bowel disease, especially Crohn's disease; also, the relatively long duration of inflammation and ulceration induced in this model provide an opportunity to study the pathophysiology of colonic inflammatory disease in a controlled fashion, and to evaluate new treatments potentially applicable to inflammatory bowel disease in humans.
Chapter 3

The TNB model has not been fully characterised in a laboratory animal other than the rat. In some recent studies, acute colitis has been induced in rabbits, but the animals have been killed shortly after administration (Percy et al., 1993; Wallace et al., 1992), no dose response experiments and minimal histology are reported. There are justifiable reasons for developing a rabbit model as well as a rat model. While the anatomy of the rat and human colons is similar, the response to inflammatory mediators display more similarities when comparing the rabbit and human response than when comparing rat and human response. Moreover, these responses are mediated by similar mechanisms (Percy et al., 1990). The rabbit also has the advantage of a larger colon than that of the rat, allowing it to be inspected endoscopically for inflammatory changes, and furthermore some antibodies for immunohistochemistry are only available to the rabbit and not the rat.
5.2 **Aim of the study**

The principal aim of the present study was to establish whether 2,4,6-trinitrobenzene sulphonate acid in an ethanol solution used in the rat would produce a histopathologically relevant chronic intestinal inflammation similar to human inflammatory bowel disease when administered in the distal colon of the rabbit. Further objectives were to determine the optimum dose and the duration of the colitis, and to assess whether the progress of the disease could be followed using occult blood detection and endoscopy.

5.3 **Materials and methods**

5.3.1 Chemicals

All chemicals were obtained from Merck, Poole, Dorset, UK and were of AnalAr grade unless otherwise stated.

5.3.2 Animals

Home Office approval was obtained for all procedures. Female New Zealand white rabbits (Froxfield Farms, Petersfield, Hants, UK) weighing between 2 and 2.5 kg were used for all experiments, and were housed in individual cages. No bowel preparation was carried out, and there was no preoperative restriction of food or water. Standard laboratory pelleted formula and tap water were provided *ad libitum*. At least three animals were used per group in experiments to determine the optimum dose for the induction of colitis and for investigation of the time course.

5.3.3 Anaesthesia

Rabbits were anaesthetised with a 0.3 cm$^3$/kg hypnorm (Jassen Pharmaceuticals Ltd, Oxford, UK) injection given intramuscularly in conjunction with a 2.5 mg/kg diazepam (Phoenix Pharmaceuticals Ltd, Gloucester, UK) injection intraperitoneally in 10 cm$^3$ of a sterile saline solution (0.9 per cent w/v). Diazepam has hypnotic, sedative, anxiolytic, amnestic, anticonvulsant, and centrally-produced muscle relaxant properties, but lacks analgesic properties. It is generally agreed that benzodiazepines exert their effects by occupying the benzodiazepine receptor that modulates gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the brain (Mohler & Richards, 1988). Hypnorm contains fentanyl citrate and fluanisone. Fentanyl provides analgesia. Fluanisone is a neuroleptic of the butyrophenone group that induces hypnosis, sedation, and antiemetic effects. Butyrophenones may occupy GABA receptors on the post synaptic membrane, thereby reducing synaptic transmission and resulting in a build-up of dopamine in the intersynaptic cleft (Reves & Glass, 1989).
5.3.4 General method for the introduction of solutions into the lumen of the distal colon

Rabbits were randomised into treatment groups and an infants' feeding tube (6 French gauge, from Portex, Hythe, Kent, UK) was inserted anally into the rectum and eased into the colon so that the tip was 20cm proximal to the anus, at approximately the left colic flexure. Insertion was facilitated by the application of K-Y lubricating jelly to the feeding tube. A 0.6cm³ volume of solution was then injected through the feeding tube into the lumen of the distal colon. The tube was flushed with 7cm³ of air, to ensure delivery of the complete dose, and then withdrawn.

5.3.5 Verification of the position of the tube in the colon

A midline incision was made in the abdomen of three control animals following insertion of the feeding tube to 20cm. The colon was exposed, and the position of the tip of the tube was found to be just distal to the left colic flexure. 0.6cm³ of 0.9 per cent saline was instilled via the tube. The abdomen was closed and the animals were allowed to recover. After three hours, the animals were killed and the entire large bowel was removed and examined. There was no evidence of damage caused by the insertion of the tube or by the instillation of saline.

For further confirmation of the position of the tube in the colon, X-ray radiographs were taken of five animals in later experiments (the feeding tubes are radio-opaque). The radiographs confirmed that the tip of the feeding tube had passed into the distal colon. In a single control animal just prior to killing, a feeding tube was inserted and 0.6 cm³ of barium sulphate solution was introduced to the lumen. The barium sulphate was observed, by means of real-time radiography, to quickly cover the walls of the bowel below the tip, and also to move up the colon proximal to the site of introduction.

5.3.6 Experiments to determine the optimum dose for the induction of colitis

5.3.6.1 Concentrations of ethanol used

Initial experiments were performed to determine the appropriate concentration of ethanol to be used in all further investigations. Animals received either a 50 per cent or a 25 per cent solution of ethanol in saline, with or without 40mg of TNB, to determine the effect on the pathology of different ethanol concentrations. These animals were killed after two weeks. The colons were removed, and examined both macroscopically and microscopically (see section 5.3.11).
5.3.6.2 Concentrations of trinitrobenzene sulphonic acid used

Experiments were performed to establish the optimum dose of 2,4,6-trinitrobenzene sulphonic acid (TNB) required for the induction of colitis. Groups of rabbits received TNB in doses of 2.5mg, 5mg, 10mg, 15mg, 20mg, 25mg, 30mg, 40mg and 50mg dissolved in a 25 per cent ethanol, 0.9 per cent w/v saline solution. For the dose response study, rabbits were generally killed two weeks after the single administration of TNB/ethanol. Pilot studies with a limited number of animals had demonstrated that transient damage in the control groups (see section 5.3.6.3 below) had been completely resolved by seven days and that damage was still present at two weeks in the animals that received TNB in ethanol. In some groups a few rabbits were endoscoped at two weeks and killed at four weeks. The colons were removed, and examined both macroscopically and microscopically (see section 5.3.11).

5.3.6.3 Controls to show that both TNB and ethanol are required for chronicity

Three groups of rabbits were set up as controls for the initial characterisation and development experiments. Rabbits were anaesthetised and received 0.6cm$^3$ of 0.9 per cent saline (control group 1), or 0.6cm$^3$ of 25 per cent ethanol (control group 2), or the same doses of TNB listed in section 5.3.6.2 in 0.6cm$^3$ of 0.9 per cent saline (control group 3).

5.3.7 Experiments to examine the time-course of the colitis induced

Changes in the pathology of the model were investigated over a six week period by setting up groups of animals with the optimum dose (see below) and killing them at different times after the induction of colitis. Control groups were set up as before (section 5.3.6.3). The animals were killed at 0h, 3h, 6h, 12h, 24h, 72h, 1 week, 2 weeks, 4 weeks, and 6 weeks. All the animals were weighed daily for the first two weeks and then once a week thereafter.

5.3.7.1 Optimum dose for the induction of chronic inflammation

For this and all further investigations colitis was induced by the introduction into the lumen of the colon of 0.6cm$^3$ of the hapten 2,4,6-trinitrobenzene sulphonic acid (40mg) dissolved in a solution of 25 per cent ethanol in 0.9 per cent (w/v) saline.

5.3.7.2 Endoscopy

Rabbits were examined endoscopically after each week under general anaesthesia (0.3cm$^3$/kg hypnorm i.m. with 2.5mg/kg diazepam i.p.), using a paediatric
bronchoscope (Olympus BF 1TR, from Keymed, Southend-on-Sea, Essex) connected to an air pump (ACM 710A, from Wappler, Germany.), with illumination provided by a cold light supply (Olympus CLE-4U, from Keymed, Southend-on-Sea, Essex). The time dependence of the development and healing of inflammation and ulceration was assessed. Damage was scored on a 0 to 3 scale (table 5.3) by the endoscopist, blind to the identity of the rabbit. Photographs were taken using a single-lens reflex camera, OM-1 (Olympus, from Keymed, Southend-on-Sea, Essex), fitted with the 1-9 focusing screen and the camera mounting adapter SM-2S needed to connect the camera to the bronchoscope. Kodak 160T film was used and photographs were taken with a shutter speed of 1/4s and with the exposure index set to 2 on the CLE-4U.

5.3.8 Occult blood detection

5.3.8.1 Principle

The method used for detecting the presence of blood in faeces depends on the peroxidase activity of haemoglobin. This type of activity has been determined by the use of chromagens such as benzdine, o-dianisidine and o-tolidine, but as these compounds are now considered to be potential carcinogens, reduced phenolindo-2,6-dichlorophenol is now employed instead of o-tolidine (Clark & Timms, 1968).

Peroheme 40 is a stable modification of the reagent that has been used for the detection of occult blood in the faeces of animal species (Dent, 1973a; Dent, 1973b). The test will detect 1 part of blood in 40,000 parts of sample.

5.3.8.2 Sampling

Pellets were collected from the rabbits prior to the induction of colitis, daily for the first week and then every other day until negative results were consistently obtained.

5.3.8.3 Procedure

The procedure recommended by the manufacturer of Peroheme 40 was followed. Each box contained a pad of 50 double test papers, a sealed bottle of reagent 1, and a sealed bottle containing a tablet of urea hydrogen peroxide (reagent 2) and two dropper attachments.

The aluminium seals and the septum plugs were removed from the reagent bottles. 5cm$^3$ of distilled water was added to the bottle containing the tablet of urea and the dropper attachments were fitted to both bottles. The test reagents were stored at 4°C when not in use.
Chapter 5

Moist faeces from the rabbits was smeared on to a piece of the absorbent paper provided. One drop of reagent 1 was added, followed by one drop of reagent 2. A red-pink coloration observed within two minutes indicated a positive result (coloration developing after two minutes was disregarded). If the colour developed instantly after the addition of reagent 2, the test was scored as very positive (++).

<table>
<thead>
<tr>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colour develops instantly</td>
</tr>
<tr>
<td>Colour develops within 2 minutes</td>
</tr>
<tr>
<td>Colour develops after 2 minutes</td>
</tr>
</tbody>
</table>

*Table 5.2: Occult blood detection scoring.*

5.3.9 Assessment of colonic inflammation and damage

The rabbits were killed with sodium pentobarbitone 200mg/cm$^3$ (Expiral, from Sanofi, Watford, Herts). The entire colon was mobilised, resected and opened along the anti-mesenteric border, then washed with saline and placed in Hank's balanced salt solution containing antibiotics (penicillin 100 units/cm$^3$, streptomycin 100mg/cm$^3$ and amphotericin B 0.25mg/cm$^3$, all from Sigma, Poole, Dorset). The colon was examined under a stereo-microscope and any abnormality scored according to the same criteria as at endoscopy (table 5.3) by two observers independently. Figure 5.2 shows a schematic drawing of the rabbit intestinal tract.

<table>
<thead>
<tr>
<th>Morphology scores</th>
<th>Corresponding appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No abnormality.</td>
</tr>
<tr>
<td>1</td>
<td>Inflammation, but no ulceration.</td>
</tr>
<tr>
<td>2</td>
<td>Inflammation and ulceration at one site (&lt;1 cm).</td>
</tr>
<tr>
<td>3</td>
<td>Two or more sites of inflammation and ulceration OR one major site of inflammation and ulceration extending further than 1cm along the length of the colon.</td>
</tr>
</tbody>
</table>

*Table 5.3: Macroscopic scoring scheme. Severity and extent of the macroscopic inflammation and ulceration was assessed on endoscopy and on killing the rabbits, and scored from 0 to 3.*
Figure 5.2: Schematic drawing of the intestinal tract of a rabbit.

 Indicates position at which tissue was removed for histology
5.3.10 Tissue sampling for microscopic assessment

Segments of colon were excised at autopsy (a) from the inflamed and ulcerated regions and (b) from normal colon; segments 0.5 cm wide were cut from positions at 3 cm, 5 cm, 9 cm, 10 cm, 15 cm, 20 cm, 30 cm and 60 cm proximal to the anus. Specimens of ileum, liver, lung, heart, and kidney were also removed for histology.

5.3.11 Histological assessment

All specimens selected at autopsy were fixed in neutral buffered formalin and embedded in paraffin (see appendix A); then 4 μm sections were cut on a microtome (Bright Instrument Co., Huntingdon, England). Sections were cleared in CNP30, rehydrated through alcohols (70 per cent to 100 per cent), stained in Harris's haematoxylin for 5 minutes, washed, differentiated and counter-stained with eosin for 5 minutes (appendix B). Slides were mounted in DPX and examined on a Leitz Laborlux S microscope (Leica, Milton Keynes, UK) using a tungsten lamp. Photomicrographs were taken on 160 ASA film for tungsten light (Kodak ektachrome).

The presence or absence of a series of histopathological features in the colon, as shown in table 5.4, was recorded.

The features chosen are representative of the pathology found in inflammatory colorectal disease, and these features are described in detail below.

5.3.11.1 Mucosal crypt architecture

An inspection of the crypt architecture at low power was performed to look for abnormalities affecting crypts along the length of mucosa, seen in various forms of colitis.

Crypt branching implies prior mucosal damage. Branching crypts may be bifid or even, after severe damage, run parallel to the surface. Few irregular crypts have no specific diagnostic significance, apart from the implication of previous damage, but when crypt branching is a dominant feature it is diagnostic of ulcerative colitis in humans. Branching may also be seen in a healing mucosa after any bout of severe ulceration, for example, in ischaemia. However, in infective colitis and Crohn's disease the basic crypt alignment is usually maintained.
### Histological Features investigated

| Mucosal Crypt Architecture | Crypt branching and bifurcation  
|                           | Crypt atrophy  
|                           | Crypt degeneration  
|                           | Crypt dilatation  
|                           | Crypt enlargement  
|                           | Crypt abscess  
|                           | Misplaced crypts  
| Crypt Epithelium           | Mucin depletion  
| Lamina Propria - cells     | Proportions of inflammatory cells (acute/chronic)  
|                           | Distribution of inflammatory cells  
|                           | Giant cells  
|                           | Granulomas  
| Submucosa & Muscularis    | Inflammation (acute or chronic)  
| Propria                   | Neuronal hyperplasia  
|                           | Lymphangectesia  
| Ulceration                | Erosion  
|                           | Acute ulceration  
|                           | Chronic ulceration  
|                           | Fissures  
| Other                     | Pseudopolyps  
|                           | Fibrosis  
|                           | Oedema  
|                           | Hyperplasia (regenerative)  

**Table 5.4: Histological features for the assessment of microscopic abnormalities.**

A reduction in the number of crypts was interpreted as **crypt atrophy**; this is most commonly seen in ulcerative colitis. As with branching, it may be the end result of healing after severe acute ulceration or chronic disease states. As well as showing increased lateral separation, the crypts are generally shorter than normal, with an obvious gap between their bases and the muscularis mucosae.

**Crypt degeneration** can involve either the whole crypt or the superficial half of the crypt. Damage to the superficial half of the crypt is a useful pointer to ischaemia and pseudomembranous colitis. The bases frequently survive and the remaining epithelial cells appear hyperchromatic and basophilic. Degenerative changes throughout the length of the crypts may occur in either ischaemic disease or infective proctocolitis.
Isolated crypt dilatation is not uncommon, but its presence was recorded since it may indicate the presence of other pathology. In humans, groups of dilated crypts are a useful indicator of pseudomembranous colitis.

Crypt enlargement, where the crypts appear longer and are lined by taller mucus secreting cells, is characteristic of transitional mucosa. Transitional mucosa is commonly found adjacent to tumours.

Crypt abscesses were identified by the presence of polymorphonuclear neutrophils within the lumen of a crypt. More than two crypts involved in any one section constituted active inflammation.

Although crypt abscesses are common in ulcerative colitis they are certainly not diagnostic of that condition and are seen in other forms of colitis. The crypt abscesses justify the label of 'colitis'. When crypt abscesses dominate the picture, ulcerative colitis would be a histologist's first diagnosis. Crohn's disease and bacterial diarrhoeas can produce crypt abscesses, but this is the exception rather than the rule.

Misplaced crypts within or beneath the muscularis mucosae, in human pathology, are of significance in association with any polypoid lesion in which there has been localised mucosal damage due to torsion or ischaemic infarction and in healed fissuring ulcers, as in Crohn's disease and amoebiasis.

5.3.11.2 Crypt epithelium

Mucin depletion is the result of discharge and exhaustion of goblet cells, which become morphologically indistinguishable from absorptive columnar cells. Goblet cell discharge occurs commonly, and can be simply a response to an irritant, in which case the mucin depletion is mild. Extensive depletion is seen in infective and ulcerative colitis, ischaemia and radiation colitis. Mucin depletion also occurs in Crohn's disease, but is usually focal and less conspicuous.

5.3.11.3 Cells of the lamina propria

The normal stroma of the lamina propria always contains an inflammatory component.

The clearest evidence of inflammation is an increase in cellularity of the lamina propria. The cells may be polymorphonuclear neutrophils, lymphocytes, plasma cells or eosinophils in varying proportions.

A polymorphonuclear neutrophil exudate is a manifestation of acute inflammation, with dilatation of mucosal capillaries and venules, margination of neutrophils in these vessels,
and exudation of fluid and polymorphonuclear neutrophils into the surrounding lamina propria. The presence of polymorphonuclear neutrophils is not a specific marker of a particular disease, but the number of polymorphonuclear neutrophils probably reflects the degree of tissue damage.

Plasma cells are also normal constituents of the lamina propria, and increase in all forms of colitis. They are, therefore, a non-specific marker of inflammation, although the proportion in relation to the numbers of polymorphonuclear neutrophils present has some diagnostic implications. As the acute phase of the various forms of colitis is resolved, the ratio of plasma cells to polymorphonuclear neutrophils increases. Since lymphocytes are numerous in the lamina propria of normal mucosa, an increase in numbers is difficult to assess. Lymphoid aggregates are associated with Crohn's disease.

Inflammation, if present, may be superficial, diffuse, patchy or focal. Superficial inflammation is typical of infective colitis. Diffusely distributed inflammatory cells throughout the mucosa indicate a disease process affecting the surface and crypt epithelium uniformly. Lymphoid follicles, rather than focal lymphoid aggregate, may be present. Ulcerative colitis is one disease in which this condition occurs. Variation in the intensity of inflammation from one part of the mucosa to another can occur in a wide variety of diseases. Diffuse patterns of inflammation become patchy as remission occurs. Focal mucosal inflammation, with a background of normal mucosa, is typically seen in Crohn's disease, but is not in itself specific and may represent resolving infective colitis.

Giant cells formed by the coalescence and fusion of activated macrophages are frequently seen in and around fistulae, and Crohn's disease would be the first diagnosis to exclude in this instance.

Granulomas are identified as small, 0.5mm to 2.0mm, collections of activated macrophages called 'epithelioid cells', usually surrounded by a rim of lymphocytes. These activated macrophages have abundant pale pink, plump cytoplasm, thus resembling an epithelial cell. Multinucleate giant cells may or may not be present. In human pathology, the presence of small granulomas within the bowel wall is evidence of Crohn's disease or intestinal tuberculosis. Large granulomas, with confluent sheets of histiocytes, are found in chronic granulomatous disease of childhood and in infection with Mycobacterium avium in patients with acquired immunodeficiency syndrome (AIDS). Discrete granulomas are sometimes found around ova in schistosomiasis and occasionally, related to lymphatics, in the vicinity of adenocarcinomas.
5.3.11.4 Submucosa and muscularis propria

Disproportionate inflammation in the submucosa is most often a sign of Crohn's disease. However, inflammation may extend into the submucosa beneath severe ulceration of any origin.

Abnormal, thick and irregular nerve bundles are present in the submucosa in large numbers in Hirschsprung's disease (congenital aganglinosis). Submucosal neuronal hyperplasia can also be a conspicuous feature of Crohn's disease.

Lymphangectesia is commonly observed histologically in Crohn's disease and, although it is most likely to reflect obstruction in draining lymph nodes secondary to inflammation, it has been suggested as a causative mechanism.

5.3.11.5 Ulceration

An ulcer is a local defect, or excavation, of the surface of an organ or tissue, produced by the sloughing of inflammatory necrotic tissue. In the acute stage, there is intense polymorphonuclear infiltration and vascular dilatation in the margins of the defect. With chronicity, the margins and base of the ulcer develop fibroblastic proliferation and scarring, with the accumulation of lymphocytes, macrophages, and plasma cells.

Ulceration when present was recorded as an erosion, acute ulceration or chronic ulceration. An erosion was defined as an area of superficial ulceration involving the mucosa but not the muscularis mucosae, whereas acute ulceration also involved the muscularis mucosae. An ulcer completely disrupting the muscularis mucosae and extending through the submucosa into the muscularis propria was the criterion for chronic ulceration. Deep focal ulceration penetrating the submucosa was separately identified as fissuring ulceration.

5.3.11.6 Other

Pseudopolyps (or inflammatory polyps as they are otherwise known) indicate a prior severe bout of mucosal ulceration. Inflammatory polyps may comprise granulation tissue, a mixture of granulation tissue and glands, or a tag of virtually normal mucosa, often surrounded by areas of ulcerated tissue.

The presence or absence of fibrosis, oedema and regenerative hyperplasia in the mucosa, submucosa and muscle layers were also noted.
5.4 Results

Figures for the microscopic appearances of tissue collected from the experiments, and tables summarising the histological results, are presented at the end of the results section.

5.4.1 Observations during the course of the experiments to determine the optimum dose

The animals took about three hours to recover from the anaesthetic. During the first 24 hours the animals tended not to eat or drink. Liquid or very soft stools and a ten-per cent weight loss were observed in rabbits that received TNB in ethanol over the first week (figure 5.3). The growth rates of these animals returned to normal in the second week. Neither weight loss nor diarrhoea was associated with any of the three control groups. Blood was commonly observed in the faeces of the animals that received higher doses of TNB in ethanol, but not in any of the control groups. Mortality in the rabbits treated with TNB/ethanol rose with the dose of TNB, and was the result of either faecal peritonitis following perforation or of toxic dilatation, where perforation did not occur.

5.4.2 Macroscopic assessment of the dose response experiments

5.4.2.1 Concentrations of ethanol used

Two alternative concentrations of ethanol were evaluated. 40mg of TNB in 50 per cent ethanol in saline resulted in the premature death of all six animals into which it was instilled. The animals that received 40mg of TNB in 25 per cent ethanol in saline displayed inflamed and ulcerated colons at autopsy, with an associated mortality rate of 15 per cent. (See section 5.4.2.2 for a full description of the macroscopic appearance.) The 25 per cent ethanol in saline solution was used for all further investigations.

5.4.2.2 Concentrations of trinitrobenzene sulphonic acid used

Macroscopically, there was a significant correlation (p<0.001) using the chi-squared test for trend between the score assigned and the dose of TNB instilled at two weeks (table 5.5). Where macroscopic damage was visible, it occurred at the same site irrespective of the dose, usually over an area between 5cm and 15cm proximal to the anus, and was characterised by bowel wall thickening, an erythematous appearance, and loss of vascular pattern (figure 5.4). Incomplete obstruction of the colon by stricture formation with proximal dilatation of the colon was frequently observed. Ulceration when present occurred either as narrow linear ulcers, surrounded by thickened mucosa and often
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Figure 5.3: Weights of rabbits with time. Only the TNB/ethanol group shows an initial decrease in weight. Error bars ± s.d.
Figure 5.4: Resected bowel from a rabbit killed two weeks after receiving 40mg TNB/ethanol. A region from the anus (AN) to the ascending colon (AS) is shown. The majority of the damage is present between 10cm and 15cm proximal to the anus (see figure 5.5B for magnified view), although sites of inflammation and ulceration at 8cm and 25cm (corresponding to the site of administration) are also visible. Above 15cm the bowel is dilated and thickened - a feature typical of the macroscopic damage found in the model.
Figure 5.5 A & B: Typical macroscopic views of the distal colon 10cm to 15cm proximal to the anus at two weeks (opened to reveal the mucosa uppermost). (A) The appearance of normal colon (saline control group I) showing the vessel pattern and translucent appearance of the mucosa. (B) A diseased colon from an animal that received 40mg TNB ethanol. The colon is thickened and ulcerated. White arrows indicate areas of ulceration.
extending for up to 2cm, or as skip lesions, that is, small ulcers interspersed with normal mucosa giving a 'cobblestone' appearance (figure 5.4). Extensive ulceration often involved the entire circumference and extended for more than 2cm along the colon. Damage was rarely observed proximal to the left colic flexure, except in cases where the colon had been severely distended from obstruction.

<p>| Frequency of scores at dose of trinitrobenzensulphonic acid |
|-----------------------------------------------|------|------|------|------|------|</p>
<table>
<thead>
<tr>
<th>Score</th>
<th>10mg</th>
<th>20mg</th>
<th>30mg</th>
<th>40mg</th>
<th>50mg</th>
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</tr>
<tr>
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<td>0</td>
<td>0</td>
<td>6</td>
<td>7</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 5.5: Number of times each score was assigned at various doses of trinitrobenzene sulphonic acid at two weeks. There is a significant correlation ($p<0.001$) using the chi-squared test for trend (Bland, 1987) between the score assigned and the dose of TNB instilled.

10mg TNB in 25 per cent ethanol

Three animals received 10mg of TNB, and these were endoscoped after one week. The colons displayed a small amount of erythema at about 10cm in each animal, but no other sign of damage. Two animals were killed at two weeks and the other was endoscoped; there was no evidence of macroscopic damage resulting from the introduction of the TNB/ethanol solution. The third rabbit was killed at four weeks and the colon appeared macroscopically normal.

20mg TNB in 25 per cent ethanol

Three animals received a 20mg dose of TNB and were endoscoped at one week and killed at two weeks. At endoscopy this dose of TNB was observed to give variable results. One animal scored 3, having a large area of visible ulceration surrounded by extensive inflammation, another animal had a small erythematous area in the distal colon, and the third animal appeared to be completely free from any damage. At two weeks when the animals were killed this remained the pattern. Only one animal had visible ulceration (score 2) present, and in the distal colon of the other two animals only small patches of inflamed tissue were visible.
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25mg TNB in 25 per cent ethanol

Three animals were given 25mg of TNB. After one week, endoscopy was possible in only one of the animals, in which a small area of ulceration was observed; the other two animals had very soft stool. At two weeks the animals were killed. All three had small aphthous ulcers at about 10cm proximal to the anus and surrounding inflammation. Elsewhere the colons appeared normal.

30mg TNB in 25 per cent ethanol

Ten animals were given 30mg of TNB. Two animals died in the first week with faecal peritonitis following perforation of the distal colon. The remaining animals were all endoscoped at one week. One of the animals had very soft stool and was impossible to examine; all the others had macroscopically visible ulcers and scored 2 or 3. After two weeks, four of the remaining animals were chosen at random at two weeks from the group and killed. Two of these animals scored 2 and two scored 3. The four remaining animals were endoscoped at two weeks and all had ulcerated colons at 10 to 15cm proximal to the anus. These animals were killed two weeks later to see if the ulceration persisted. At four weeks one animal still had macroscopically visible ulceration surrounded by inflamed tissue. The other colons still appeared abnormal and inflamed, but there was no visible ulceration.

40mg TNB in 25 per cent ethanol

Of thirteen rabbits in which 40mg of TNB was administered, two died in the first week and the remaining eleven were killed at two weeks. In ten cases there was still macroscopically visible ulceration in the distal colon (figure 5.5B). Although as with 30mg there was an equal proportion of animals that scored 2 or 3, the damage was more extensive in the animals that received the 40mg dose, with no rise in the mortality rate.

50mg TNB in 25 per cent ethanol

The three animals receiving 50mg of TNB in this group all had diarrhoea for the course of the experiment, and became very dehydrated, each losing 0.5kg in weight. None of the animals could be endoscoped at one week. The condition of one animal deteriorated in the second week and it was destroyed. The two remaining were killed at two weeks; both animals had peritonitis and multiple adhesions, making the colons difficult to isolate. This dose was therefore unsafe for use in further studies.
5.4.2.3 Control groups

At two weeks no macroscopic abnormalities were observed in the rabbits that received saline alone (control group 1) (figure 5.5A), ethanol alone (control group 2) or TNB in saline (control group 3).

5.4.3 Microscopic assessment of the dose response experiments

5.4.3.1 TNB/ethanol

Microscopically the severity of the changes from normal colon increased with higher doses of TNB (table 5.6). With a 10mg dose of TNB, only mucin depletion and infiltration of inflammatory cells into the lamina propria occurred. With 20mg of TNB, there were more extensive changes including crypt abscesses (for example see figure 5.10), architectural abnormalities and lamina propria oedema. Erosion of the mucosa was observed in only one rabbit. At 30mg TNB, all rabbits had ulcers and many of the other features, but it was only at 40mg TNB that extensive chronic ulceration occurred regularly, and at this dose there was a higher incidence of the other characteristic features of inflammatory colon pathology (figure 5.12). Rabbits given 50mg of TNB displayed acute transmural inflammation and ulceration, but this dose was not further investigated because of difficulty experienced in resecting the colons and the poor condition of these rabbits.

5.4.3.2 Controls

No microscopic abnormalities were found in control groups 1 or 2 (figure 5.8). In those animals that received 40mg TNB in saline (control group 3), a small increase in the numbers of acute inflammatory cells within the lamina propria was observed, but no other feature was apparent.

5.4.4 The optimum dose

The most consistent results were obtained with 40mg TNB in 25 per cent ethanol. This optimum dose was chosen for use in all further experiments. The associated mortality rate was 15 per cent.

5.4.5 Time-course experiments

5.4.5.1 Appearance at endoscopy

Rabbits maintained for more than one week were endoscoped weekly throughout the experimental period (figure 5.6A&B). Endoscopy showed damage consistent with that
observed in rabbits killed at the same time, and so the macroscopic score given at
endoscopy was combined with that assigned to rabbits killed at the same time. This
showed that the score remained at two and three from 24 hours to two weeks. After this
time, there was a gradual decrease in the score until six weeks. It was also observed that
an endoscopically normal mucosa may still have histological abnormalities.

5.4.5.2 Results of the occult blood tests

Tests were carried out to detect occult blood in the fresh stool of the animals receiving
TNB in ethanol for up to three weeks after the challenge; the results (positive or
negative) gave a good indication of the degree of ulceration within the colon in the
experimental animals. At one week all animals that scored 3 had positive tests for occult
blood. However, in tests for occult blood carried out after three weeks there was no
occult blood present, although macroscopic ulceration was still apparent.

5.4.6 Time-dependent macroscopic assessment

5.4.6.1 TNB/ethanol

In the three animals killed at t0, the resected colon was bright yellow in colour from the
point of delivery (at 20cm) down to the anus. (The TNB solution is bright yellow).
Although no ulcers were apparent, most of the mucosa had suffered a chemical burn. At
three hours (n=3) and twelve hours (n=3) the appearance was little changed, although it
was evident at 12 hours that the TNB solution had accumulated in the region 10 to
15cm proximal to the anus. Some areas appeared to have been spared, possibly by folds
in the mucosa.

Macroscopically at one day (n=3) and three days (n=3), the damage at post mortem was
characterised by extensive hyperemia throughout the entire colon and areas of
coagulative necrosis and ulceration in the distal colon. The bright yellow colour had
disappeared, except within areas of necrotic tissue.

Fourteen rabbits were killed at one week; ten of these scored 3 and the remainder scored
2. Only small patches of coagulative necrosis were seen. All of the animals had by this
time developed areas of grossly visible bowel wall thickening, inflammation, and
ulceration. The main site of inflammation and ulceration was typically found at the
junction of the descending colon and the sigmoid colon at 10cm proximal to the anus.
At one week, no inflammation or ulceration was found above the left colic flexure,
except in cases where the colon had been chronically distended. In addition to severe
transmural inflammation, fibrinous adhesions to the small bowel were sometimes
observed. After two weeks, the diffuse inflammation present initially had become patchy.
Figure 5.6A & B: Endoscopic view of the rabbit distal colon 10cm proximal to the anus one week after treatment. (A) The appearance of normal colon (saline control group 1) showing the vessel pattern and pale appearance of the mucosa. (B) A diseased colon from an animal that received 40mg TNB/ethanol. There is a loss of vascular pattern and the mucosa has a granular appearance. White arrows indicate an area of inflammation and ulceration. Note: the black dots on the photographs are the result of broken fibres in the endoscope.
and associated with one or more sites of extensive ulceration, often displaying a 'cobblestone' appearance. By four weeks, the extent and size of ulceration had diminished, and in three of the six colons examined the macroscopic damage had been completely resolved. Three of six colons examined at six weeks still had erythematous patches and were often thickened circumferentially. One animal killed at six weeks still had a 0.5cm diameter ulcer 15cm proximal to the anus.

5.4.6.2 Controls for the time course experiments

Animals receiving saline alone (control group 1) at no time displayed any evidence of damage resulting from the instillation of saline through the feeding tube. Those animals given ethanol alone (control group 2) displayed diffuse inflammation, but no ulceration, in the distal colon at one day, with 25 per cent ethanol (n=3) and 50 per cent ethanol (n=2). At three days (n=3), this transient inflammation had been completely resolved. At one day the appearance of the colons in those animals (control group 3) that received 40mg TNB in saline (n=3) was similar to those that had received the TNB in ethanol, although the damage was less extensive. By three days only patchy inflammation remained (n=3). The colons appeared normal in this control group at one week (n=3) and at two weeks (n=3).

5.4.7 Time-dependent microscopic assessment

5.4.7.1 TNB/ethanol

At t₀, tissue examined from sites 5cm, 10cm, 15cm and 20cm proximal to the anus was oedematous, but otherwise appeared normal, except that the epithelial cells were very eosinophilic. At three hours, the appearance was little changed. By six hours, polymorphonuclear neutrophils could be seen marginated in dilated vessels packed with red blood cells within the mucosa and submucosa. Although the overall structure of the mucosa and crypts appeared to be intact, the epithelium and a region immediately underneath it that had been exposed to TNB was clearly necrotic, having been 'fixed'. At twelve hours, the epithelium was missing on some sections from 5-15cm, and there was an increased number of polymorphonuclear neutrophils in the mucosa. However, in no instance did the inflammation penetrate the muscularis mucosae.

One day after the introduction of TNB in ethanol, acute ulcers with a large underlying infiltrate of polymorphonuclear neutrophils and other architectural abnormalities were present (table 5.7). Crypt abscesses (for example see figure 5.10), mucin depletion and oedema were observed in all the animals studied after one day.
At three days there was a marked contrast between the relatively normal appearance of the TNB/saline control group (with only mucin depletion and oedema) and the damage observed in the TNB/ethanol group, in which most of the features associated with gastrointestinal inflammation occurred. After one week the muscularis mucosae was, in all cases in the TNB/ethanol group, disrupted by deep ulceration that extended to the submucosa (for example see figure 5.16) and an inflammatory cell infiltrate in both the lamina propria and submucosa. Regenerative hyperplasia and pseudopolyps were features present at this time which had not previously been seen (table 5.7). In addition, an increase in the number of extravascular inflammatory cells, particularly polymorphonuclear leukocytes, was commonly observed at sites up to 20cm away from any visible macroscopic damage.

At two weeks, the pattern in TNB/ethanol was similar to that after one week, although the overall histology score was greater (figure 5.7) since features such as giant cells and granulomas (which had not previously occurred) were present in some rabbits (figures 5.14 & 5.18). The inflammatory cell infiltrate was now of a chronic nature, as indicated by evidence of fibrosis and infiltration of macrophages, fibroblasts and a greater proportion of plasma cells in addition to the acute inflammatory cells (figure 5.12). Four weeks after administration of the single dose of TNB, abnormalities of the mucosal crypt pattern, oedema and inflammatory cell infiltrate in both the submucosa and lamina propria were still present in all animals, and in most (but not all) ulceration was seen (figure 5.20). The microscopic damage persisted for six weeks (figure 5.22), by which time changes such as hyperplasia, branching and bifurcation compatible with regeneration were present. In addition to acute inflammatory cells, there was an increase in the fibroblastic component of the lamina propria and the submucosal tissue, and scar tissue formation was evident. Crypt atrophy and architectural distortion were often seen in otherwise normal tissue up to 15cm from the main area of damage.

The damage produced by TNB in ethanol was restricted to the colon; tissue taken from the liver, lung, kidney, caecum and ileum showed no histological abnormalities.

5.4.7.2 Controls

At no time were any histological abnormalities observed in animals that received 0.6cm$^3$ saline into the lumen of the distal colon.

Microscopically, the histological appearance of the tissues examined one day after the administration of TNB/ethanol did not differ from that of the tissues taken one day after the administration of either 25 per cent ethanol alone or of 40mg TNB in saline, although the inflammatory cell infiltrate was greatest in the group that received
TNB/ethanol. By three days the ethanol alone animals (group 2) had virtually normal histology, the only abnormality being an acute inflammatory cell infiltrate in the lamina propria of one rabbit. Animals killed three days after receiving 40mg TNB in saline displayed mucin depletion and oedema, but appeared otherwise normal. The improvement in the TNB/saline and ethanol groups demonstrates the colonic mucosa's potential capacity for rapid regeneration.

After one week, no histological abnormalities were observed in any of the control groups.
Table 5.6: Histological features with dose of TNB at 2 weeks. This table shows the frequency with which each histological feature appeared in each group of animals killed following administration of saline, ethanol or different doses of TNB in saline or 25 per cent ethanol. Crypt branching and bifurcation, atrophy, degeneration, dilatation are grouped under the heading architectural abnormalities. Histological features referred to in the methods section (table 5.4) but not appearing in this table were not observed. 'LP inflam. cell infiltrate' signifies an increased lamina propria inflammatory cell infiltrate and 'SM inflam. cell infiltrate' signifies a presence of submucosal inflammatory cells. (n=number of animals in each group.)
Table 5.7: Histological features with time. The table shows the frequency with which each histological feature appeared within each group of animals killed from 24 hours to six weeks after receiving 40mg of TNB at 5-20cm proximal to the anus. Crypt branching and bifurcation, atrophy, degeneration, dilatation are grouped under the heading 'Architectural abnormalities'. Histological features referred to in the methods section (table 5.4) but not appearing in this table were not observed. 'LP inflam. cell infiltrate' signifies an increased lamina propria inflammatory cell infiltrate and 'SM inflam. cell infiltrate' signifies a presence of submucosal inflammatory cells. (n=number of animals in each group.)
Figure 5.7: The average number of histological features as a function of time and substance instilled.
**Figure 5.8 (TOP):** The normal histological appearance of rabbit distal colon. Original magnification, x100.

**Figure 5.9 (BOTTOM):** Explanatory diagram for figure 5.8.
Figure 5.10 (TOP): Crypt abscesses. Photomicrograph of an area of inflamed mucosa from a rabbit killed two weeks after receiving 40mg TNB/ethanol. There are many polymorphonuclear neutrophils in the lamina propria and three crypt abscesses are present. (The presence of polymorphonuclear neutrophils within the lumen of a crypt constitutes a crypt abscess.) Note: rabbit polymorphonuclear neutrophils contain eosinophilic granules (cytoplasm stains pink). Original magnification, x400.

Figure 5.11 (BOTTOM): Explanatory diagram for figure 5.10.
Figure 5.12 (TOP): Full-thickness photomicrograph of a region of inflamed colon from a rabbit killed two weeks after receiving 40mg TNB/ethanol. A pseudopolyp containing relatively normal mucosa is surrounded on either side by deep ulcers that disrupt the muscularis mucosae. Each layer has been infiltrated with inflammatory cells and is oedematous. The smooth muscle is hypertrophied and evidence of fibrosis is present in the submucosa. Original magnification, x100.

Figure 5.13 (BOTTOM): Explanatory diagram for figure 5.12. U, region of ulceration.
Figure 5.14: Photomicrograph of a granuloma in the mucosa which contains a multinucleate giant cell. The granuloma, a collection of modified macrophages, is surrounded by a cuff of lymphocytes.

Original magnification, x400.

Figure 5.15: Explanatory diagram for figure 5.14. **GC**, giant cell.
Figure 5.16: Fissuring ulceration in a rabbit killed 2 weeks after receiving 40mg TNB/ethanol. The ulceration has penetrated the muscularis mucosa extending into the submucosa. The base of the ulcer is surrounded by an infiltrate of acute inflammatory cells and fibrosis.

*Original magnification, x200.*

Figure 5.17: Explanatory diagram for figure 5.16. **U**, focal ulceration.
Figure 5.18 (TOP): Large diffuse granulomas in the mucosa of a rabbit killed two weeks after receiving 40mg TNB/ethanol. Original magnification, x40.

Figure 5.19 (BOTTOM): Explanatory diagram for figure 5.18. G, large granulomas.
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Figure 5.20 (TOP): Transmural chronic inflammation and ulceration four weeks after the administration of 40mg TNB/ethanol. The inflammation is characterised by the presence of acute and chronic inflammatory cells. Crypt architectural abnormalities are present at the margins of the ulcer. Original magnification, x100.

Figure 5.21 (BOTTOM): Explanatory diagram for figure 5.20.
Figure 5.22 (TOP): Transmural chronic inflammation and ulceration six weeks after the administration of 40mg TNB/ethanol. The inflammation is characterised by the presence of fibroblasts and plasma cells in addition to acute inflammatory cells. Original magnification, x100.

Figure 5.23 (BOTTOM): Explanatory diagram for figure 5.22. U, ulceration; F, fibrosis.
5.5 Discussion

In a review of animal models for colitis, Kim and Berstad (1992) state that the ideal model, whether naturally occurring or induced, should in every aspect resemble the human disease; that is, it should have the same aetiology and pathophysiology, exhibit the equivalent clinical spectrum, and be treated by same therapeutic agents. In addition, the ideal model should be a practical study tool, providing easy access and experimental manipulation (Kim & Berstad, 1992). Obviously for human ulcerative colitis and Crohn’s disease, where the aetiology is unknown, these criteria cannot be fully met.

Although spontaneous colitis does occur in some animal species, the incidence is generally too low to be of practical use as discussed in the introduction to this chapter. An idiopathic colitis that develops spontaneously in 50 per cent of cotton top tamarins kept in captivity (Madara et al., 1985) has many similarities to human ulcerative colitis, including responsiveness to sulphasalazine, active and remission phases, and similar biochemical changes. However, this model does not meet the criteria of Kim and Berstad as an endangered species cotton top tamarins are not readily available, making their use as a practical research tool extremely limited. Hence, for Crohn’s disease and ulcerative colitis, experimentally-induced colitis models have to be used.

A wide range of approaches has been used to induce colitis, encompassing sulphated polysaccharides (Marcus & Watt, 1969), chemotactic peptides (LeDuc & Nast, 1990), tissue from patients with inflammatory bowel disease (Mitchell & Rees, 1970), manipulation of the immune system (Hodgson et al., 1978a), and chemical irritation of the colon (Fabia et al., 1992). The TNB/ethanol model in the rat has advantages over many other models of inflammatory bowel disease in its simplicity of induction and the prolonged effect following a single administration (Morris et al., 1989). While acetic acid or formalin in a single dose produce inflammation and ulceration, the inflammatory changes last for only a matter of days (Fabia et al., 1992). Models with persistent inflammation generally require lengthy protocols sometimes lasting several weeks (Marcus & Watt, 1971).

Although TNB has now been used in rabbits (Percy et al., 1993; Wallace et al., 1992) as well as rats (Morris et al., 1989; Rachmilewitz et al., 1989), there has been no systematic study that compares the temporal relationship between mucosal injury and inflammation in the rabbit models. Although the anatomy of the rabbit colon differs from that of the human colon (rabbits being herbivores have a very large caecum), the work of Percy and co-workers has demonstrated that the responses of human and rabbit distal colonic muscle to inflammatory mediators are similar, while distal colonic muscle from rats
reacts differently to these mediators (Percy et al., 1990). This suggests that colitis induced in the rabbit rather than in the rat may be a better model to study some aspects of colonic inflammation and ulceration. In the investigation reported here it was discovered that the administration of 40mg TNB, with 25 per cent ethanol as the 'barrier breaking solution', to rabbits as a single intracolonic dose produced inflammation and ulceration of the distal colon which persisted for at least six weeks. The acute mucosal injury produced by the ethanol was quickly resolved, as observed in control experiments. TNB in saline alone produced only transient damage, including some short-lived ulceration that had healed by one week. In keeping with the results reported by Morris (1989) and Yamada (1992) on the colitis induced in the rat by TNB, the present study revealed that in the rabbit the combination of TNB and ethanol produced more colonic injury after one week than TNB or ethanol alone.

5.5.1 Comment on the clinical course

The dose of TNB and the concentration of ethanol used in most of the present study were based on the results of the dose-response study. Although the dose selected consistently produced a severe form of colonic inflammation, it did not significantly affect the long-term weight gain of the animals. In rabbits that received TNB in ethanol, liquid or soft stool and weight loss were observed only after the first week. The growth rates of these animals returned to normal in the second week. This pattern of weight loss and diarrhoea was also observed in the rat model (Morris et al., 1989). Occult blood was detected in fresh stool of the animals receiving TNB in ethanol only for tests conducted up to three weeks after the challenge, despite the continued presence of ulcers in some animals. This suggests that those ulcers present are non-bleeding. The sensitivity of the test may also be insufficient to detect all bleeding-ulceration.

An advantage of using the rabbit over the rat is that the larger size has allowed changes in the appearance of the colon to be examined endoscopically. The scores on endoscopy correlated with those of animals killed at the same time, allowing the progress of the disease to be monitored in individual rabbits, a valuable tool for following the effects of any therapies. However, an endoscopically or macroscopically normal mucosa at post mortem does not exclude histological abnormalities.

5.5.2 Comment on the macroscopic appearance

In the rabbit, characteristic gross lesions of hyperaemia, oedema, mucosal thickening, and haemorrhage were all confined to the distal colon where the TNB had been administered. Ulceration was present in all the animals killed one week after receiving the optimum dose. These early lesions were characterised by an abrupt transition from
normal to inflamed mucosa, a typical feature of human Crohn's disease. In later lesions the luminal surface of the colon appeared irregular at endoscopy, and corrugated and ulcerated at post mortem. In some instances the colon became strictured as in Crohn's disease, and exhibited a reduced lumen diameter, with a loss of elasticity. However, evidence of healing was apparent from two weeks onwards. There was also a broad spectrum of injury at two weeks. In some instances there was a substantial difference in the amount of ulceration observed. An explanation for this discrepancy may rest with the different batches of TNB used. Over the course of the experiments it was necessary to use different lots of TNB with noticeable variation between them. Another group observed that different lots from the same vendor produced varying degrees of injury and inflammation using the same sex and strain of rat (Yamada et al., 1992). The model was also very sensitive to the concentration of ethanol. An increase in the ethanol concentration while maintaining the concentration of TNB resulted in a rapid increase in mortality, probably associated with deeper penetration by the ethanol. Rats treated with 30mg TNB were able to tolerate a 50 per cent ethanol concentration (Morris et al., 1989), emphasising inter-species differences.

The macroscopic and microscopic features of resolution were well advanced at six weeks, suggesting that further experiments investigating damage at later time points would not be relevant. The damage induced in this rabbit model was not as persistent as that in the rat model of TNB-induced colitis reported elsewhere, where ulceration was still visible at eight weeks (Morris et al., 1989).

It has been suggested that the presence of altered populations of colonic bacteria may have a role in the severity and persistence of TNB-induced colitis (Gardiner et al., 1993). However, in this model, administration of TNB alone to the rabbit produced some ulceration, but this did not develop into a chronic inflammation despite the undoubted presence of endogenous colonic bacteria. This result suggests that the presence of intraluminal bacteria was not responsible for the transition from acute to chronic inflammation which was observed in the colon of animals treated with TNB and ethanol together. Similarly, in the rat model of TNB-induced colitis (Morris, 1989), the control groups treated with 50 per cent ethanol or 30mg TNB in saline exhibited a transient acute colonic inflammation one day after administration, but resembled the control saline-treated rats by one week.

The damage induced in this rabbit model was confined to the distal colon as in the rat model of chronic colitis (Morris et al., 1989). However, TNB has also been used to induce ulceration and inflammation elsewhere in the gastrointestinal tract of rats. Oral administration of 50mg of TNB in 0.75cm³ of 50 per cent ethanol resulted in the formation of discrete ulcers in the antral region of the stomach (Morris et al., 1984).
5.5.3 Comment on the microscopic appearance

At 12 hours following the administration of TNB/ethanol to rabbits, the lamina propria had been infiltrated with large numbers of polymorphonuclear neutrophils. Oedema of the mucosa was also a feature. In more advanced lesions, there was an infiltration of the lamina propria and submucosa with macrophages and lymphocytes, which is more consistent with Crohn's disease. Inflammation observed in ulcerative colitis tends to be limited to the mucosa, although in chronic ulcerative colitis there is considerable overlap with Crohn's disease. The characteristic histological changes resulting from a single administration of TNB shared many of the features of human colitis including ulceration, architectural distortion, fissures, crypt abscesses, mucin depletion, oedema, fibrosis, and, in a few cases, granuloma formation. The reported incidence of granuloma in Crohn's disease varies between zero and 30 per cent and is dependent on the site of the disease (Talbot & Price, 1987). General thickening and regenerative hyperplasia were also prominent features, as was hypertrophy of the muscularis propria in the chronic stages of the disease. The presence of granulomas and fissuring ulceration is common to both the model and Crohn's disease. The model also displayed features more commonly associated with ulcerative colitis: architectural distortion and a diffuse pattern of inflammation. Crypt abscesses, oedema, mucin depletion, fibrosis, and epithelial exfoliation are histological features shared by ulcerative colitis, Crohn's disease and the model.

The rabbit model, therefore, has histological features similar to both human ulcerative colitis and Crohn's disease. It does not precisely mirror either disease.

The histological findings in the rabbit are in most respects similar to those found in the rat, although fewer granulomas were observed in the rabbit model, and the inflammation does not appear to be quite as persistent. The rat model was characterised by an infiltration of polymorphonuclear neutrophils, macrophages and lymphocytes into the mucosa and submucosa (Morris et al., 1989). Granulomas were observed in 57 per cent of the rats killed three weeks after induction of inflammation. Langhan's giant cells were also observed in the mucosa, and small ulcers were found up to eight weeks after initiation.

5.5.4 Comparison with other rabbit models

Rabbits have commonly been used in the development of colitis models. In the immunological type III hypersensitivity models, researchers injected preformed immune complexes of human serum albumin and anti-human serum albumin into non-sensitised rabbits to give a severe inflammation (Hodgson et al., 1978a); however, a chronic lesion
did not develop. Subsequently, rabbits were immunised with the common enterobacterial antigen of Kunin and haemagglutinating antibodies before inducing mild irritation of the rectum with dilute formalin followed by the injection of soluble immune complexes (Mee et al., 1979). The rabbits developed a persistent inflammation, but no ulceration was observed. Similar results were obtained in a more recent model of the same type, where rabbits were sensitised to lipopolysaccharide (Hotta et al., 1986). Likewise, administration of n-formyl-methionyl-leucyl-phenylalanine, a neutrophil chemotactic, induced colitis that persisted for three days, but without ulceration (LeDuc et al., 1993). The above models have been of limited use because the induced colitis was of short duration, and in some cases did not show ulceration.

Both carageenan and the delayed hypersensitivity reaction after sensitisation to DNCB produce inflammatory bowel disease-like lesions in the rabbit (see section 5.1). The carageenan model is distinct in that epithelial dysplasia was apparent in the majority of animals killed at 28 months. However, despite a number of histopathological similarities to human inflammatory bowel disease, both these models have disadvantageously lengthy protocols, making them costly to use.

The TNB-induced model of inflammatory bowel disease seems to offer the most convenient method of induction in the rabbit, without compromising the similarity of the associated pathological features.

5.5.5 The mechanism by which TNB induces colitis

The mechanism by which TNB in the presence of ethanol initiates inflammation in unsensitised animals is unclear. The results of the present study are consistent with the hypothesis that chronic inflammation of the intestine may occur as a consequence of increased permeability of the mucosa to a luminal antigen which cannot be readily cleared by the immune system (Shorter et al., 1972; Ward, 1972). Whether or not the administration of antigens normally found in the mammalian gut, in combination with a 'barrier breaker', would yield similar results has yet to be determined. It should be noted that bacterial cell wall fragments from Streptococcus pyogenes and Streptococci faecium were capable of eliciting chronic inflammation when injected into the bowel wall of the rat (Sartor et al., 1988), and that uptake of bacterial cell wall polymers was enhanced by acute injury to the colon (Breeling et al., 1988; Hotta et al., 1986). Studies on the carrageenan model for ulcerative colitis have also demonstrated the importance of bacterial populations in the development of experimental colitis (Onderdonk et al., 1978).
Although TNB is often used as a hapten, it is generally thought that animals need to be sensitised to a hapten before a hypersensitivity immunologic response is possible. In the original report on the rat TNB-induced model the researchers suggest that the mechanism involves macrophage-mediated recognition of protein-TNB conjugate on cells within the mucosa. Beck and co-workers reported that some of the colonic injury induced by TNB-ethanol may be attenuated by prior tolerisation of the animals to TNB, suggesting cell-mediated tissue injury (Beck et al., 1988). Although previous sensitisation to TNB is not required for induction of colonic inflammation in this model, Morris and co-workers mention preliminary studies suggesting that relapse can be induced by intracolonic administration of TNB alone several weeks after challenge with TNB-ethanol. It may be hypothesised that the animals become sensitised to the damaging effects of TNB. This ability to induce relapse by repeated intraluminal administration of TNB may provide a method to mimic remission and relapse - another important characteristic of inflammatory bowel disease (see Chapter 8). Morris's data suggest an immune cell-dependent component of TNB-induced colonic inflammation; however, the mechanism for TNB-induced injury and inflammation may be more complex than has been previously appreciated. Recently, it has been shown that TNB may be metabolised to yield both proinflammatory and cytotoxic oxidants that could initiate colonic inflammation (Grisham et al., 1991). TNB in the absence of ethanol is toxic to intestinal epithelial cell monolayers \textit{in vitro} (Yamada et al., 1992), confirming that some of the inflammation induced by TNB undoubtedly results from this cytotoxic effect.

5.5.6 Summary

In summary, the TNB-induced model in the rabbit fulfils the aims set out at the beginning of the study. The simplicity of induction, the severity, and the persistence of the inflammation are characteristics of the new model that make it attractive for the investigation of the pathophysiology and the treatment of intestinal inflammation. Moreover, the model is histopathologically relevant, in that many of the features of human inflammatory bowel disease, particularly Crohn's disease, are present. This study has also demonstrated that endoscopy can be used to examine the course of the disease and represents a useful procedure for evaluating the effects of potential new therapies.

Until the aetiologies of Crohn's disease and ulcerative colitis are better understood there will be no perfect model for inflammatory bowel disease. However, this TNB-induced model in the rabbit shows great promise as a valuable aid for our understanding of the processes of chronic inflammation in the colon.
THE IMMUNOHISTOCHEMICAL LOCALISATION
OF MATRIX METALLOPROTEINASES AND TIMP
IN THE MODEL

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6.1 Introduction

The extracellular matrix is degraded by the matrix metalloproteinases, collagenase, stromelysin and gelatinase; the activity of these is usually strictly controlled by tissue inhibitor of metalloproteinase (TIMP). Matrix degradation, measured as collagen turnover, is typically a very slow process in structures like skin and tendon of adult animals. However, increased degradation of connective tissue matrix is a normal process during some physiological events associated with morphogenesis and growth, as well as in processes such as angiogenesis, cell migration, cervical softening, uterine involution, and tissue repair. It has been demonstrated that in these circumstances the matrix metalloproteinases together with their inhibitors are present.

All inflammatory conditions are associated with lysis of connective tissue to a greater or lesser extent, and the acute inflammatory cell types recruited to sites of inflammation have potent proteolytic potential. Failure of the normal regulatory systems may be responsible for abnormal remodelling processes in some diseases (see chapter 4). In inflammatory bowel disease intestinal inflammation is usually accompanied by changes in bowel wall thickness. Increases in thickness may be due to excessive deposition of collagen. The intestinal strictures seen in Crohn’s disease are due to both the proliferation of smooth muscle cells and the accumulation of collagen. Conversely, excessive connective tissue degradation can lead to ulcer formation if the inflammatory lesion is close to a surface. In Crohn’s disease especially, the formation of deep fissuring ulceration is a typical feature. The matrix metalloproteinases are likely to be involved in these events.

Previous experiments, based mainly on biochemical techniques, have demonstrated the ability of colonic explants from inflammatory bowel disease patients to produce matrix metalloproteinase enzymes in vitro (Horowitz et al., 1987; Sturzaker & Hawley, 1975). However, although such studies are valuable, they do no more than indicate the capacity of the cells and tissues examined to synthesise these enzymes under the experimental conditions used. Furthermore, the cellular heterogeneity of tissues makes it difficult to know which cells are responsible for matrix metalloproteinase synthesis, or to determine the distribution of matrix metalloproteinases within different parts of the bowel wall.
Immunolocalisation studies with specific antibodies raised against the matrix metalloproteinases and tissue inhibitor of metalloproteinases (TIMP) have the potential to redress these deficiencies, allowing an assessment to be made of the \textit{in vivo} role of the matrix metalloproteinases and TIMP in this model of inflammatory bowel disease. The application of immunohistochemical techniques also allows an assessment of the distribution of the metalloproteinases and TIMP over an extended period of time. A recent study has demonstrated the presence of matrix metalloproteinases in resected specimens from Crohn's disease patients (Bailey, 1992).

Before examining the pattern of matrix metalloproteinase expression in the model, the characteristics of the each polyclonal antiserum used need to be considered. The polyclonal antiserum to collagenase was prepared against rabbit bone collagenase as antigen. Besides binding rabbit interstitial collagenase, the antiserum also cross-reacts with human and pig collagenase; however, it has been shown by inhibition studies not to cross-react with either rabbit stromelysin or gelatinase. The antiserum recognises both the latent and active forms of collagenase and inactivated collagenase bound to TIMP (Hembry \textit{et al.}, 1986). Specificity was determined by immunoblotting and immunoassay. The antibody has also been tested in a biological system, which allows a correlation to be made between biochemically and immunochemically detectable enzyme. Cultured chondrocytes provide such a system. Under normal circumstances these cells make negligible quantities of collagenase. However, once the cells are stimulated by the addition of conditioned medium from human blood mononuclear cells, large amounts of biochemically detectable collagenase are produced (Trechsel \textit{et al.}, 1982). When unstimulated and stimulated chondrocyte cultures were probed with the anti-collagenase antibody used in this study the amount of immunoprecipitable collagenase was found to correspond with the expected changes in biochemically detectable enzyme (Hembry \textit{et al.}, 1986).

Failure of immunolocalization observations to match biochemical data has been observed previously, and has cast doubts on the specificity of the antibodies used in previous immunolocalisation experiments. In a biochemical study of the rat uterus, Woessner observed that there was negligible collagenase activity in both the non-gravid uterus and in the uterus at the time of parturition (Woessner, 1979). During post-partum involution, however, collagen degradation increased markedly and was accompanied by a rise in collagenase activity. A subsequent immunohistochemical study of collagenase in the rat uterus failed to confirm these findings (Montfort & Perez, 1975). Contrary to the researchers' expectations, the non-gravid uterus demonstrated considerable staining for collagenase, which then diminished markedly during the first 24 hours after parturition. By comparison, when a similar experiment was conducted in
the rabbit with the anti-collagenase antibody used in this study, Woessner's finding were verified, confirming the specificity of the anti-collagenase antiserum (Hembry et al., 1986).

The antiserum to stromelysin used in this study was raised against purified rabbit bone stromelysin and immunoprecipitated both the latent and active forms of the enzyme (Murphy et al., 1986). The anti-stromelysin antiserum was shown by means of immunoblotting and immunoprecipitation not to recognise purified rabbit bone collagenase or gelatinase. In vitro the antiserum detected metalloproteinase activities degrading casein and proteoglycan found in human fibroblast culture media and pig synovial culture media (Murphy et al., 1986).

The antiserum used in the present study for immunolocalisation of gelatinase A and B was raised against gelatinase from rabbit bone and was predominantly of the lower molecular weight form; however, crossed immunoblotting analysis has shown that the antiserum contains different species of IgG that react with each form of the enzyme individually (Murphy et al., 1989).

The antiserum to gelatinase B, raised against the polymorphonuclear leukocyte gelatinase, recognises high molecular weight gelatinases from human granulocytes, pig monocyes and rabbit connective tissue cells, but not gelatinase A from connective tissue cells (Murphy et al., 1989).

The specificity of the polyclonal antiserum raised against purified rabbit TIMP-1 was investigated by immunoblotting (Gavrilovic et al., 1987). The antibody recognised a single band which ran parallel to the antigen, purified rabbit bone TIMP. The antiserum against TIMP does not react with purified human or bovine TIMP or with rabbit TIMP-2 (Hembry, 1994). However, the staining pattern with cells cultured either on glass or on collagen films was identical to that previously described for anti-human TIMP antibody (Hembry et al., 1985). The anti-TIMP antibody induced degradation of collagen by chondrocytes by inhibiting the collagenase binding ability of TIMP (Gavrilovic et al., 1987). This degradation could be reversed with an anti-collagenase antibody.
6.2 **Aim of the study**

The aim was to investigate the expression of the matrix metalloproteinases and their inhibitor, tissue inhibitor of metalloproteinase, at all stages of the disease process in the rabbit model of chronic inflammatory bowel disease.

6.3 **Materials and methods**

6.3.1 **Production of the polyclonal antibodies**

The antibodies used for the immunolocalisation of the matrix metalloproteinases and TIMP were all developed and generously supplied by Drs J J Reynolds and R M Hembry, Strangeways Research Laboratory, Cambridge, UK.

Each of the polyclonal antibodies was raised according to an established method (Hembry et al., 1985). In outline, a purified sample of the whole antigen was mixed with an equal volume of Freund's complete adjuvant and injected intramuscularly into an adult sheep. At varying intervals, depending on the antibody being produced, blood samples were taken, the serum was removed and the immunoglobulin fraction was purified. The specificities of the antisera obtained were verified by electrophoretic immunoblotting, inhibition studies and immunolocalisation studies. In addition, the antibodies used in the experiments described here have been further validated in a number of investigations (Bailey, 1992; Brown et al., 1989; Gavrilovic et al., 1987; Gavrilovic et al., 1985; Hembry et al., 1986).

6.3.2 **Tissue collection**

After the macroscopic assessment of damage, full thickness segments of the rabbit colon were removed from sites 3cm, 5cm, 10cm, 15cm, 20cm, 30cm and 60cm proximal to the anus, from animals killed from 0h to 6 weeks after the induction of colitis (see figure 5.2).

Each piece of colon was washed by soaking and intermittent agitation in five changes of Hank's balanced salts solution, the first three containing ten times the normal concentration of antibiotics (appendix C). The segment was transferred to a tissue culture hood while in the fifth wash, and dissected with sterile instruments. Each piece of tissue was cut transversely into 3 strips; one of the strips was placed in short term tissue culture and the other two were processed immediately.

Each strip of colon, cultured or not, was oriented with the mucosal surface uppermost on a nitrocellulose filter (Whatman, Maidstone, UK), placed in a 7ml bijou (Bibby
Sterilin, Stone, UK), and embedded in 7 per cent gelatin in 0.9 per cent saline, containing 0.02 per cent sodium azide as an antibacterial agent (appendix D). Tubes containing tissue and gelatin were frozen for 90 seconds in liquid nitrogen and stored at -20°C until required.

6.3.3 Tissue culture

All work involving tissue for culture was carried out in a Bassaire dust-free assembly cabinet (John Bass Ltd, Southampton, UK) using sterile instruments.

Strips of tissue for short-term culture were placed on stainless steel grids in separate 3.5cm diameter wells of a six-well tissue culture plate, each containing 4ml of Dulbecco's Modification of Eagle's Medium (ICN Flow, High Wycombe, UK) with supplements (appendix C) including 5μM monensin. Monensin is a monovalent ionophore that prevents the translocation of secretory proteins (Brown & McFarland, 1992); this causes the accumulation of metalloproteinases and TIMP in the Golgi apparatus of cells that are actively synthesising enzyme or inhibitor, making the cell types easier to identify microscopically.

The culture plates were incubated in a humid atmosphere at 37°C for three hours. The tissue was then removed from the culture and processed in the same way as uncultured tissue as previously described (6.3.2).

6.3.4 Immunohistochemistry

Frozen sections, 4μm thick, were cut on a cryostat (Bright, Huntingdon, UK) and fixed for 30 minutes in 4 per cent paraformaldehyde in phosphate-buffered saline (PBS) (appendix D). After fixing, sections were washed in PBS. All washing stages comprised three changes of PBS at intervals of 5 minutes. The tissue was then permeabilised in 0.1 per cent Triton X-100 in PBS for 5 minutes, to allow subsequent penetration of antibody into cells. After washing, sections were treated for 20 minutes with 4-chloro-1-naphthol (Sigma Chemicals, Poole, UK), 2.8mM, in methanol/PBS (1:5) containing 0.01 per cent hydrogen peroxide (appendix D), to prevent the non-specific binding of fluorescein isothiocyanate (FITC) antibody by inflammatory cells (Johnston & Bienenstock, 1974). Sections were washed again and incubated at room temperature in a humid atmosphere for 30 minutes with 70μl (50μg/ml) per section of either anti-collagenase, anti-gelatinase (72kDa & 95kDa), anti-gelatinase (95kDa), anti-stromelysin, or anti-tissue inhibitor of metalloproteinases (TIMP). The antiserum IgG was made up in PBS and 2.5 per cent normal donkey serum (Sigma Chemicals, Poole, UK) to block non-specific binding. Slides were washed to remove excess first antibody and incubated for 30 minutes with 2.75mg of second antibody, rabbit anti-sheep FITC
F(ab')2 (Southern Biotechnology Associates, Inc., Birmingham, Alabama, USA; supplied by Euro-Path, Bude, UK) made up in a PBS solution containing 2 per cent rabbit albumin (Sigma chemicals, Poole, UK) and 3 per cent normal donkey serum. The sections were washed once more before staining them for 2 minutes with methyl green (1mg/ml) as a nuclear counter-stain. The slides were then given a final wash and mounted in glycerol/PBS mounting fluid containing additives to reduce fading of fluorescence (Citifluor Ltd., University of Kent, Canterbury, UK).

Control sections were prepared in an identical manner except that the primary antibody was replaced by normal (non-immune) sheep serum (NSS) (Sigma Chemicals, Poole, UK), also used at a concentration of 50μg/ml.

Sections were examined by epifluorescence, using a Leitz Laborlux S photomicroscope (Leica, Milton Keynes, UK), with standard wide and narrow band filters. Sections were examined under low power with a rhodamine filter to map the layout of the tissue, and then under higher powers with the FITC filter to determine the localisation of fluorescent antibody within the tissue. Slides incubated with NSS served as controls for the non-specific uptake of FITC antibody. Fluorescent photomicrographs were taken on AGFA agfachrome 1000ASA RS colour film and processed commercially.

Selected sections were re-stained with haematoxylin and eosin (appendix B) for direct comparison of the findings, since areas and cells that had shown expression of metalloproteinases and TIMP could be identified more readily using haematoxylin and eosin staining. For re-staining of frozen sections, the cover slips were removed by soaking in PBS and the slides were washed in tap water. Sections were stained for 1 minute in Harris’s haematoxylin, washed in water and counter-stained for 1 minute with eosin. Slides were dehydrated in alcohol, cleared in CNP and mounted in DPX. Examination and photography were as for the formalin-fixed tissue.

6.3.5 Fluorescence photometry

The slides were stained for the matrix metalloproteinase and TIMP as previously described, with the following exception: after washing off the second antibody the sections were air dried for 10 minutes before being examined photometrically. No mounting medium or cover slips were placed on the sections.

The staining was then examined using an inverted microscope (Olympus IMT-2) with epifluorescence and phase contrast attachments. Fluorescence excitation came from a cold light supply (Olympus CLE-4U). The beam was delivered by a fibre optic light guide through a 480nm narrow band filter (Omega Optical Inc., Vermont, USA.). The FITC fluorescence signal was detected through a 510nm OG filter (Omega Optical Inc.,
Vermont, USA) by means of a highly sensitive cryogenically cooled CCD (charge-coupled device) camera (Model 1, resolution 400x600 pixels, from Wright Instruments, London, UK) fitted to the microscope. This signal was processed by means of an IBM personal computer into a falsely colour-coded microscopic image of the section depicting the mean signal counts per pixel. The software also allowed quantitative analysis of the signal. The combination of phase contrast microscopy and conventional light microscopy of the subsequently H&E stained section enabled accurate identification of the various microscopic structures of the colon. Fluorescence was measured arbitrarily as counts per pixel, and corrected for the background on the non-immune sheep serum stained controls. Both the falsely coloured coded fluorescence image and the light microscopic image of the serial sections were photographed for comparison.
6.4 Results

Immunohistochemical findings were consistent in animals investigated at the same time following induction of colitis. In any one tissue block, areas of staining for the metalloproteinases and TIMP tended to appear and disappear gradually over 15 or 20 serial sections; the descriptions given below, therefore, represent the ‘average’ pattern of staining throughout the tissue.

6.4.1 Saline controls

Three saline control treated animals (sa6, sa7, sa8) killed at 24 hours did not display any bright green fluorescence localising expression of metalloproteinases or TIMP on any of the sections taken from the colon, whether cultured with monensin or not. Occasional yellow fluorescence was seen, the product of treatment with 4-chloro-1-naphthol, identifying small numbers of scattered inflammatory cells normally present in colon; this was more clearly seen in the animals that received trinitrobenzene sulphonic acid, in which large numbers of inflammatory cells were present.

6.4.2 Ethanol controls

Three ethanol control rabbits were also killed at 24 hours and in each case there was no matrix metalloproteinase or TIMP expression on any of the sections taken from the colon, whether cultured with monensin or not.

6.4.3 Trinitrobenzene sulphonic acid in saline controls

Sections from three animals receiving 40mg of trinitrobenzene sulphonic acid in saline and killed at one week were stained for matrix metalloproteinase and TIMP expression. Histologically, none of the animals studied had ulcerations in their colon, and no staining was found on any of the sections except for a few isolated cells containing granules in the cytoplasm that stained with anti-gelatinase A&B (GLA+B) at 10cm in one animal.

6.4.4 Expression of the matrix metalloproteinases and TIMP zero hours after the initiation of colitis

None of the three animals (tnb90, tnb91, tnb92) receiving 40mg TNB in 25 per cent ethanol and killed immediately afterwards displayed any collagenase (CL), stromelysin (SL), gelatinase B (GLB) or TIMP staining on sections taken from 5cm, 10cm, 15cm or 20cm proximal to the anus, regions where the TNB solution had been in contact with the mucosa (table 6.1). Culturing the tissue with monensin also failed to reveal any early
expression of collagenase, stromelysin or TIMP. Histologically, the colons appeared to be slightly oedematous and the epithelium was necrotic in areas that had been in contact with the TNB.

Of the three animals killed at 0 hour, one animal displayed intracellular gelatinase staining (with anti-Gelatinase A&B) in a few isolated cells in the mucosa at 10cm proximal to the anus, which was the area of maximal damage. This staining, identified at higher powers, could be seen as bright speckles of green fluorescence within the cytoplasm, showing secretory granules around nuclei stained red.

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<thead>
<tr>
<th>Animal</th>
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<th>CL</th>
<th>SL</th>
<th>GLA+B</th>
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Table 6.1: Expression of the matrix metalloproteinase enzymes and TIMP in animals killed 0 hours after initiation of colitis with 40mg of trinitrobenzene sulphonic acid in a 25 per cent ethanol/saline solution. +/-ve, isolated expression associated with only a few cells; -ve, no staining. Note 1: intracellular only.

6.4.5 Expression of the matrix metalloproteinases and TIMP three hours after the initiation of colitis

At three hours after the introduction of 40mg TNB in 25 per cent ethanol, the three animals (tnb72, tnb73, tnb74) studied at this time all displayed intracellular gelatinase staining with anti-gelatinase A&B (table 6.2). However, the histological appearance remained unchanged from the 0 hour animals. The staining was observed in both the mucosa and submucosa under the damaged tissue at 5cm, 10cm and 15cm proximal to
anus, appearing as bright speckles of green fluorescence within the cytoplasm around nuclei counter-stained red in cells that had a fibroblast-like appearance. However, none of the other enzymes or TIMP were found to be expressed at this time in any layer or any region of the colon studied at this time. Three-hour culture in the presence of monensin enhanced the intracellular gelatinase staining in those segments expressing gelatinase without culture, but did not reveal any new staining.

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Table 6.2: Expression of the matrix metalloproteinase enzymes and TIMP in animals killed three hours after initiation of colitis with 40mg of trinitrobenzene sulphonic acid in a 25 per cent ethanol/saline solution. +ve, one site of expression on sections; +/-ve, isolated expression associated with only a few cells; -ve, no staining. Note 1: intracellular only in the mucosa and submucosa.

6.4.6 Expression of the matrix metalloproteinases and TIMP six hours after the initiation of colitis

At six hours the mucosa was oedematous, the epithelium was often discontinuous, and the mucosa contained some necrotic areas; however, the normal crypt architecture was, at this stage, well preserved. In the three rabbits (tnb66, tnb67, tnb68) studied at this time, all the enzymes were expressed (table 6.3). From 5cm to 15cm the mucosa contained collagenase on the interstitial matrix. The green fluorescence outlined the superficial mucosal crypts, especially under or immediately adjacent to regions of
necrotic tissue (figure 6.1). Collagenase was not observed at the site 20cm proximal to the anus and was found in only one animal at 3cm (tnb68). Despite extensive and bright collagenase staining, it was confined to the mucosa. Cells secreting collagenase could be identified by their bright red nuclei and speckles of green fluorescence within the cytoplasm showing the secretory granules. These cells, which were fibroblast-like in appearance and frequently pericryptal, were found in the same areas as extracellular collagenase, and could be seen in tissue both with and without monensin treatment. Intracellular collagenase was never seen within epithelial cells, and in the rabbit neutrophil collagenase is not detected by the antisera used.

Stromelysin staining was less extensive than collagenase staining, appearing on sections closer to the focus of damage, mainly as bright green extracellular enzyme, but also as intracellular granules in fibroblast-like cells that were restricted to the mucosa (figure 6.1). In tnb67 stromelysin was expressed only in the 5cm segment and was not seen in tissue from the ulcerated region or the region directly proximal to the ulceration. However, in tissue cultured for three hours, the enzyme was expressed at a relatively low level in these segments. Stromelysin was not expressed in any of the segments taken from 20cm.

Intracellular and extracellular staining by the antisera against gelatinase A and gelatinase B was found in all animals at this time in the mucosa and submucosa (up to 15cm). However, it was not present on all sections obtained from the regions of damaged tissue and the fluorescence, where present, was less bright than for any other enzyme. As with stromelysin and collagenase, no gelatinase was observed in the segments removed from 20cm proximal to the anus.

Gelatinase B was expressed in some polymorphonuclear cells within the mucosa in segments from the ulcerated tissue and adjacent sites. In tnb68, gelatinase B was demonstrated only in the 5cm segment of the three-hour culture tissue.

Importantly, TIMP was not observed either intracellularly or extracellularly in any layer or in any segment of the colon studied at this time (figure 6.1).
Figure 6.1A: Haematoxylin and eosin stain of mucosa.

Figure 6.1A to F: Immunolocalisation of the matrix metalloproteinases in the colonic mucosa of a rabbit killed 6 hours after receiving 40mg TNB/ethanol. Extracellular green FITC-immunofluorescence (large arrows) shows the presence of enzyme on the matrix in areas of damaged tissue, and small granules of perinuclear fluorescence (small arrows) indicate intracellular enzyme. The nuclei are counter-stained red. Original magnification, ×400.

Serial sections were stained with the following antisera (or stain):

(A) haematoxylin and eosin

(B) normal sheep serum (control)

(C) collagenase

(D) stromelysin

(E) gelatinase AB

(F) TIMP
Figure 6.1B: Normal sheep serum (control).

Figure 6.1C: Collagenase.
Figure 6.1D: Stromelysin.

Figure 6.1E: GelatinaseAB.
Figure 6.1F: TIMP.
Table 6.3: Expression of the matrix metalloproteinase enzymes and TIMP in animals killed six hours after initiation of colitis with 40mg of trinitrobenezene sulphonic acid in a 25 per cent ethanol/saline solution. +++ve, extensive staining on the majority of the section; ++ve, two or more sites of expression on sections; +ve, one site of expression on sections; -ve, no staining.

6.4.7 Expression of the matrix metalloproteinases and TIMP 12 hours after the initiation of colitis

At 12 hours the pattern of expression of the enzymes was similar to that at 6 hours. Four segments of distal colon were stained: 5cm, 10cm, 15cm, and 20cm proximal to the anus. Histologically, this time point was characterised by the appearance of acute inflammatory cells.

Extensive bright collagenase staining was present in all the 5cm and 10cm segments; however, this staining was always confined to the mucosa surrounding crypts, and was mainly present as extracellular enzyme (table 6.4). No expression was found in the 20cm segments, but two of the three animals studied showed extensive extracellular expression in the 15cm segments.
Stromelysin was expressed in a similar pattern to collagenase; however, it was always less bright and less extensive within any one section. Occasionally, stromelysin was not observed despite extensive collagenase staining. In most cases, the staining was both intra- and extra-cellular, and there was no further enhancement by three-hour culture with monensin.

Gelatinase A&B staining was present on all the slides that exhibited the collagenase staining, but was never as widespread or as bright. In addition to the extracellular staining in the mucosa gelatinase was also present as intracellular enzyme in the submucosa, and appeared to be polymorphonuclear neutrophil in origin.

A few isolated cells containing intracellular gelatinase B were observed in the mucosa of all the 10cm segments.

No positive green fluorescence was observed with anti-TIMP on any of the sections studied, either intracellular or extracellular.

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Table 6.4: Expression of the matrix metalloproteinase enzymes and TIMP in animals killed twelve hours after initiation of colitis with 40mg of TNB in a 25 per cent ethanol/saline solution. +++ve, extensive staining on the majority of the section; ++ve, two or more sites of expression on sections; +ve, one site of expression on sections; +/-ve, isolated expression associated with only a few cells; -ve, no staining. Note 1: intracellular and extracellular in mucosa, but intracellular only in submucosa. Note 2: intracellular only in mucosa.
6.4.7.1 **Fluorescence Photometry 12 hours after the initiation of colitis**

To obtain a quantitative assessment of the amount of FITC marker present on each section the number of emitted photons was counted and expressed as counts per pixel and then corrected for the background on the non-immune sheep serum stained controls. Sections cut from blocks containing tissue from 10cm in the three animals were stained for the matrix metalloproteinases and TIMP. Falsely coloured fluorescence images and a light microscopic image of the serial sections were photographed for visual comparison, and the average number of counts per pixel in four fields on each of the serial sections was determined.

Visual comparison of the images confirmed the order of intensity and extensiveness of staining that had been observed at fluorescence microscopy. In each animal the order of intensity of the staining was collagenase>stromelysin>gelatinase>TIMP (figure 6.2). When the relative intensity of the staining was compared there was no significant difference between the expression of TIMP and that in the NSS control for all three animals (p>0.05). The intensity of the gelatinase staining was significantly different from the control only in TNB95 (see table 6.5). There was up to five times more FITC bound to the collagenase stained sections (28.7 counts/pixel) than to the stromelysin stained sections (4.6 counts/pixel) (p=0.04).

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**Table 6.5: Fluorescence photometry results.** The figures represent the mean of four measurements made on a randomly chosen high power field. The field was selected on the control section and the equivalent field was identified on the corresponding matrix metalloproteinase stained serial sections. One count is equivalent to an arbitrary, but constant, number of photons collected per pixel. The counts are displayed less the background control value. (Figures in brackets are the standard errors.)
Figures 6.2A to F: Immunolocalisation of the matrix metalloproteinases by means of fluorescence photometry in the colonic mucosa of a rabbit killed 12 hours after receiving 40mg TNB/ethanol. The images are colour-coded according to the mean counts per pixel. White and blue colours indicate regions containing more FITC-probe than red and black regions. Original magnification, ×400.

Serial sections were stained with the following antisera:

(A) phase contrast of NSS section

(B) normal sheep serum (control)

(C) collagenase

(D) stromelysin

(E) gelatinase AB

(F) TIMP
Figure 6.2B: Normal sheep serum (control).

Figure 6.2C: Collagenase.
Figure 6.2D: Stromelysin.

Figure 6.2E: GelatinaseAB.
Figure 6.2F: TIMP.
6.4.8 Expression of the matrix metalloproteinases and TIMP 24 hours after the initiation of colitis

At 24 hours, the histology was characterised by acute inflammation and ulceration accompanied by architectural abnormalities and crypt abscesses; these features were generally present from 5 to 15cm proximal to the anus. Three animals (tnb60, tnb61, tnb64) were studied at 24 hours and the following segments were cut and stained: 3cm, 5cm, 10cm, 15cm, 20cm, and 3 hour culture blocks from 5cm, 10cm, and 15cm.

Collagenase was found in segments 3cm, 5cm, and 10cm proximal to the anus as bright extensive extracellular staining, but was restricted to the mucosa (table 6.6). Collagenase was also observed in two of the three animals in the 15cm and 20cm segments, although in these instances the expression was limited to single sites of extracellular staining on each section.

Stromelysin was also present in the ulcerated segments, but it was not as widespread or as bright as collagenase on serial sections. The distribution of gelatinase A&B was similar to that of stromelysin, but the fluorescence was less intense.

TIMP was observed for the first time in the ulcerated mucosa, although it was expressed at a low level compared to the other enzymes and was almost entirely intracellular in fibroblast-like cells.

Three-hour culture with monensin did not reveal any additional intracellular staining.
### Table 6.6: Expression of the matrix metalloproteinase enzymes and TIMP in animals killed twenty-four hours after initiation of colitis with 40mg of trinitrobenezene sulphonic acid in a 25 per cent ethanol/saline solution.

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+++ve, extensive staining on the majority of the section; ++ve, two or more sites of expression on sections; +ve, one site of expression on sections; +/-ve, isolated expression associated with only a few cells; -ve, no staining; nd, not done. Note 1: intracellular only.

### 6.4.9 Expression of the matrix metalloproteinases and TIMP 72 hours after the initiation of colitis

Three animals (tnb62, tnb63, tnb65) were killed at 72 hours and the pattern of expression of the enzymes and TIMP was essentially the same at 72 hours as at 24 hours (table 6.7). Haematoxylin and eosin staining also revealed a similar pattern of histological features at 72 hours which was similar to that at 24 hours. Five segments (3cm, 5cm, 10cm, 15cm and 20cm) were examined for matrix metalloproteinase expression, as were three segments of monesin-cultured tissue from the ulcerated regions at 5cm, 10cm and 15cm.

Collagenase was the most extensively expressed enzyme on the sections and was usually brighter than the other two enzymes, although they occurred in the same area. The enzymes were mostly confined to the regions of ulceration (5cm and 10cm) in the
mucosa, muscularis mucosa and, less often, the submucosa; they were not found in the 15cm or 20cm segments. In two of the three animals, collagenase and stromelysin were still present in the 3cm segment. Gelatinase was observed in all the 3cm segments as bright intracellular enzyme that appeared to be associated with polymorphonuclear neutrophils. Three-hour culture with monensin did not reveal any additional intracellular staining.

TIMP seemed to be co-ordinately expressed with the other enzymes, although the FITC staining was not very bright.

A consistent trend from 6 hours to 72 hours was seen in the extensiveness of expression of the three enzymes in damaged tissue: collagenase expression was greater than stromelysin and stromelysin greater than gelatinase. The principal difference between the staining patterns in the mucosa of the animals killed at 6 hours and 12 hours compared to the patterns in animals killed at 24 hours and 72 hours was the appearance of TIMP at 24 hours.

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Table 6.7: Expression of the matrix metalloproteinase enzymes and TIMP in animals killed 72 hours after initiation of colitis with 40mg of trinitrobenzene sulphonic acid in a 25 per cent ethanol/saline solution. +++ve, extensive staining on the majority of the section; ++ve, two or more sites of expression on sections; +ve, one site of expression on sections; +/-ve, isolated expression associated with only a few cells; -ve, no staining; nd, not done.
6.4.10 Expression of the matrix metalloproteinases and TIMP one week after the initiation of colitis

At one week ulcers, often penetrating the submucosa, were associated with a dense inflammatory cell infiltrate composed of both neutrophils and round cells. Around these ulcers the tissue often appeared to be macroscopically normal, although an increase in inflammatory cells was often observed microscopically. The pattern of matrix metalloproteinase expression changed dramatically in the nine animals studied at this time point (table 6.8). Collagenase was no longer expressed throughout the mucosa from the regions of maximal damage, but was confined to the area immediately adjacent to ulcerated tissue or to the base of the ulcer. The staining was almost entirely extracellular. Bright extracellular stromelysin appeared to be more predominant than the other enzymes, although the actual intensity of expression appeared to be little different from that observed at earlier time points. Stromelysin expression was also closely associated with the areas of ulceration and regions of smooth muscle regeneration. Gelatinase was observed in the same regions as stromelysin, but was less intense and was distributed more superficially in the mucosa than were the other two enzymes. Additionally, intracellular gelatinase was present in the submucosa within polymorphonuclear neutrophil-like cells. TIMP, of the same intensity as that observed at earlier time points, was seen at the same location as the other enzymes.

Table 6.8 (Next page): Expression of the matrix metalloproteinase enzymes and TIMP in animals killed one week after initiation of colitis with 40mg of trinitrobenzene sulphonyl acid in a 25 per cent ethanol/saline solution. +++, extensive staining on the majority of the section; ++, two or more sites of expression on sections; +, one site of expression on sections; +/−, isolated expression associated with only a few cells; −, no staining; nd, not done. Note 1: intracellular in the mucosa only. Note 2: intracellular and extracellular in the mucosa, but intracellular only in the submucosa.
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6.4.11 Expression of the matrix metalloproteinases and TIMP two weeks after the initiation of colitis

Five animals were killed at two weeks, and it was observed that the healing process had advanced sufficiently in some areas to restore epithelial continuity across the ulcerations. Deeper in the wall granulation tissue, containing fibroblasts and collagen, was present in regions of ulceration or in regions where ulceration had recently been resolved. In all the animals, collagenase was observed around chronic ulcers in the distal colon (table 6.9). Extracellular staining was present at the junction of normal mucosa with ulcerated tissue, and just below the surface in ulcers. At the base of some ulcers, intracellular staining was observed, although only in a few fibroblast-like cells. Both stromelysin and gelatinase were expressed at the same site and the staining was brighter and more extensive than collagenase (figure 6.3). Gelatinase appeared, on a few sections, to be much brighter than both of the other enzymes.

TIMP was also confined to those regions directly under or immediately adjacent to the ulceration and was present both intracellularly, within fibroblast-like cells, and extracellularly.
**Figure 6.3A to F: Immunolocalisation of the matrix metalloproteinases in the colonic mucosa of a rabbit killed two weeks after receiving 40mg TNB/ethanol.** Extracellular green FITC-immunofluorescence (large arrows) shows the presence of enzyme or TIMP on the matrix in areas of damaged tissue, and small granules of perinuclear fluorescence (small arrows) indicate intracellular enzyme or TIMP. The nuclei are counter-stained red. Original magnification, \( \times 400 \).

Serial sections were stained with the following antisera:

(A) normal sheep serum

(B) collagenase

(C) stromelysin

(D) gelatinase AB

(E) gelatinase B

(F) TIMP
Figure 6.3B: Collagenase.

Figure 6.3C: Stromelysin.
Figure 6.3D: GelatinaseAB.

Figure 6.3E: GelatinaseB.
Figure 6.3F: TIMP.
Table 6.9: Expression of the matrix metalloproteinase enzymes and TIMP in animals killed two weeks after initiation of colitis with 40mg of trinitrobenzene sulphonylic acid in a 25 percent ethanol/saline solution. +++ve, extensive staining on the majority of the section; ++ve, two or more sites of expression on sections; +ve, one site of expression on sections; +/-ve, isolated expression associated with only a few cells; -ve, no staining; nd, not done.

6.4.12 Expression of the matrix metalloproteinases and TIMP four weeks after the initiation of colitis

At 4 weeks only the segments that contained ulcers had any metalloproteinase expression on them (table 6.10). Ulcers were found on tissue from tnb58 and tnb59 at 10cm proximal to the anus. No metalloproteinase or TIMP expression was found in segments of bowel at 5cm, 10cm, 15cm, or 20cm proximal to the anus. Four other rabbits killed at 4 weeks had no ulceration on the segments selected for metalloproteinase staining.

In tnb58, extracellular collagenase and gelatinase were found at the junction of normal mucosa with ulcerated tissue. Stromelysin staining seemed the most prominent, but was
also confined to the regions immediately surrounding the ulcers in the mucosa and submucosa. TIMP was also present at the same sites, but the fluorescence was much less intense.

In tnb59, extracellular stromelysin was immunolocalised, surrounding the base of a small ulcer. Extracellular TIMP was also expressed at this site.

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Table 6.10: Expression of the matrix metalloproteinase enzymes and TIMP in animals killed four weeks after initiation of colitis with 40mg of trinitrobenzene sulphonic acid in a 25 percent ethanol/saline solution that displayed positive staining. +ve, two or more sites of expression on sections; +ve, one site of expression on sections; +/-ve, isolated expression associated with only a few cells; -ve, no staining; nd, not done.

6.4.13 Expression of the matrix metalloproteinases and TIMP six weeks after the initiation of colitis

At 6 weeks no collagenase expression was observed in any of the animals studied (table 6.11). TIMP was found in two of six animals, appearing as intracellular enzyme in fibroblast-like cells within the mucosa where there was evidence of previous injury, such as crypt architectural abnormalities. Gelatinase was found in the mucosa of one animal at 10cm and 20cm. It was mainly intracellular; some extracellular at 20cm was associated with a crypt abscess. Intracellular stromelysin was also observed in the mucosa of the same animal at 10cm.
Table 6.11: Expression of the matrix metalloproteinase enzymes and TIMP in animals displaying positive staining killed six weeks after initiation of colitis with 40mg of trinitrobenzene sulphonic acid in a 25 per cent ethanol/saline solution. +++ve, extensive staining on the majority of the section; ++ve, two or more sites of expression on sections; +ve, one site of expression on sections; +/-ve, isolated expression associated with only a few cells; -ve, no staining; nd, not done. Note 1: mucosal intracellular staining only.

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6.4.14 Summary of the results

The results are best summarised graphically (figure 6.4).

Figure 6.4: Expression of the matrix metalloproteinase enzymes and TIMP with time. Ribbon plot showing the relative expression of each matrix metalloproteinase and TIMP in ulcerated regions of colon against time. Score is as follows: 3, extensive expression of matrix metalloproteinase or TIMP across the majority of the frozen sections; 2, more than one site of expression on sections; 1, isolated expression associated with only a few cells.
6.5 Discussion

Matrix metalloproteinases are a tightly regulated family of enzymes that degrade components of the extracellular matrix and basement membrane; they play important roles in growth and development, and in invasion and metastasis of tumours. They are, therefore, likely to participate in the dynamic structural changes occurring in inflammatory bowel disease. This study documents for the first time the changes in matrix metalloproteinase and TIMP expression and distribution that take place in the wall of the colon up to six weeks after the induction of colitis in an animal model of inflammatory bowel disease.

6.5.1 Advantages and limitations of the technique

An immunofluorescence technique was adopted for this study for two principal reasons: firstly, immunofluorescence can detect the very small levels of antigen (matrix metalloproteinases) that are often present; and, secondly, only immunofluorescence can provide information about the microenvironmental pattern of synthesis. While biochemical assays have made a substantial contribution to our understanding of matrix breakdown and are quantifiable, they often involve homogenisation or culture of tissue, resulting in artificially high or low values. The influence which an in vitro technique can have on collagenase expression has been clearly shown; a short period of tissue culture (2 to 4 days) can significantly increase the amount of immunolocalisable collagenase in tumour tissue (Woolley & Grafton, 1980) and switch on the expression of collagenase in normal rabbit colonic tissue (Chowcat et al., 1990). In situ hybridisation could also have been used to investigate the pattern of matrix metalloproteinase mRNA expression; to date, however, the technique lacks resolution. Moreover, the presence of mRNA does not necessarily imply expression of the protein.

The major disadvantage of the immunofluorescence technique is that the data is qualitative and any assessment of the relative amounts of activity in the sections can only be subjective. To address this problem, fluorescence photometry was performed with a non-bleaching incident source, and emitted photons were detected quantitatively so that the relative amounts of enzyme could be compared. This technique was well suited to the earlier time points, when the matrix metalloproteinases were extensively expressed throughout the mucosa. A field within the mucosa could be chosen at random and the relative fluorescence measured. In three animals killed at twelve hours following the induction of colitis, the fluorescence photometry confirmed the immunofluorescence microscopy findings. However, at subsequent time points it was necessary to select microscopic fields containing representative staining patterns, because of the focal nature
of the staining. This added a further element of subjectivity and results from these time points were not included. Another disadvantage with the immunofluorescence technique is that it is not always possible to identify the cell types synthesising the matrix metalloproteinases or to know whether the synthesised enzyme is in the active form, pro form, or complexed with TIMP. Double labelling experiments could overcome the cell identification problem; cell markers could be used in conjunction with the matrix metalloproteinase markers on the same sections.

6.5.2 Principal observations

The principal observations made were as follows: (1) collagenase expression is predominant in the acute stages of disease and then declines, (2) stromelysin expression is consistently expressed until the ulceration in the colon resolves, (3) gelatinase expression peaks two weeks following initiation of colitis, (4) TIMP is not expressed until 24 hours after initiation of colitis, suggesting early uncontrolled tissue degradation.

These results must be compared with the histological findings at each time point, with studies involving human tissue, and with other animal models of inflammatory disease. They must also be evaluated in the light of biochemical and molecular data relating to the expression of the matrix metalloproteinases in inflammatory diseases.

6.5.3 Immunolocalisation of collagenase

The antiserum employed in this study has been shown to be specific for collagenase, without cross reactivity for stromelysin or gelatinase. The antisera detects pro-enzyme, active enzyme, and the enzyme-TIMP complex.

In the model collagenase was the most dominantly expressed enzyme after the induction of colitis, during the phase of ulcer development. It was seen extracellularly in the mucosa, bound to the matrix, with smaller areas of fluorescence in the submucosa in areas of inflamed tissue. Since only active collagenase binds to collagen fibres, the enzyme observed bound to the matrix had to be active (Hembry et al., 1986; Stricklin et al., 1978). Biochemical data has also suggested that when collagenase is present on the matrix the enzyme is active (Welgus et al., 1985). Collagenase dominance during the acute stage probably reflects its very specific action which initiates degradation of collagen in its triple-helical region. Once collagenase has destabilised the helix the collagen molecule becomes susceptible to degradation by many other proteases. The absence of any TIMP at six and twelve hours, and the presence of collagenase bound to matrix is a strong indication of uncontrolled collagen degradation suggesting that collagenase may be responsible, at least in part, for the generation of the ulcers observed
in the model. The delay in the expression of TIMP is likely to facilitate the breakdown and removal of damaged tissue required for tissue remodelling to take place.

Cells containing collagenase were observed in all layers of the bowel wall and each had a fibroblast-like appearance. Others have demonstrated that rabbit polymorphonuclear neutrophils contain collagenase (Robertson et al., 1972). However, collagenase-containing polymorphonuclear neutrophils were not identified in the present study. The polymorphonuclear neutrophil collagenase, unlike other collagenases, is stored and is not actively synthesised by the mature cell (Harris et al., 1984). As the polymorphonuclear neutrophil collagenase is a different gene product from interstitial collagenase (Mainardi et al., 1987; Weiss & Peppin, 1986) it is not surprising that the anti-collagenase antiserum used in the present study is not able to detect the polymorphonuclear neutrophil collagenase.

Acute inflammatory cells are responsible for the release of cytokines that stimulate the production of the matrix metalloproteinases. Moreover, granulocytes release other activators of the pro-enzymes: oxygen radicals, plasminogen activator and the serine proteinases (cathepsin G and elastase) (Weiss & Peppin, 1986). Elastase can indirectly potentiate matrix metalloproteinase activity through the inactivation of TIMP (Okada et al., 1988). However, peak enzyme expression of collagenase at six hours preceded infiltration of the acute inflammatory cells into the submucosa and mucosa. The resident cells, therefore, appear to be able to initiate the remodelling processes in the absence of an inflammatory cell infiltrate. The acute cells, present from 12 hours in the rabbit model of colitis, are likely to be of significance in the persistence of the expression of the matrix metalloproteinases in the model.

Collagenase was still present in the model from one week until the ulcers had been resolved, but with much less enzyme in the mucosa, mostly immediately adjacent to ulcers in regions of remodelling and repair. Hayrinenimmonen detected a similar pattern in biopsies removed from patients with recurrent aphthous ulcers in the oral cavity (Hayrinenimmonen et al., 1993). This group reported that interstitial collagenase and stromelysin were found in macrophages and fibroblast-like mononuclear cells in the lamina propria laterally adjacent to the ulcerous lesions. A small number of interstitial collagenase and stromelysin positive cells was also noted in control biopsies obtained from the clinically uninvolved control mucosa of the same patients.

During the present study, collagenase was never observed in normal tissue in control animals or in non-inflamed tissue from the colitis animals. The absence of matrix metalloproteinases in normal tissue conflicts with the results of Hawley who reported higher levels of collagenolytic activity in normal colon than in other regions of the
gastrointestinal tract (Hawley, 1970). These levels rose even further following sham laparotomy. However, Hawley did not demonstrate that the activity was specifically due to mammalian collagenase. The lysis of collagen gels could have been due to bacterial collagenase or another protease, e.g., cathepsins. Another possibility is that the activity is specific for that collagenase which has been stimulated by the culture conditions required for the gel. Chowcat and co-workers (1990) showed that colonic biopsies of normal colon maintained in culture secreted collagenase into the media following a lag period of 24 hours, whereas no collagenase was released by uncultured adjacent biopsies at zero hours. In a study examining the distribution of collagenase and TIMP in colorectal tumours and in the connective tissue of normal mucosa by immunostaining (Hewitt et al., 1991), collagenase was observed in normal mucosa. However, only active collagenase binds to collagen. Hewitt's findings imply active resorption of the extracellular matrix of normal colonic mucosa which would be surprising as the turnover of formed collagen fibrils in quiescent tissues is thought to be exceedingly slow (Gross et al., 1981). Immunolocalisation studies have failed to confirm the presence of collagenase in other normal tissues such as the rabbit colon (Chowcat et al., 1988); a similar result was obtained in a study of the distribution of matrix metalloproteinases in human colorectal cancer (Gallegos, 1992). Furthermore, Oyamada, using a biochemical assay, failed to detect collagenase activity in normal rabbit colon unless it was cultured (Oyamada et al., 1983). Other studies indicating that collagenase may be present in explants of normal colonic mucosa, in which the lysis of collagen gels has been used as a measure of collagenase activity, often refer to a few cases only. Horowitz et al., for example, detected collagenase activity in only 3 out of 34 biopsies of normal colonic mucosa, and then only after 72 hours incubation with the gel (Horowitz et al., 1987).

There has been only one previous immunolocalisation study of the distribution of the matrix metalloproteinases on tissue from human Crohn's disease patients, which has been published as a short communication (Bailey et al., 1990) and in a thesis (Bailey, 1992). No work has been published on tissue from patients with ulcerative colitis. In her thesis, Bailey examined tissue from Crohn's disease patients with polyclonal antisera to the matrix metalloproteinases and TIMP-1 (also provided by Strangeways Research Laboratory). Collagenase staining was confined to very few isolated mononuclear cells distributed throughout the bowel wall. In Crohn's disease no difference in collagenase staining was observed between non-diseased and diseased tissue. Collagenase, however, may have been present in the earlier stages of the human disease as in the rabbit model. This highlights the problem inherent in examining tissue at one instant in its natural history because of the transient nature of matrix metalloproteinase expression.
Collagenolytic activity has been measured by others (Horowitz et al., 1987), using a biochemical assay technique, in colonic mucosa specimens from 35 patients with inflammatory bowel disease and from 34 normal subjects. Increased collagenolytic activity was present in 94 per cent of the specimens taken from the inflamed mucosa, compared to only nine per cent of the control group. In another study, for 21 of 27 ulcerative colitis patients collagenolytic activity was found to be increased in explants of colonic mucosa when compared to controls (Sturzaker & Hawley, 1975). Increases in collagenolytic activity appear to be accompanied by a reduction in the plasma concentration of the protease inhibitor $\alpha_2$-macroglobulin (Bohe et al., 1986). This is probably due to its removal by complex formation with proteases.

Matthes and co-workers have presented results of in situ hybridisation experiments on inflammatory bowel disease tissue in abstract form only (Matthes et al., 1992b). They report that mRNA for collagenases type I (collagenase) and type IV (gelatinase) is present in 15 to 25 per cent of the cells of the lamina propria, muscularis mucosa, and muscularis propria in normal tissue. In Crohn’s disease the results were similar. However, in ulcerative colitis patients the number of positive cells rose to between 65 and 86 per cent, and the intensity of the staining was increased. The presence of large numbers of positive cells in the normal tissue is surprising given the low level of collagen turnover. The authors did not perform immunolocalisation experiments to detect the presence of the enzymes themselves, so it is impossible to know whether the mRNA observed was actually being transcribed, or whether extracellular enzyme was present.

In other experimental models of gastrointestinal ulceration, collagenolytic activity has been shown to be enhanced. In acetic acid-induced ulcers of dogs, the gastric mucosa displayed significantly increased collagenolytic activity, although the specific enzymes involved were not identified (Hasebe, 1987; Hasebe et al., 1987). The peak of activity on the third day was coincident with the maximum ulcer index, which supports the hypothesis that the matrix metalloproteinases are important in the generation of ulceration. However, in a study of acetic acid-induced gastric ulcers in the rat, the investigators found that the activity was most likely to reflect degradation by stromelysin rather than collagenase (Ito et al., 1989).

Involvement of collagenase in the healing process following colonic anastomosis has been demonstrated elsewhere. Polyclonal antisera to collagenase and TIMP were applied to colonic anastomoses in rabbits to investigate the role of the enzyme during healing (Chowcat et al., 1988). Within twelve hours of operation, secreting cells and extracellular collagenase were identified at the everted edges of the bowel wall. After 24 hours, collagenase activity was accompanied by TIMP secretion in the same localised regions, and by the third postoperative day very few cells were still synthesising enzyme.

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in these areas, although extracellular staining remained visible. The appearance of TIMP at 24 hours in this rabbit model of colonic anastomosis is similar to the results observed in the colitis model. Although the nature of the insults inflicted is very different in the two models, the similar results obtained may be indicative of a limited number of responses to injury that the gastrointestinal tract is able to mount.

6.5.4 Immunolocalisation of stromelysin

The antiserum to stromelysin used in this study recognises both the latent and active forms of the enzyme (Murphy et al., 1986).

In the model, stromelysin was first observed on the matrix and in fibroblast-like cells at the same time and place as collagenase, although at a lower level of expression. This is unsurprising, since both enzymes may be induced by interleukin-1 alone or in combination with fibroblast growth factor. Many other stimulators of collagenase and stromelysin synthesis are common to both enzymes.

Both the active and pro forms of the stromelysin have been shown to bind to the matrix. Collagenase and stromelysin may be activated by the same route; thus, the presence of active, matrix bound, collagenase suggests that a proportion of the stromelysin present is also likely to be active. Activation in vivo is thought to occur through a urokinase-dependent pathway where the plasminogen activator urokinase converts plasminogen into plasmin (He et al., 1989). Plasmin is capable of activating purified procollagenase and prostromelysin. Plasmin-dependent activation of procollagenase generates an enzyme species, by amino-terminal processing, identical to species produced by limited proteolysis of procollagenase with trypsin or by treatment with organomercurial compounds. In vitro, the enhanced secretion of urokinase has been shown to facilitate the metastatic process (Yu & Schultz, 1990) and like the matrix metalloproteinases, urokinase has also been immunolocalised to fibroblast-like cells in colonic cancers (Hansen et al., 1991). The co-expression of stromelysin with collagenase at six hours following the induction of colitis may be needed for the full activation of collagenase. Catalytic amounts of activated stromelysin convert plasmin- or trypsin-activated collagenase into a fully active enzyme in vitro by removal of approximately 15 amino acid residues from the carboxyl end of the enzyme (He et al., 1989; Murphy et al., 1987), resulting in a five- to eight-fold increase in collagenase specific activity. Stromelysin alone in both latent and activated forms is not capable of efficient activation of human fibroblast interstitial procollagenase.

There has been only one report studying the expression of stromelysin in inflammatory bowel disease patients (Bailey et al., 1990). In bowel removed from Crohn's disease
patients a few positive staining isolated inflammatory cells were found in apparently normal regions of tissue. However, in strictured tissue a large increase in the numbers of positive cells was observed. Furthermore, stromelysin was detected bound to the extracellular matrix in regions of mucosal degradation and smooth muscle cell proliferation. A consistent observation was that stromelysin predominated during the chronic phase of the colitis model, and was apparently associated with the remodelling process. Histological examination was consistent with this hypothesis, since by two weeks there was evidence of granulation tissue and repair.

Stromelysin's ability to degrade type III collagen (Galloway et al., 1983) may, in part, explain the presence of this enzyme throughout the healing process. In the initial stages of tissue repair, type III collagen fibres are more numerous than type I fibres (Goldberg & Rabinovitch, 1988). For instance, in active ulcerative colitis, type III procollagen mRNA has been shown to be over-expressed compared to other procollagen transcripts (Matthes et al., 1992a). Also, immunolocalisation of types I, III, IV, V, and VI collagens on sections of normal-looking and diseased intestines from patients with Crohn's disease revealed accumulation of collagen types I and III in the submucosa and sometimes in the main muscle layer (Bailey, 1992). In a guinea pig model of intestinal wound repair, epithelial migration to reseal the wound can be inhibited by the addition of antibodies to collagens type III and IV, but not to collagen type I or to fibronectin and laminin (Moore et al., 1992). The collagen initially deposited during healing is largely responsible for restoring the tensile strength of the damaged tissue. Following this initial step, developing granulation tissue is subjected to remodelling by enzymatic turnover and deposition of new fibres. In the later stages of repair type I collagens predominate (Goldberg & Rabinovitch, 1988), and the presence of stromelysin may be largely responsible for removal of the type III collagen that was initially laid down. Further experiments to investigate the nature of the collagen deposited in the colon following the induction of colitis might add support to this hypothesis.

6.5.5 Immunolocalisation of gelatinase B

The antiserum to gelatinase B, 95kDa, (Murphy et al., 1989) was acquired towards the end of the present study, and this resulted in the omission of some of the time points; however, gelatinase B was first observed at six hours following the initiation of colitis and was still present in two animals at six weeks. Gelatinase B was confined to neutrophils in which were seen secretory granules containing the enzyme. No gelatinase B was observed in fibroblast-like cells and no extracellular gelatinase B was observed. These observations confirm that gelatinase B is not constitutively expressed by fibroblasts (Hipps et al., 1991).
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The presence of gelatinase B in neutrophils is probably a requirement for the cells to be able to cross the basement membranes of capillary endothelium, which exhibit high type IV collagen content.

6.5.6 Immunolocalisation of gelatinase A and B

Gelatinase was present at similar levels of fluorescence throughout the period of study. It was first observed as intracellular enzyme in very few cells in the mucosa at 0 hour in one animal. The presence of this enzyme probably represents constitutive expression of gelatinase A, the 72kDa enzyme, in fibroblasts or macrophages, since no gelatinase B was observed at this time. Similarly, intracellular enzyme was observed in all animals killed at three hours; again no gelatinase B was observed at this time, and so the staining can be attributed to the presence of gelatinase A. This result confirms previous studies demonstrating that human fibroblasts constitutively synthesise gelatinase A (Hipps et al., 1991), but that gelatinase B is inducible in fibroblasts by agents such as cytokines.

At 12 hours and 24 hours, positive staining was seen in similar locations to the other matrix metalloproteinases, mainly in the mucosa (both intracellularly and extracellularly), with a few cells containing the enzyme in the submucosa and muscle. Gelatinase staining was often located in the same area as collagenase; however, at times the expression tended to be closer to the lumenal surface in the mucosa, and associated with the inflammatory cell infiltrate in the base of the ulcers. From one week gelatinase expression most frequently mirrored stromelysin expression, that is, it occurred immediately adjacent to areas of ulceration in regions of cell proliferation and repair. Like stromelysin, both latent and active forms of gelatinase can bind to the matrix, and the antiserum used binds both forms; thus, whether or not the enzyme present is active cannot be established from these experiments (Murphy et al., 1989).

Both gelatinase synthesis and activation are regulated by different mechanisms from those associated with collagenase and stromelysin. This phenomenon was demonstrated in a study of matrix metalloproteinase immunolocalisation in colorectal cancer, when adjacent sections stained for stromelysin and gelatinase A were compared and the distributions of the enzymes were found to differ. This observation suggests that their synthesis and secretion are not co-ordinately regulated, at least spatially (Gallegos, 1992). The mechanism whereby progelatinase is activated in vivo remains to be elucidated (Murphy et al., 1990). Recent in vitro work, however, suggests that the activation of gelatinase A may be initiated by a fibroblast membrane-bound molecule which induces a conformational change in the enzyme followed by self-cleavage (Ward et al., 1991). There is some evidence that neutrophil gelatinase may be activated by a HOCl-dependent process, since HOCl activates the latent enzyme in both a cell-free
supernatant and in a highly purified state (Peppin & Weiss, 1986), but as yet there are no clues as to the in vivo stimulator of synthesis.

In Bailey's study of the immunolocalisation of the matrix metalloproteinases in Crohn's disease tissue, a large number of polymorphonuclear neutrophils containing gelatinase were observed throughout the gut wall, but particularly in the mucosal and submucosal regions (Bailey et al., 1990). The presence of gelatinase-staining polymorphonuclear cells within the mucosa and submucosa was also a feature of the rabbit model. The only other human study of the role of the gelatinases in chronic ulceration was a comparison of non-healing leg ulcers with mastectomy wounds (Wysocki et al., 1993). Samples of blood and wound fluid from acute mastectomy and chronic leg ulcer wounds were collected, and metalloproteinase profiles of the samples were determined by means of gelatin zymography. Compared to serum, the acute wound fluid was found to contain markedly increased levels (five- to ten-fold) of gelatinases A and B. In chronic wound fluid these enzyme levels were increased another five- to ten-fold compared to mastectomy fluid. These results may be of relevance in inflammatory bowel disease, where chronic ulcers also develop in an environment containing high levels of gelatinase; this may contribute to chronic tissue turnover and failure of wound closure.

6.5.7 Immunolocalisation of TIMP

In the present study TIMP was observed first on the matrix at 24 hours and thereafter until the ulceration had been resolved. This suggests that degradation is regulated from early in the time course. The other implication from the staining is that where TIMP is observed on matrix, it has to be present in a TIMP-enzyme complex, since TIMP itself cannot bind directly to the matrix.

6.5.8 Implications of the findings

Chronic inflammatory bowel diseases are associated with an altered collagen metabolism, resulting in the deposition of both interstitial collagen and basement membrane collagen in the mucosal and muscular layers. Such alterations of collagen metabolism are reflected in the development of strictures, and are a frequent complication of Crohn's disease. At the other end of the spectrum, excessive collagen degradation in both ulcerative colitis and Crohn's disease may lead to ulcer formation. The deep fissuring ulcers observed in Crohn's disease are thought to be the precursors of fistula formation.

In the new model of inflammatory bowel disease described in this thesis, all the metalloproteinases and their inhibitor TIMP have been shown to be present. These enzymes and their inhibitor are undoubtedly involved in the remodelling process following the induction of the lesions. Comparison with an immunolocalisation study in
human Crohn's disease has shown close agreement with the later time points in the model (from one week onwards). Since the study of the temporal expression of the matrix metalloproteinases is impractical with human tissue, the use of this animal model may provide a valuable tool for studying the individual roles of these enzymes with time.

As yet, the precise mechanisms responsible for the transcription and activation of these enzymes *in vivo* is not known. The present study has shown that stromelysin and collagenase are frequently co-expressed. Many other researchers have noted the co-expression of the matrix metalloproteinase family members (Aggeler *et al.*, 1984; Cambray *et al.*, 1981a; Murphy *et al.*, 1981; Sellers *et al.*, 1978), and it is accepted that there is a common pathway leading to gene expression. Therefore, their presence together at the same site in the model is not surprising. In the present study, however, dominance of one enzyme and then the other suggests that other non-co-ordinately regulated pathways of activation of synthesis may also be operating; there is some support for this hypothesis from other sources (Chandrasekhar & Harvey, 1992; Murphy *et al.*, 1986). When rabbit articular chondrocytes were treated with interleukin-1 either alone or in combination with fibroblast growth factor, and the accumulated steady-state mRNA levels, for both collagenase and stromelysin, were measured (Chandrasekhar & Harvey, 1992), the latter treatment was more effective, inducing increased levels of mRNA within a shorter time. Cycloheximide added one hour post-stimulation with interleukin-1 and fibroblast growth factor failed to block stromelysin mRNA expression, but was able to block collagenase steady-state mRNA levels, indicating different regulatory pathways.

Cytokines may be important in the development or perpetuation of immune events in inflammatory bowel disease. As indicated in the study of Chandrasekhar and Harvey (1992), research has focused on the role of cytokines, such as interleukin-1 and tumour necrosis factor, and various growth factors as possible mediators of matrix metalloproteinase gene expression *in vivo*. Exposure of quiescent MRC-5 human fibroblasts to epidermal growth factor, basic fibroblast growth factor, or embryonal carcinoma-derived growth factor resulted in the induction of mRNA transcripts encoding procollagenase and prostromelysin and TIMP without increasing the production of collagen (Edwards *et al.*, 1987). Interleukin-1 accelerates both TIMP and matrix metalloproteinase production co-ordinately (with the possible exception of gelatinase A). Tumour necrosis factor, another cytokine released primarily from monocytes, also stimulates collagenase and stromelysin production, but inhibits TIMP production (Ito *et al.*, 1990). Therefore, interleukin-1 and tumour necrosis factor play, predominantly, a catabolic role in tissue responses to injury. The significance of these observations *in vivo* and in other tissue must be considered in the context of the
inflamed colon. The presence of interleukin-1 has been investigated in human inflammatory bowel disease. Enhanced spontaneous interleukin-1 production by peripheral blood mononuclear cells has been reported in Crohn's disease (Satsangi et al., 1987) and in vitro intestinal macrophages from patients with active Crohn's disease or ulcerative colitis secreted more interleukin-1 than cells isolated from histologically normal mucosa (Mahida et al., 1989). Moreover, inflamed colonic mucosa of inflammatory bowel disease patients has been found to release much higher quantities of interleukin-1 than normal mucosa (Mahida et al., 1991b). Enhanced generation of colonic interleukin-1 has also been shown in the rat model of chronic inflammatory bowel disease induced by TNB (Rachmilewitz et al., 1989). Reports of tumour necrosis factor involvement in human inflammatory bowel disease are conflicting. An increase in tumour necrosis factor production has been observed in some studies (Maeda et al., 1992; Murch et al., 1991), whereas a decrease or no change has been observed in others (Hyams et al., 1991; Mazlam & Hodgson, 1992). The stimulus for matrix metalloproteinase production mediated by interleukin-1 is, therefore, feasible in the colon of inflammatory bowel disease patients, although the role of tumour necrosis factor is inconclusive.

A local imbalance of matrix metalloproteinase expression may lead to either excessive degradation or excessive deposition of collagen, as in the strictures of inflammatory bowel disease patients. Opposite regulation of TIMP and the matrix metalloproteinases by transforming growth factor beta (TGFβ) has been reported and may favour increased deposition of collagen. TGFβ is a homodimeric protein with autocrine and paracrine activities, influencing cellular proliferation and differentiation, and is thought to be an important mediator in bone remodelling and wound healing (Shull et al., 1992). After tissue injury, TGFβ is released from degranulating platelets and autoinduces TGFβ production in local cells. TGFβ induces TIMP expression, but represses growth factor induction of the metalloproteinases stromelysin and collagenase in cultured fibroblasts (Overall et al., 1989). TGFβ mRNA steady-state expressions are higher in inflammatory bowel disease specimens than in normal intestine (McCabe et al., 1993). In animal models, disruption of the TGFβ1 gene in mice by homologous recombination results in the development of normal-looking animals that succumb to a wasting syndrome accompanied by severe gastric ulceration (Shull et al., 1992); in a model of glomerulonephritis in rats the therapeutic administration of the natural inhibitor of TGFβ, decorin, has been found to protect against scarring. Localised imbalance of the factors controlling the transcription of the matrix metalloproteinases may, therefore, be responsible for the broad spectrum of connective tissue lesions observed in inflammatory bowel diseases.
In the present study the distribution of TIMP in the model did not always follow the same pattern of expression as the other metalloproteinases, suggesting that matrix metalloproteinase activity may be uncontrolled in these areas. The widespread secretion of the matrix metalloproteinases associated with the heavy infiltration of inflammatory cells and oedema of the bowel wall together with lack of TIMP further suggests that excessive matrix metalloproteinase activity may favour initial ulcer formation in human inflammatory bowel disease as in the rabbit model of colitis. The extent of ulceration in inflammatory bowel disease could possibly be reduced by the administration of inhibitors of metalloproteinases. The natural inhibitors of the metalloproteinases have already been shown to be powerful anti-invasive agents when added to various model systems, both in vitro and in vivo. Thus, adding recombinant TIMP to cultures of melanoma cells in an amnion adhesion assay can totally inhibit their invasion (Schultz et al., 1988). Furthermore, co-injecting TIMP with melanoma cells significantly inhibits their metastasis to the lung. Reducing the concentration of TIMP produced by 3T3 cells with antisense construct increases the metastatic potential and tumorigenicity of these cells when injected back into nude mice (Khokha et al., 1989). It is claimed that glucocorticosteroids are also inhibitors of the matrix metalloproteinases both in vivo and in vitro (McGuire et al., 1983; Pelletier et al., 1987). However, glucocorticosteroids also have a large number of other effects in vivo and it seems likely that matrix metalloproteinase inhibition is a secondary effect.

In the colon of rabbits, collagenolysis has been inhibited in vivo by administration of the inhibitors aprotinin, soy bean trypsin inhibitor, and lima bean trypsin inhibitor (Lewin et al., 1986). Studies of anastomotic healing have been reported in which aprotinin improved the post-operative bursting pressure in rabbits (Delaney & Lalor, 1976) and rats (Bary et al., 1976). Aprotinin does not inhibit the matrix metalloproteinase directly, but inhibits the activators of the enzymes, such as trypsin. Despite these advantages shown in animals, a clinical trial of aprotinin in colonic anastomotic healing was inconclusive (Young & Wheeler, 1984).

Several synthetic inhibitors have been described that are based on chelation of the active site zinc atom of the metalloproteinase enzymes by moieties carried on peptide mimetics reflecting the substrate specificities of the enzymes (Lelievre et al., 1990). Other low molecular weight inhibitors have been described that are efficacious in vitro, but fail to have in vivo effects. Recently, a novel generalised inhibitor of the matrix metalloproteinases, BB-94, has been described which appears to improve the condition of nude mice with human ovarian carcinoma xenografts (Davies et al., 1993).

However, while considering the possible benefits of matrix metalloproteinase inhibition the potential side effects must also not be overlooked. Remodelling of the collagenous
extracellular matrices of tissues is a normal homeostatic activity. Such homeostatic remodelling presumably involves the matrix metalloproteinases. Thus the continuous administration of matrix metalloproteinase inhibitors, by preventing remodelling and ulcer formation, may induce severe side effects. To date, no published data has appeared on the toxicology of matrix metalloproteinase inhibitors. The likely side effects of matrix metalloproteinase inhibitors will be on the function of tissues with rapid connective tissue matrix turnover such as bone and lung. The severity of such side effects can only be ascertained when systemically active compounds become available. The potential deleterious effects of matrix metalloproteinase inhibition may be curtailed by developing agents which selectively inhibit only one of the metalloproteinases. For example, in inflammatory bowel disease a suitable therapeutic agent might inhibit collagenase only, while leaving gelatinase and stromelysin free to act.

6.5.9 Summary

In recent years, evidence has accumulated to support the hypothesis that connective tissue matrix metalloproteinases are responsible for the destruction of connective tissue in a number of chronic diseases. The differential expression of these enzymes with TIMP may also be significant in the pathogenesis of human inflammatory bowel disease.
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7 PROPHYLACTIC THERAPY IN THE MODEL WITH GLUCOCORTICOSTEROIDS

7.1 Introduction

7.1.1 General

Not only are the aetiologies of ulcerative colitis and Crohn’s disease unknown; the soluble mediators that amplify and modulate the inflammatory response have not been fully explored. The large number of potentially important mediators (as discussed in Chapter 3) complicates the treatment of inflammatory bowel disease in that pharmacological agents directed against one mediator may have no effect on others.

At present there is a variety of anti-inflammatory medications, ranging from sulphasalazine and its derivatives to glucocorticosteroids, all of which appear to derive at least a part of their effectiveness from their ability to limit the body’s synthesis of the prostaglandins and leukotrienes. In ulcerative colitis and Crohn’s disease, however, the efficacy of available medications is limited. In addition, side effects remain a problem, even with newer medications. In patients with active ulcerative colitis and Crohn’s disease, intestinal prostaglandin generation is increased (Donowitz, 1985). (In remission, however, colonic prostaglandin generation is similar to that in normal subjects.) The increased colonic generation of prostaglandins in inflammatory bowel disease may also contribute to the diarrhoea experienced by patients (Rachmilewitz, 1980). Non-steroidal anti-inflammatory drugs are able to suppress prostaglandin production, by inhibition of cyclooxygenase (Ferreira et al., 1971; Smith & Willis, 1971; Vane, 1971). However, the suggestion that inhibition of colonic prostanoid synthesis is an important mechanism to explain the therapeutic efficacy of the drugs used in inflammatory bowel disease has been challenged by the observation that non-steroidal anti-inflammatory drugs are of no therapeutic benefit, and even induce clinical deterioration. There are, however, now known to be at least two types of cyclooxygenase: cyclooxygenase-1, which is constitutively expressed, and cyclooxygenase-2, which is inducible (O’Neill & Ford-Hutchinson, 1993). The selective inhibition of cyclooxygenase-2 may give rise to new anti-inflammatory drugs with a therapeutic action in inflammatory bowel disease, while maintaining the homeostatic role of low-level prostaglandin release.
The disappointing results obtained with cyclooxygenase inhibitors have heightened interest in the role of leukotrienes in inflammation. It has been demonstrated by many workers that the leukotriene products of 5-lipoxygenase are present in the mucosa of patients with ulcerative colitis or Crohn's disease (Boughton-Smith et al., 1983; Peskar et al., 1987; Sharon & Stenson, 1984). Both sulphasalazine and the glucocorticosteroids have 5-lipoxygenase inhibiting activity. The value of specific 5-lipoxygenase inhibition has yet to be demonstrated in human clinical trials; however, promising results have been obtained with a variety of compounds in various animal models (Laursen et al., 1990; Morteau et al., 1993; Wallace & Keenan, 1990; Wallace et al., 1989).

In 1955 the efficacy of cortisone was proven in a large placebo-controlled clinical trial (Truelove & Witts, 1955). At present, however, intravenous use of high-dose glucocorticosteroids remains an important therapeutic approach for patients with ulcerative colitis or Crohn's disease (Rosenberg et al., 1990). In the following section the use of these potent antiinflammatory drugs and the mechanism by which they exert their effects are considered.

7.1.2 Glucocorticosteroid action

When considering the mode of action of these drugs, it must remembered that endogenously produced glucocorticosteroids are part of the normal regulation of the immune system, which explains why adrenalectomised animals tend to produce exaggerated responses to environmental trauma such as infection or injury (Peers & Flower, 1992). In humans, hydrocortisone is the predominant endogenous glucocorticosteroid, but in other animals a different, but related, steroid may be released. Until recently, glucocorticosteroids were thought to initiate the inflammatory response; however, it is now clear that they are actually part of the control system which prevents our 'defence mechanisms' from threatening homeostasis. The glucocorticosteroids influence many systems within the body, including the immune system, carbohydrate metabolising enzymes, and bone catabolism. Suppressive actions on the hypothalamus and pituitary may lead to adrenal insufficiency. The glucocorticosteroids are potent and effective anti-inflammatory agents, used in the treatment of a wide range of conditions, from skin irritation to asthma, multiple sclerosis, and rheumatoid arthritis. They are still the most effective single class of drugs available for the treatment of inflammatory bowel disease. Controlled therapeutic trials have demonstrated their effectiveness in the treatment of acute ulcerative colitis and active Crohn's disease (Summers et al., 1979). They are more effective than sulphasalazine and similar drugs in the treatment of active Crohn's disease, and it is only in mild cases of ulcerative colitis that 5-aminosalicylate therapy may be as effective. There is also evidence that a low dose of methylprednisolone (8mg/day) is of value in maintaining remission in Crohn's disease patients (Brignola et
al., 1988). However, it is still debated whether the favourable clinical results for ulcerative colitis and Crohn’s disease, where glucocorticosteroids induce remission of symptoms or decrease the activity index, are associated with a concomitant decrease in the inflammatory pattern (Modigliani et al., 1990). Both naturally-occurring steroids or related synthetic analogues, such as methylprednisolone (figure 7.1), are used clinically, and have similar wide-ranging biological activities. Hence, when glucocorticosteroids are used as anti-inflammatory agents they possess the range of unwanted side effects associated with over-production of endogenous corticosteroids seen in Cushing’s syndrome.

![Figure 7.1: A synthetic glucocorticosteroid. The structure of methylprednisolone or 11,17,21-trihydroxy-6-methyl-1,4-pregnadiene-3,20-dione.](image)

Although the glucocorticosteroids are among the most potent and widely used anti-inflammatory agents, the mechanisms by which they reduce inflammation in vivo are still uncertain. Traditionally, glucocorticosteroids are thought to produce their anti-inflammatory effects by inhibiting eicosanoid production at the level of phospholipase A2, through stimulating the production of lipocortin which suppresses phospholipase A2 activity (Flower, 1988). Lipocortin is a member of a family of glucocortico-inducible proteins with phospholipase A2 inhibitory activity, originally purified from a variety of biological sources. One member of the family, lipocortin-1, has been produced using recombinant technology, and most of our knowledge relates to this peptide. Recent work has suggested that the inhibition of arachidonic acid synthesis by lipocortin may be due to sequestration of the phospholipid substrate by lipocortin. Lipocortins are calcium lipid-binding peptides and seem to block the access of phospholipase A2 to the phospholipid substrate; this is apparently easily overcome by increasing the substrate concentration (Davidson & Dennis, 1989).
Lipocortin production by glucocorticosteroids appears to occur at the transcriptional level via glucocorticosteroid receptors (Beato, 1989). Occupancy of these receptors results in the switching on (or off) of a number of genes, not just those encoding the lipocortins. Most mammalian cells have up to $10^5$ glucocorticosteroid receptors per cell. The glucocorticosteroid receptors are members of a gene 'superfamily', the products of which are ligand-sensitive transcription factors, and include receptors for thyroid hormone, vitamin D and retinoic acid. The steroid receptors have a variable N-terminal region modulating transactivation and a well-conserved central domain which appears to be partly responsible for intranuclear localisation of the receptor. This region is also important for interaction with the 90kD heat shock protein (see Figure 7.2). The C-terminal domain is relatively well conserved and has complex functions, since, as well as being involved in glucocorticosteroid binding and nuclear translocation, it may have transactivation and dimerization functions. Interaction of the glucocorticosteroid with the receptor leads to dissociation of the heat shock protein and a change in receptor conformation, which allows it to bind to chromatin. The hormone-receptor complex binds to specific hormone response elements (HREs) on certain genes (Beato, 1989), including those encoding the lipocortins, and stimulates transactivation by a mechanism not yet fully understood. (Some genes appear to be repressed by glucocorticosteroids, and these have an HRE distinct from that of genes whose transcription is enhanced by glucocorticosteroids.) Since glucocorticosteroid action requires mRNA and protein synthesis, drugs such as actinomycin D or cycloheximide can block steroid effects (Tsurufuji et al., 1979).

Besides induction of lipocortin, alternative hypotheses for the anti-inflammatory effects of glucocorticosteroids have been proposed. These include: allosteric effects on enzymes (Samuels & Tomkins, 1970); redirection of lymphocyte traffic (Parrillo & Fauci, 1979); modulation of cyclooxygenase transcription (Raz et al., 1989); inhibition of transcription of various cytokines (II-1, TNFα, and II-6) (Bochner et al., 1987; Chensue et al., 1991; Kirnbauer et al., 1991) and matrix metalloproteinases (Hunter et al., 1984); and the stabilisation of lysosomal and other cellular membranes. Recent evidence also suggests that the glucocorticosteroid receptor regulates leukocyte adhesion to endothelial cells and the expression of both endothelial-leukocyte adhesion molecule 1 (ELAM-1) and intercellular adhesion molecule 1 (ICAM-1) (Cronstein et al., 1992). It is likely that glucocorticosteroids mediate their antiinflammatory effects through multiple pathways.
Figure 7.2: The actions of glucocorticosteroids. (a) The anti-inflammatory actions of glucocorticosteroids include: inhibition of oedema by stimulating vasoconstriction of small blood vessels; inhibition of the production of the vasodilatory prostaglandins; inhibition of the recruitment and activity of polymorphonuclear neutrophils, mast cells, lymphocytes and monocytes; and Il-6 receptor induction on fibroblasts and hepatocytes. (b) Glucocorticosteroids (GCS) bind to the steroid receptor and displace two 90kD heat shock proteins (HSP). Receptor-bound GCS is translocated to the nucleus, where it associates with specific regions of DNA, inducing the transcription of certain species of mRNA, including that coding for lipocortin. One function of lipocortin is to antagonise the action of TNF and IL-1 by inhibiting phospholipase A2 (PLA2) activity, thereby inhibiting the production of eicosanoids. Note: Red arrows indicate inhibitory effect and blue arrows indicate stimulatory effect.
7.1.3 Summary

Oral glucocorticosteroids were first used in inflammatory bowel disease in the early 1950s. In 1955 the efficacy of cortisone was proven in a large placebo-controlled clinical trial (Truelove & Witts, 1955). However, our understanding of the mechanism of action of these drugs remains incomplete. Although animal models of inflammatory bowel disease cannot be used to identify the initiating aetiological factors leading to chronic idiopathic colitis in humans, they can be used to evaluate the efficacy of therapeutic agents, the role of inflammatory mediators, and the mechanisms of tissue injury. However, such models must present a spectrum of responses to current therapeutic agents which reflects the human response to those agents.
7.2 Aims of the study

The present experiments were carried out to evaluate the effect of methylprednisolone, a drug used in the treatment of inflammatory bowel disease (Brignola et al., 1988; Rosenberg et al., 1990), on the cellularity, on the generation of arachidonic acid metabolites, and on macroscopic damage, in TNB-induced chronic colitis in the rabbit. The present study also aims to determine the effect of glucocorticosteroid administration on matrix metalloproteinase expression, following reports that glucocorticosteroids decrease matrix metalloproteinase production by connective tissue cells in culture (Hunter et al., 1984).

7.3 Materials and Methods

7.3.1 Animals

Thirty female New Zealand white rabbits (see section 5.3.2) were randomised into four treatment groups:

Group I: Saline control

Five rabbits were anaesthetised, and 0.6cm³ of 0.9 per cent saline was introduced into the colon. Twenty-four hours prior to the introduction of saline, the rabbits were injected intramuscularly with 0.0125cm³/kg vehicle (distilled water).

Group II: Glucocorticosteroid-treated saline control

Five animals were treated with a daily intramuscular injection of an aqueous solution of methylprednisolone acetate, 0.5mg/kg ('Depo-Medrone', 40mg/cm³, from Upjohn Ltd, Crawley, West Sussex). 24 hours after the first injection, 0.6cm³ of 0.9 per cent saline was introduced to the colon. This group acted as a control for the detection of any adverse steroid effects.

Group III: Colitis

Beginning 24 hours prior to the induction of colitis (section 5.3.4), and daily thereafter, ten rabbits were injected intramuscularly with 0.0125cm³/kg distilled water (vehicle) as a control. Colitis was induced with 40mg TNB in 25 per cent ethanol.

Group IV: Glucocorticosteroid-treated colitis

Beginning 24 hours prior to the induction of colitis (section 4.3.4), and daily thereafter, ten rabbits were injected intramuscularly with an aqueous solution of
methylprednisolone acetate, 0.5mg/kg ('Depo-Medrone', 40mg/cm³, from Upjohn Ltd, Crawley, West Sussex). Colitis was induced with 40mg TNB in 25 per cent ethanol.

7.3.2 Choice and dose of glucocorticosteroid

In a pilot study both oral prednisolone and intramuscular methylprednisolone were evaluated for use in these experiments. Doses of each drug were chosen that were equivalent in mg/kg to a high human dose which might be used to treat acute inflammatory bowel disease. The use of oral prednisolone conferred no advantage over intramuscular methylprednisolone, and administration may have been more stressful to the rabbits. Higher doses of methylprednisolone (2mg/kg/day) and prednisolone (3mg/kg/day) were not tolerated by the rabbits. Therefore, 0.5mg/kg/day methylprednisolone, administered intramuscularly, which is equivalent to ~70mg/day in humans, was chosen as the preferred treatment.

7.3.3 Occult blood

Occult blood was detected in the stool using Peroheme 40-C. Pellets were collected from the rabbits each day prior to the induction of colitis, and each day for the following week. (Section 4.3.12)

7.3.4 Erythrocyte sedimentation rate

7.3.4.1 General

If blood is drawn and mixed with an anticoagulant so that it remains fluid, the erythrocytes will gradually settle to the bottom of the container. Normally sedimentation takes place slowly, but in a variety of disease states the rate is rapid, and in some cases proportional to the severity of the disease. Measurement of the sedimentation rate is a useful test to assist in the diagnosis of occult disease or to follow the course of manifest disease. The sedimentation rate is expressed as the distance (in mm) that erythrocytes fall per unit of time (usually 1 hour, although interval measurements show that the rate of sedimentation is not constant throughout the 1-hour period) (Puccini et al, 1977).

Measurement of the erythrocyte sedimentation rate was adopted by modern medicine in 1918 when Fahraeus published his observations on the erythrocyte sedimentation rate in pregnancy. He first proposed it as a test for pregnancy, but in 1921 he wrote a more detailed report, in which the phenomenon was related to many factors other than pregnancy.

The sedimentation rate is affected by properties of the erythrocytes, by properties of the plasma, and by mechanical or technical factors.
The spontaneous sedimentation velocity of a single spherical body falling freely through a simple fluid may be expressed by the Stokes equation:

\[ V = \frac{2r^2(d_1 - d_2)g}{9\eta} \]

Where:
- \( V \) = Sedimentation velocity
- \( r \) = Radius of the sphere
- \( d_1 \) = Density of the sphere
- \( d_2 \) = Density of the fluid
- \( g \) = Force of gravity
- \( \eta \) = Viscosity of the fluid

The Stokes equation can be modified to apply to a disc-shaped body falling through plasma by changing the denominator to \( 7.65\eta \) and substituting \( ac \) for \( r^2 \), where \( a \) is the radius and \( c \) is the thickness of the body (Ponder, 1948). Sedimentation velocity therefore increases with mass of the sedimenting particle and is proportional to the difference in density between the particle and the fluid. It is inversely proportional, however, to the viscosity of the fluid. In normal blood, erythrocytes suspended in plasma form few if any aggregates. The mass of the sedimenting particle is therefore small, and the sedimentation velocity tends to be low. In abnormal blood, rouleau formation or erythrocyte agglutination may take place, increasing the particle mass and the sedimentation velocity. Rouleau formation depends on the protein composition of the plasma, particularly with regard to fibrinogen and globulin.

Erythrocytes are negatively charged and normally repel each other. The negative charge is expressed as the zeta potential. All protein molecules and other macromolecules decrease the zeta potential (Chien, 1976; Pittz et al, 1977). Thus when the plasma concentrations of fibrinogen and immunoglobulin are increased, the zeta potential of the erythrocytes is decreased; this leads to an increase in rouleau formation. The greatest effect is exerted by fibrinogen. The sedimentation rate is, therefore, increased in inflammatory bowel disease and other conditions characterised by hyperfibrinogenemia (tissue damage or infection) or elevated immunoglobulin.

7.3.4.2 Method

2 cm\(^3\) of peripheral venous blood was collected from the ear vein of each animal 24 hours after dosing with methylprednisolone or the distilled water vehicle and just before insertion of the feeding tube into the colon. A further 2 cm\(^3\) of blood was collected
immediately prior to killing each animal. The blood was collected into dry EDTA tubes and diluted with sterile trisodium citrate, 109mmole/l, anticoagulant solution in the proportion of 1 volume of citrate to 4 volumes of blood.

Plastic erythrocyte sedimentation rate tubes, 230mm in length and having an internal diameter of 2.55mm, with a graduated mm scale extending over the lower 200mm (Bibby Sterilin, Stone, Staffs), and a specially made rack with adjustable levelling screws (Westergren erythrocyte sedimentation rate stand AT2, from Bibby Sterilin, Stone, Staffs) for holding the sedimentation tubes firmly in an exactly vertical position, were used to obtain the rate. (Minor degrees of tilting have a marked accelerating effect on the sedimentation rate.) The sedimentation rate tests were performed at room temperature and were set up within two hours of collecting the blood. The blood sample was mixed thoroughly and then drawn up into the Westergren tube to the 200mm mark by means of a teat. The tubes were set in the vertical position and left undisturbed for 60 minutes; then the height of the clear plasma above the upper limit of the column of sedimenting cells was read to the nearest 1mm. When the test is applied to human blood this 60-minute measurement is the Westergren erythrocyte sedimentation rate. However, the rate in rabbit blood is much slower, and so an average hourly rate, computed from the change observed over 24 hours, was recorded. A separate test confirmed that the rate remained sensibly constant over this period.

7.3.5 Morphometric analysis of macroscopic appearance

Transparencies taken of the first 20cm of the colon with 160T ASA film were examined using monochrome image analysis. Size-calibrated black and white images were captured with a television camera connected to an IBM personal computer and areas of ulceration were selected by grey thresholding using image analysis software (Sight Systems). The areas of the selected regions were calculated and expressed as percentages of the total surface area of the first 20cm of each colon.

7.3.6 Histology

Segments of colon were selected at autopsy from 5cm, 10cm, 15cm, 20cm, 30cm, and 60cm proximal to the anus. These were fixed in formol saline and embedded in paraffin (appendix A); then 4μm sections were cut on a microtome and stained as previously described with haematoxylin and eosin (appendix B). The presence or absence of each of a series of histopathological features as shown in table 5.4 (chapter 5) was recorded for each group and the results were compared.
<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Not inflamed</td>
</tr>
<tr>
<td>1</td>
<td>Mild inflammation</td>
</tr>
<tr>
<td>2</td>
<td>Mild to moderate inflammation</td>
</tr>
<tr>
<td>3</td>
<td>Moderate inflammation</td>
</tr>
<tr>
<td>4</td>
<td>Moderate to severe</td>
</tr>
<tr>
<td>5</td>
<td>Severe inflammation</td>
</tr>
</tbody>
</table>

*Table: Grading of the inflammation. The extent of the microscopic inflammation was assessed by a histopathologist specialising in gut pathologist and graded on a continuous scale.*
Grading of the inflammation

Microtome sections from the ulcerated regions of distal colon at approximately 10cm proximal to the anus in the colitis groups III & IV and from the same position in the control groups I & II were stained with haematoxylin and eosin. A pathologist (V.S.), specialising in gut pathology and blinded to the identity of the microscope slides, graded them on a continuous scale from 0 (no inflammation) to 5 (severe inflammation), according to the degree of acute inflammation. The different animal groups were then compared.

7.3.7 Immunochemical assays for eicosanoids

7.3.7.1 Tissue incubation prior to eicosanoid assay

Three 0.5g strips labelled A, B and C of adjacent tissue from each of four sites at 5cm, 10cm, 15cm, and 20cm proximal to the anus (12 strips of tissue in total) were collected from each colon into individual 3.5cm-diameter wells of a six-well tissue-culture plate each containing 5cm³ Kreb's solution, and gassed with a mixture of 95 per cent O₂ and 5 per cent CO₂ at 37°C for 30 minutes (basal incubation).

After the 30 minutes the strips of tissue were transferred to new dishes each containing 10⁻⁵M A23187 (calcium ionophore), which stimulates eicosanoid release, in 5cm³ Kreb's solution (see appendix E for composition). As before, the tissue was gassed with a mixture of 95 per cent O₂ and 5 per cent CO₂, at 37°C for 30 minutes (stimulated incubation). The incubating medium for B-labelled strips also contained 3x10⁻⁴M MK886, a lipoxygenase inhibitor, and the medium of the C-labelled strips was supplemented with 3x10⁻⁵M indomethacin, a cyclooxygenase inhibitor.

The tissue was then removed and weighed before being fixed in formalin and embedded in paraffin (appendix A); sections were cut for routine H&E staining as previously described (appendix B).

The Kreb's solution from each well was transferred to a test tube and placed in a boiling water bath for 5 minutes, in order to destroy interfering factors. The tubes were cooled and six, 0.5cm³, aliquots were removed from each sample. The aliquots were stored at -20°C until required.
7.3.7.2 Enzyme linked immunosorbent assay technique for the determination of the concentration of leukotriene B₄ (LTB₄) and 6-keto prostaglandin F₁α (PGF₁α)

**Principle of the enzyme linked immunosorbent assay (ELISA)**

This assay is based on the competition between free LTB₄ (or 6-keto PGF₁α) and acetylcholinesterase (AChE)-linked LTB₄ (or 6-keto PGF₁α) tracer for limited specific antiserum binding sites. The antiserum-LTB₄ (or 6-keto PGF₁α) complex (either free or tracer) then binds a mouse monoclonal anti-IgG antibody coating the wells of a 96 well plate. The plate is washed and Ellman’s Reagent is added to the well. The AChE tracer cleaves the Ellman’s Reagent. This reagent contains acetylthiocholine, which is cleaved to give free thiocholine. Thiocholine then reacts with the dimer of 5-thio-2-nitrobenzoic acid, the second component of Ellman’s reagent, producing a distinct yellow colour which is measurable spectrophotometrically (figure 7.3).

The density of the colour is proportional to the amount of tracer bound to the well, which is inversely proportional to the amount of free 6-keto PGF₁α (or LTB₄) present in the well during the incubation.

![Diagram summarising the steps of an ELISA assay for eicosanoids.](image)

**Figure 7.3: Diagram summarising the steps of an ELISA assay for eicosanoids.**

**Coating the Plates**

Before running the assay, microtitre plates (NUNC Certified Maxi-Sorp) were coated with the mouse monoclonal anti-IgG. Since the coated plates are quite stable when stored, several plates were coated at the same time. The mouse monoclonal anti-IgG was dissolved in 100 cm³ of 0.05M potassium phosphate buffer, pH 7.4 (see appendix F). 200μl of this solution was transferred to each well, and the plates were covered with plastic film. The plates were incubated for at least 18 hours at room temperature. The following day 100μl of the Saturation Buffer (See appendix F) was added to each well.
The plates were re-covered with plastic film and incubated for at least 18 hours at 4 °C, after which time they were ready for use.

Reconstitution of the lyophilized tracer and antisera
The LTB₄ or 6-keto PGF₁α acetylcholinesterase tracer was reconstituted with 30 cm³ of EIA buffer and stored at 4 °C for use within two weeks. The LTB₄ or 6-keto PGF₁α antiserum was reconstituted with 30 cm³ of EIA buffer and stored at 4 °C for use within four weeks.

Running the assay
The coating monoclonal, and saturation, solutions were emptied from the wells and each well was rinsed three times with Wash Buffer (see appendix F).

A standard curve was prepared by dissolving lyophilized LTB₄ or 6-keto PGF₁α standard in 1 cm³ of deionized water, giving a stock solution with a concentration of 5 ng/cm³. Different concentrations of LTB₄ or 6-keto PGF₁α needed for the standard curve were obtained by serial dilution of the stock solution in nine clean microcentrifuge tubes, numbered for identification (table 7.1).

<table>
<thead>
<tr>
<th>Standard Number</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
<th>S6</th>
<th>S7</th>
<th>S8</th>
<th>S9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc’n (pg/cm³)</td>
<td>20</td>
<td>39</td>
<td>78</td>
<td>157</td>
<td>313</td>
<td>625</td>
<td>1250</td>
<td>2500</td>
<td>5000</td>
</tr>
</tbody>
</table>

Table 7.1: Table displaying the concentrations of standard used to construct the standard curves.

Plate setup
The 96 well plates used in the assays were set up as shown in Table 7.2. Each standard and each unknown was duplicated.
Table 7.2: The 96 well plates used in the assays were set up as shown in this table. Here, TA = total activity, NS = non-specific binding, B0 = maximum binding, S1-9 = standards, U1-36 = unknowns.

The standard curve was prepared by adding 25\textmu l of standard, 25\textmu l of tracer and 25\textmu l of antiserum to each well. Each plate had its own set of standards. 25\textmu l of unknown sample was added per well in addition to 25\textmu l of tracer and 25\textmu l of antiserum. The concentration of an unknown sample was determined by reference to the standard curve derived from the same plate. Since a very small amount of tracer binds to the well even in the absence of specific antiserum, a non-specific binding pair was included on the plate to measure this low-level binding. The level of non-specific binding was determined by adding 25\textmu l LTB_4 or 6-keto PGF_1\alpha tracer and 50\textmu l EIA buffer to the two wells.

Maximum binding (B0), representing the maximum amount of eicosanoid that can bind to the antisera, was determined by adding 25\textmu l of tracer, 25\textmu l of antisera and 25\textmu l of EIA buffer to each of two wells.

The plates were covered with plastic film and incubated for at least 18 hours at room temperature.

**Development of the plates**

The plates were washed six times with Wash Buffer and vacuum dried. The Ellman’s Reagent was prepared by reconstituting with 50cm^3 of deionized water, and was protected from light by wrapping it in foil. Ellman’s Reagent is unstable and was used only on the day of preparation. 200\textmu l of Ellman’s Reagent was added to each well, and 5\textmu l of tracer was added to the total activity wells. This was done to check the level of enzymatic activity of the tracer. A rapid development of yellow colour indicated that the
acetylcholinesterase enzyme linked to the LTB$_4$ or 6-keto PGF$_{1\alpha}$ had full catalytic activity. The plates were covered with plastic film and left overnight in the dark.

Reading the plate

The plates were read on a plate-reading spectrophotometer at 412nm (Molecular Devices, CA, USA). Before reading each plate, the bottom of the plate was wiped with a clean tissue to remove finger prints, dirt, etc.

Calculating the results

The results were analysed by means of an IBM PC running 'SOFTMAX 2.01' software which performed the following operations:

1. The absorbance readings from the NS wells were averaged.
2. The absorbance readings from the B$_0$ wells were averaged.
3. The NS average was subtracted from the B$_0$ average to give corrected B$_0$.
4. The absorbance readings from each duplicate sample or standard were averaged.
5. Percentage B/B$_0$ was calculated using the equation:

$$\frac{B}{B_0} \times 100 = \left( \frac{\text{absorbance sample or standard} - \text{absorbance NS corrected B$_0$}}{\text{absorbance NS corrected B$_0$}} \right) \times 100$$

6. A standard curve was plotted by the computer of percentage B/B$_0$ of the standards against concentration.

Determination of the concentration of samples

The percentage B/B$_0$ value was calculated for each sample, and the concentration of each sample was determined by reading the percentage B/B$_0$ value from the standard curve. The results were expressed as release in ng/g of tissue.

7.3.7.3 Prostaglandin E$_2$ radioimmunoassay (RIA) technique

Principle of the RIA

Radioimmunoassay involves the competition between a radioactively-labelled antigen and a non-labelled antigen in the sample or standard for a limiting amount of antisera. After equilibration, unbound labelled antigen is separated from the bound labelled antigen by the addition of dextran-coated activated charcoal, on to which the unbound labelled antigen binds. Following centrifugation, the supernatant containing antigen-antibody complexes is removed and the radioactivity determined. The radioactivity of
the supernatant is inversely proportional to the amount of antigen contained in the standard or sample. The concentration of antigen in the sample can be estimated from a standard curve.

**Reconstitution of the lyophilized antisera**

Lyophilized anti-PGE₂ (ICN Immunobiochemicals, High Wycombe, Bucks) was reconstituted with 5.0 cm³ of distilled water to give the stock antiserum solution. This was then diluted 1 in 10 with RIA buffer (appendix G) for use in the assay.

**Running the assay**

A solution of 1 µg/cm³ PGE₂ (ICN Immunobiochemicals, High Wycombe, Bucks) in absolute ethanol was prepared. This was then diluted to 30 ng/cm³ for a working dilution. A standard curve was prepared by serial dilutions of the 30 ng/cm³ solution (see table 7.3).

<table>
<thead>
<tr>
<th>Standard Number</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
<th>S6</th>
<th>S7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. (ng/cm³)</td>
<td>0.03</td>
<td>0.1</td>
<td>0.3</td>
<td>1.0</td>
<td>3.0</td>
<td>10.0</td>
<td>30.0</td>
</tr>
</tbody>
</table>

*Table 7.3: Standard concentrations of PGE₂ for use in RIA.*

3.4 µl of the ^3^HPGE₂ (100 Ci/m mole) tracer (Amersham International plc, Bucks) was diluted with 5 cm³ RIA buffer for use in the assay. The reagents were then added to duplicate-labelled 1.5 cm³ microcentrifuge tubes (Eppendorf) according to table 7.4. The whole assay was carried out at 4°C.
Table 7.4: Composition of RIA tubes for the measurement of PGE2 concentration

The contents of the tubes were mixed and incubated overnight at 4°C (figure 7.4).

Dextran-coated charcoal solution (appendix G) at 4°C was added as in table 7.4. The tubes were mixed and left for 10 minutes, and the charcoal was sedimented by centrifuging at 4000 rpm for 1 minute in a bench-top centrifuge (Eppendorf).

350μl of the supernatant was removed from each tube and placed in a separate glass scintillation vial. 8cm³ Picofluor 30 liquid scintillant (Canberra-Packard, Pangbourne, Berks) was added, the contents of the vials were mixed on a vortex mixer, and the radioactivity was measured in a β scintillation counter (Denley, Billingham, Sussex).
50μl Standard or Sample

250μl PGE₂ Antiserum
50μl 3H PGE₂ Tracer

4°C Overnight

100μl Dextran Coated Charcoal 10 minutes

Centrifugation at 4000 rpm for 1 minute

Supernatant collected and counted for beta emission

*Figure 7.4: Diagram to show the steps in an RIA assay for prostaglandin E₂.*
Calculating the results
The results were processed by means of MINITAB software running on a VAX/VMS computer at Glaxo, Ware.

The following operations were performed by a routine of grouped commands within MINITAB.
1. dpm readings from the NS vials were averaged.
2. dpm readings from the Bq vials were averaged.
3. The NS average was subtracted from the Bq average to give corrected Bq.
4. dpm readings from each duplicate or standard were averaged.
5. Percentage B/Bq was calculated using the equation:

\[
\% \frac{B}{Bq} = \frac{\text{dpm sample or standard - dpm NS}}{\text{corrected Bq}} \times 100
\]

6. A standard curve was plotted by the computer of percentage B/Bq of the standards against concentration.

Determining the concentration of PGE2 in the samples
The standard curve was linearised by a logit/log transformation in MINITAB. Linear regression analysis was then performed on the logit/log transformation and sample concentrations were calculated from the resulting analysis. Concentrations of immunoreactive PGE2 were expressed as release in ng/g of tissue.

7.3.8 Myeloperoxidase Assay

7.3.8.1 Extraction of Myeloperoxidase

Introduction
Myeloperoxidase (MPO) is found in the azurophilic granules of neutrophils. There is much more MPO in neutrophils than in other leukocytes, and it can therefore be used as an indicator of neutrophil presence. Tissue levels of MPO may give an indication of leukocyte infiltration into the tissue; MPO has also been used as a biochemical marker for polymorphonuclear neutrophil infiltration and inflammation in the gastrointestinal tract (Smith and Castro, 1978).
Method

MPO was extracted by a modification of a previously reported method (Eliakim, Karmeli, Okon, & Rachmilewitz, 1992). Strips of tissue of approximately 1g were collected from sites 5cm, 10cm, 15cm and 20cm proximal to the anus in the distal colon, snap-frozen in liquid N\(_2\), and stored at -20°C until required.

Thawed tissue was homogenised in 5cm\(^3\) phosphate buffer in 10cm\(^3\) plastic centrifuge tubes for five 10-second periods at 4°C with a polytron (Kinematica GmbH, Kriens-Luzern, Switzerland). The homogenate was centrifuged at 3,000rpm for 15 minutes at 4°C and the supernatant was discarded. The pellet was resuspended in 1.5cm\(^3\) of ice-cold 0.5 per cent hexadecyltrimethylammonium bromide (Aldrich, Gillingham, Dorset) in 50mM phosphate buffer, pH 6.0 (see appendix H), and transferred on ice to 4.5cm\(^3\) tubes (NUNC, Gibco, Paisley, Scotland). The samples were sonicated for 10 seconds, freeze-thawed three times in liquid nitrogen, and sonicated for a further 10 seconds before centrifuging in a bench-top centrifuge (Eppendorf) at 11,500 rpm for 20 minutes to achieve maximal release of MPO from the granules. The supernatant was collected and stored at -20°C until assayed for MPO activity.

7.3.8.2 Peroxidase assay method using pyrogallol

Principle of the assay
The addition of a peroxidase to hydrogen peroxide solution results in the evolution of oxygen which oxidizes pyrogallol, a chromagen, producing a colour change as a measurable indicator of the extent of reaction. Pyrogallol changes from a colourless solution to yellow.

Method
A spectrophotometer (Perkin-Elmer Ltd, Beaconsfield, UK) was set to the optimum wavelength of 420nm and allowed to stabilise for 30 minutes. 5cm\(^3\) clear plastic test tubes were used to mix the reagents. 0.9cm\(^3\) of the chromagen solution was pipetted into a tube and allowed to temperature stabilise. 0.1cm\(^3\) extract was added and stirred. The spectrophotometer was set to zero absorbance. Finally, 16\(\mu\)l of a hydrogen peroxide solution (for 0.005 per cent) was introduced and the mixture was stirred. 0.9cm\(^3\) of the reaction mixture was drawn into the spectrophotometer; the change in absorbance (\(\Delta A_{420}\)) was monitored for two minutes and an average rate of reaction determined. The rate remained constant over the two-minute period, although a five-second delay was introduced at the beginning of the recording time to eliminate artefact generated by turbidity in the sample after drawing it in. The chamber was flushed with pyrogallol solution after each run.
Calculating the results

The results were calculated as enzyme units (E.U.) per mg of tissue, where one unit is defined as forming 1.0mg of purpurogallin from pyrogallol in 20 seconds at pH 6.0 at 20°C.

$$\text{E.U./mg} = \frac{\Delta A_{420/20\text{seconds}}}{\varepsilon \times (\text{mg tissue as solid/cm}^3 \text{ sample})}$$

where $\varepsilon$, the extinction coefficient = 12

7.3.9 Matrix metalloprotease staining of tissue

Full-thickness segments of rabbit colon were removed after the macroscopic assessment of damage from sites 5cm, 10cm, 15cm, 20cm, 30cm and 60cm proximal to the anus from each animal in the four experimental groups.

Each segment of colon was processed and stained for the metalloprotease enzymes and TIMP as previously described (section 6.3).

The staining pattern in each group was recorded and compared.
7.4 Results

7.4.1 Erythrocyte sedimentation rate

An erythrocyte sedimentation rate was calculated from multiple observations over a 24-hour period. The rate remained sensibly constant throughout this period.

The erythrocyte sedimentation rate of blood collected 24 hours after pre-dosing did not differ significantly between each of the four groups (Student's t-test), and was within the normal range, 0.2-0.8 mm/h (Okerman, 1988).

The erythrocyte sedimentation rate was significantly increased (P<0.05, Student's t-test) at one week in the glucocorticosteroid-treated control group II (0.53mm.h⁻¹ s.e.±0.08), the colitis group III (0.63mm.h⁻¹ s.e.±0.11), and treated colitis group IV (0.97mm.h⁻¹ s.e.±0.27) with respect to the untreated saline control group I (0.22mm.h⁻¹ s.e.±0.01) (table 7.5). There was, however, no significant difference between groups II, III and IV when similarly compared with each other by means of a t-test (figure 7.5).

<table>
<thead>
<tr>
<th>Group</th>
<th>ESR (mm/h)</th>
<th>Degrees of freedom</th>
<th>P-value: comparison with Group I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>0.22</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Group II</td>
<td>0.53</td>
<td>8</td>
<td>0.001</td>
</tr>
<tr>
<td>Group III</td>
<td>0.63</td>
<td>9</td>
<td>0.002</td>
</tr>
<tr>
<td>Group IV</td>
<td>0.97</td>
<td>9</td>
<td>0.013</td>
</tr>
</tbody>
</table>

Table 7.5: Analysis of erythrocyte sedimentation rate experiments. The table shows the comparison between the erythrocyte sedimentation rate results at the end of the steroid experiments and those for control group I (Student's t-test).

Comparison of the erythrocyte sedimentation rates in individual groups at the beginning and end of the experiments, possibly a better reflection of the effects of the various treatments, revealed that a significant increase (P=0.044, paired t-test) in erythrocyte sedimentation rate was only achieved in the group III animals, i.e., the untreated colitis group.
Figure 7.5: Bar chart showing erythrocyte sedimentation at the start of the steroid experiments and one week later. There was no significant difference between the groups at start (Student’s t-test). At one week the erythrocyte sedimentation rate was significantly raised in group III (n=10), using a paired t-test.

7.4.2 Occult blood results

All animals were tested for the presence or absence of occult blood each day. None of the animals in either group I or group II, the non-colitis controls, tested positive at any time. An analysis of the results, using a chi-squared test, comparing group III (positive readings on 40 days) and group IV (positive readings on 46 days) animals showed that there was no significant difference (P=0.18) between the number of positive or negative readings over the one-week experimental period (n=7 in both groups) (table 7.6). Therefore, administration of methylprednisolone to the group IV rabbits did not alter the outcome of the occult blood tests.
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Table 7.6: Occult blood results for the steroid experiments. This contingency table shows the total number of positive or negative readings in the two colitis groups; all animals in both groups were tested on each day (eight tests on each rabbit). Chi-squared analysis probability, $P=0.18$.

7.4.3 Macroscopic assessment

At one week the animals were killed and the macroscopic appearance of the colons was scored (table 7.7). The appearance of the colons was normal for all rabbits in both the control groups I and II.

All animals in groups III and IV had macroscopically visible ulcers present in the distal colon and scored either 2 or 3. A chi-squared test shows no association between scores for the glucocorticosteroid-treated or untreated groups ($P=0.65$).

Table 7.7: Macroscopic scores in the steroid experiments. Contingency table showing the frequency of the macroscopic scores at one week in untreated colitis animals (group III) compared to scores in glucocorticosteroid treated animals (group IV). The chi-squared probability assuming no association is $P=0.65$.

7.4.4 Morphometric analysis of the macroscopic appearance

Comparison of the percentage area of ulceration over the first 20cm, employing monochrome image analysis (figure 7.6), showed that there was no significant difference ($P=0.97$) between groups III and IV using a t-test. The technique did not pick out any ulcerated areas of mucosa in the control groups I and II. The mean area of ulceration was 3.3 per cent in group III animals and was 3.5 per cent in group IV animals (figure 7.7). Daily administration of 0.5mg/kg methylprednisolone, therefore, did not influence the size of ulceration.
Figure 7.6: Image capture. Thermal print of a view of the VDU screen following selection of ulcerated regions by the image analysis program. The ulcers are masked, and appear as white regions on the print (U). The area in mm$^2$ of the masked tissue can be calculated to give the area of ulceration.
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Figure 7.7: Image Analysis Results. Bar chart showing the effect of glucocorticosteroid on the percentage area of ulceration induced in the four experimental groups by TNB. No significant difference was found comparing group III to group IV (Student's t-test, P=0.97). Bars: ±1 standard error. The low percentage ulceration figures reflect the large reference region (0-20cm proximal to the anus). Most ulceration was localised to a region 5 to 15cm proximal to the anus; however, the first 20cm was chosen in order to include all ulceration that might be present.

7.4.5 Histology

Neither group I nor group II exhibited any abnormal histological features. There was one very striking difference between group III and group IV in the numbers of the acute inflammatory cells that had infiltrated the mucosa and the submucosa of the distal colon (figure 7.9 A and B). However, most of the other histological features
Figure 7.9A: The typical appearance of a region of mucosa and submucosa directly under an area of ulceration in rabbits treated with 40mg TNB/ethanol and a daily intramuscular injection of vehicle. Note the large inflammatory cell mass and the destruction of the muscularis mucosae.

Original magnification, x200.

Figure 7.9B: The typical appearance of a region of mucosa and submucosa directly under an area of ulceration in rabbits treated with 40mg TNB/ethanol and a daily intramuscular injection of an aqueous solution of methylprednisolone acetate. Compared to figure 7.1A there is a relative absence of acute inflammatory cells.

Original magnification, x200.
examined were unchanged by glucocorticosteroid treatment. All the animals in group III and IV exhibited architectural abnormalities and crypt abscesses from 5cm to 20cm. Other histological features investigated were present in similar proportions in both colitis groups.

7.4.5.1 Grading of the inflammation

The sections examined by the pathologist (V.S.) were graded on a continuous scale from 0 (no inflammation) to 5 (severe inflammation), according to the degree of acute inflammation and placed in an ascending order of inflammation (figure 7.8). A significant decrease (P=0.026) in acute inflammation, as evidenced by leukocytosis, was found when groups III and IV were compared (Mann-Whitney U test). The tissue of all animals from groups I and II was graded as ‘non-inflamed’.

Figure 7.8: Univariant scattergram of the acute inflammation scores assigned to group III and group IV animals. The scores are an indication of the level of acute inflammation observed in microtome sections from ulcerated regions of distal colon by a pathologist blinded to the identity of the microscope slides. A significant decrease (P=0.026) in acute inflammation was found in the group IV animals (Mann-Whitney U test). However, there was no significant difference (P=0.90) between the macroscopic scores in same two sets of animals as evaluated by a Fisher’s exact test with a continuity correction.
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7.4.6 Results of the immunochemical assays for eicosanoids

7.4.6.1 Results of the LTB\textsubscript{4} ELISAs

The release of immunoreactive LTB\textsubscript{4} was the same in both control groups (group I = 0.35ng/g s.e.±0.12; group II = 0.40ng/g s.e.±0.13). Animals in colitis group III exhibited a significant six-fold increase (2.91ng/g s.e.±0.63) in the mean immunoreactive release (figure 7.10) compared to the saline control group I. Administration of 0.5mg/kg methylprednisolone did not reduce LTB\textsubscript{4} release in the treated colitis group IV animals (3.05ng/g s.e.±0.93) compared to the untreated colitis group III animals. \textit{Ex-vivo} inhibition of LTB\textsubscript{4} was achieved in the B-labelled strips incubated with the lipoxygenase inhibitor MK\textsubscript{886} (3 x 10\textsuperscript{-4}M), which brought the mean release in the group III animals back down to the level of the controls (0.33ng/g s.e.±0.05) (figure 7.10). The function of the B-labelled strips was to act as a control in order to show that the immunoreactivity was due to a lipoxygenase product. The release of LTB\textsubscript{4} from C-labelled strips appeared to be reduced (1.54ng/g s.e.±0.29) by incubation with the cyclooxygenase inhibitor indomethacin (3 x 10\textsuperscript{-5}M), but the result was not significant.

7.4.6.2 Results of the 6-Keto prostaglandin \textit{F\textsubscript{1\alpha}} ELISAs

The release of immunoreactive 6-keto PGF\textsubscript{1\alpha} was the same in both control groups (group I = 16.93ng/g s.e.±1.76; group II = 13.90ng/g s.e.±4.23). No increase in the mean immunoreactive release was observed in the group III animals (18.08ng/g s.e.±4.23) compared to group I and II animals (figure 7.11). Administration of 0.5mg/kg methylprednisolone did not influence 6-keto PGF\textsubscript{1\alpha} release in the treated animals (21.65ng/g s.e.±4.36) compared to the colitis group III animals. \textit{Ex-vivo} inhibition of 6-keto PGF\textsubscript{1\alpha} was found in the C-labelled strips incubated with the cyclooxygenase inhibitor indomethacin (3 x 10\textsuperscript{-5}M), which reduced the mean release in the group III and group IV animals to a third of the level of the controls (4.69ng/g s.e.±1.17). This inhibition indicated that the immunoreactivity was due to a cyclooxygenase product. The release of 6-keto PGF\textsubscript{1\alpha} from B-labelled strips was unaffected (14.75ng/g s.e.±3.05) by incubation with the lipoxygenase inhibitor MK\textsubscript{886} (3 x 10\textsuperscript{-4}M).

7.4.6.3 Results of the Prostaglandin \textit{E\textsubscript{2}} radioimmunoassays

There was no significant difference in the release of immunoreactive PGE\textsubscript{2} in the two control groups (group I = 3.99ng/g s.e.±0.64; group II = 8.08ng/g s.e.±2.14). Animals in group III (19.35ng/g s.e.±2.87) and group IV (27.20ng/g s.e.±5.47) exhibited a significant increase in the mean immunoreactive release of PGE\textsubscript{2} compared to animals in group I (figure 7.12). However, administration of 0.5mg/kg methylprednisolone did not alter PGE\textsubscript{2} release in the treated colitis group IV animals compared to the untreated
colitis group III animals (P>0.05). *Ex-vivo* inhibition of PGE$_2$ was achieved in the C-labelled strips incubated with the cyclooxygenase inhibitor indomethacin (3 x 10^{-5}M), which reduced the mean release in the group III animals (7.75ng/g s.e.$\pm$1.91) and group IV animals (6.45ng/g s.e.$\pm$1.50) to the levels of the controls. The release of PGE$_2$ from B-labelled strips was unaffected by incubation with the lipoxygenase inhibitor MK886 (3 x 10^{-4}M).

![Bar chart showing the immunoreactive release of LTB$_4$ from strips of colon incubated for 30 minutes in Kreb's solution. Strips from group III were also incubated in the presence of the cyclo-oxygenase inhibitor indomethacin or the lipoxygenase inhibitor MK886. Bars a and b are significantly different (P<0.05, Student's t-test) from group I and group III respectively. Bars: ±1 standard error.](image)

**Figure 7.10: Leukotriene assays.** Bar chart showing the immunoreactive release of LTB$_4$ from strips of colon incubated for 30 minutes in Kreb's solution. Strips from group III were also incubated in the presence of the cyclo-oxygenase inhibitor indomethacin or the lipoxygenase inhibitor MK886. Bars a and b are significantly different (P<0.05, Student's t-test) from group I and group III respectively. Bars: ±1 standard error.
Figure 7.11: 6-keto prostaglandin F$_{1\alpha}$ assays. Bar chart showing the immunoreactive release of 6-keto PGF$_{1\alpha}$ from strips of colon incubated for 30 minutes in Kreb's solution. Strips from group III were also incubated in the presence of the cyclooxygenase inhibitor indomethacin or the lipoxygenase inhibitor MK886. Bars a and b are significantly different (p<0.05, Student's t-test) from group I and group III respectively. Bars: ±1 standard error.
Table 7.12: Prostaglandin E\textsubscript{2} assays. Bar chart showing the immunoreactive release of PGE\textsubscript{2} from strips of colon incubated for 30 minutes in Kreb's solution. Strips from group III were also incubated in the presence of the cyclooxygenase inhibitor indomethacin or the lipoxygenase inhibitor MK886. Bars \textit{a} and \textit{b} are significantly different (p<0.05, Student's t-test) from group I and group III respectively. Bars: ±1 standard error.
7.4.6.4 Summary of the results of the assays for products of arachidonic acid metabolism

LTB$_4$ and PGE$_2$ release were significantly increased in animals with colitis at one week. There was no change in the release of 6-Keto PGF$_{1\alpha}$ in these animals.

The administration of 0.5mg/kg methylprednisolone did not affect the immunoreactive release of LTB$_4$, PGE$_2$ or 6-Keto PGF$_{1\alpha}$ in either the control group II or in the treated colitis group IV.

7.4.7 Results of the myeloperoxidase assays

Table 7.8 (below) summarises the results of the MPO assays. Although the first column shows a wide variation in mean enzyme activity, there was no significant difference between the groups (Student's t-test). In all groups a large spread of results existed.

<table>
<thead>
<tr>
<th>Group</th>
<th>mean (mEU/g)</th>
<th>standard error</th>
<th>number of assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (saline control)</td>
<td>23.44</td>
<td>3.29</td>
<td>12</td>
</tr>
<tr>
<td>Group II (treated saline control)</td>
<td>25.36</td>
<td>4.09</td>
<td>12</td>
</tr>
<tr>
<td>Group III (colitis)</td>
<td>41.77</td>
<td>10.72</td>
<td>23</td>
</tr>
<tr>
<td>Group IV (treated colitis)</td>
<td>34.81</td>
<td>6.33</td>
<td>26</td>
</tr>
</tbody>
</table>

Table 7.8: Results of the MPO assays. The table shows mean enzyme activity and the standard error as measured in a tissue extract from each of the four groups. No significant difference in activity was observed.

7.4.8 Matrix metalloproteinase staining

No matrix metalloproteinase expression or TIMP was observed in the non-colitis animals (groups I and II).

Treatment with glucocorticosteroids did not alter the pattern of expression of the matrix metalloproteinases or TIMP in the colitis groups III and IV. The enzymes were found only in the distal colon at the sites of damage. In animals with ulcers, the predominant enzymes were stromelysin and gelatinase A and B. Collagenase was also present, but was less extensively expressed. The enzymes tended to be expressed most at the junction of 'normal' and ulcerated tissue, in a region undergoing extensive remodelling. No evidence of increased or decreased expression of TIMP was found (see chapter 6 for a detailed description of the staining pattern at one week).
7.5 Discussion

The prophylactic effect of methylprednisolone, a drug relevant to the treatment of inflammatory bowel disease, given 24 hours before the initiation of colitis in the rabbit model, was studied using a range of parameters to assess its effect on the pathogenesis.

7.5.1 Clinical course

A paired t-test revealed a significant rise in erythrocyte sedimentation rate over the week in the untreated colitis group III. Animals that received 0.5mg/kg methylprednisolone daily also displayed a significantly increased erythrocyte sedimentation rate in both the colitis IV and non-colitis II groups with respect to the saline-treated control group I at one week. Administration of the glucocorticosteroid, however, did not alter the outcome of the erythrocyte sedimentation rate measurements in the colitis groups (group IV compared to group III at one week).

Although the erythrocyte sedimentation rate is a non-specific phenomenon, its measurement is clinically useful in inflammatory bowel disease, where it has been shown to be significantly related to abnormal $^{111}$In-labelled leukocyte faecal excretion (Fischbach & Becker, 1991). So far, excretion of $^{111}$In-labelled leukocytes has been shown to be the most reliable marker of gut inflammation, apart from histological evaluation (Saverymuttu, 1986). The erythrocyte sedimentation rate in inflammatory bowel disease patients is used as a marker of disease activity, and has been used to assess the usefulness of other putative markers of disease. Recently, the erythrocyte sedimentation rate in inflammatory bowel disease patients has been correlated with serum phospholipase A$_2$ activity (Minami et al., 1992), $\alpha_1$-proteinase inhibitor-bound elastase (Adeyemi & Hodgson, 1992), and neopterin concentration in urine (Judmaier et al., 1993), none of which would have offered any advantage over the erythrocyte sedimentation rate measurement for the present study. The plasma viscosity, which could have been used as an alternative, is related to disease activity in Crohn's disease, but is insufficiently sensitive to replace erythrocyte sedimentation rate as a measure of the acute-phase response in Crohn's disease (Lobo et al., 1992).

Measurement of the erythrocyte sedimentation rate in this model provided a useful indicator of disease severity. No difference was observed between the erythrocyte sedimentation rates of methylprednisolone-treated colitis animals (group IV) and the untreated colitis animals (group III); this may reflect a lack of sensitivity, the small sample size resulting in large standard errors. Previous evidence suggests that the administration of glucocorticosteroids might be expected to reduce the erythrocyte sedimentation rate (Hall & Malia, 1990).
The observation that the number of occult blood positive readings were not reduced by administration of glucocorticosteroids suggests that ulcer resolution was not enhanced in the treated animals. Occult blood was not detected in animals without ulcers, and therefore the presence of occult blood provides a useful marker of mucosal ulceration.

Subjectively, the methylprednisolone-treated rabbits appeared less unwell than the untreated rabbits. This was evidenced by increased activity in the treated group, and would suggest that the glucocorticosteroid may increase the sense of well-being experienced by these animals, despite a lack of improvement in the macroscopic appearance. Overall, these general findings suggest that the glucocorticosteroid has no effect on the disease activity produced by TNB.

7.5.2 Morphological assessment

When severity of the ulceration, determined by the macroscopic score, was compared in the two colitis groups, there was no significant difference. In addition, the quantitative image analysis also confirmed that administration of methylprednisolone did not improve the macroscopic appearance of the tissue. This method was based on measurement of the actual area of ulceration, and thus the subjectivity inherent in the macroscopic scoring system was eliminated.

In humans, glucocorticosteroids provide a feeling of well-being to the inflammatory bowel disease patient, but do not necessarily produce an improvement in the macroscopic appearance. Some patients in clinical remission still show abnormal biochemical findings, and in barium studies (Goldberg et al., 1979) they are found to have active disease. A recent multicentre prospective study included 142 patients with active colonic or ileocolonic Crohn’s disease. Oral prednisolone induced clinical remission in 92 per cent of patients within 7 weeks; however, only 29 per cent of patients in clinical remission also achieved endoscopic remission, and in 12 patients the endoscopic score actually worsened (Modigliani et al., 1990). Furthermore, it was demonstrated over 20 years ago that in rats glucocorticosteroids significantly impair the healing of cutaneous wounds (Ehrlich & Hunt, 1968), and that resection and anastomosis of the intestine in patients treated with glucocorticosteroids heightens the risk of anastomotic leak, particularly in the colon (Rousselot & Slatery, 1964). In previous animal models of inflammatory bowel disease, the reported results of glucocorticosteroid therapy on healing have been conflicting. In the dinitrochlorobenzene rat model of colitis, pretreatment of the animals with dexamethasone did not significantly alter the damage score (Murthy & Biondi, 1992), and in a TNB-induced colitis in the rat, prednisolone did not reverse the course of established colonic inflammation and tissue damage (Boughton-Smith et al., 1988a). In
contrast, in guinea pigs with carageenan-induced colitis, the administration of dexamethasone-β-D-glucoside or dexamethasone reduced the severity of ulceration observed in the colon (Friend et al., 1991). Oral or intrarectal administration of glucocorticosteroids (dexamethasone, hydrocortisone) also attenuated the parameters of acetic acid-induced colitis in the rat (Fitzpatrick et al., 1990). Subsequent experimental work has shown that prostaglandin inhibition jeopardises healing in animal colitis (Empey et al., 1992). However, leukotriene inhibition is beneficial, and the administration of misoprostol, a prostaglandin analogue, with a 5-lipoxygenase inhibitor acts synergistically to ameliorate disease activity (Empey et al., 1992).

Variation in the response to glucocorticosteroids in these models may reflect inter-species differences, differences in the mode of colitis induction, or differences in the doses and specific glucocorticosteroids used. In this study, methylprednisolone was chosen for its relevance to the treatment of human inflammatory bowel disease, and for its suitability for use in the rabbit. The dose used in this study was chosen to correspond to a high human therapeutic dose (~70mg). Oral prednisolone, which is also commonly used in human inflammatory bowel disease, was used in a pilot study, but did not confer any advantage. It is possible, however, that different doses and different glucocorticosteroids might have resulted in a different outcome in this model.

7.5.3 Histology

Although inflammatory bowel disease is viewed as a chronic inflammatory process, it is to some extent, histologically and functionally, a prolonged acute inflammatory response. The acuteness of the inflammatory response correlates with the presence of numerous neutrophils in the mucosa.

In the present study, comparison of the histology of the treated and untreated colitis groups shows that methylprednisolone produces a significant decrease in acute inflammation, and this suggests that the mucosal dose was efficacious. This inhibition of leukocyte recruitment to the inflamed areas by methylprednisolone may have resulted from the down-regulation of the expression of adhesion molecules on endothelial cells. A recent study has shown that pretreatment of endotoxin-stimulated endothelial cells (HUVEC) with nanomolar concentrations of dexamethasone inhibits the expression of the adhesive molecules ICAM-1 and ELAM-1 (Cronstein et al., 1992). These adhesion molecules are critical for neutrophil adhesion and subsequent movement into the tissue. The inhibition of expression by dexamethasone was reversed by a steroid antagonist.

Neutrophils are thought to play a critical role in the genesis of microvascular dysfunction in postischaemic skeletal muscle (Carden et al., 1990) and cardiac muscle (Hernandez et
Furthermore, neutrophils play an important role in ischaemia-reperfusion-induced vascular injury in the small intestines of cats. Pretreatment with the CD18-specific monoclonal antibody (IB4) against the leukocyte adhesion glycoprotein affords protection against ischaemia-reperfusion-induced microvascular injury (Kurtel et al., 1992). Recently, IB4 has been used to investigate the role of neutrophils in acute colitis induced with 30 per cent ethanol and 60mg TNB in small 1kg rabbits which were killed not more than four days after initiation (Wallace et al., 1992). In Wallace's experiments, the infiltration of the mucosa and submucosa by neutrophils was most apparent in animals killed more than six hours after the administration of the TNB, a result consistent with the present study. However, in contrast to the observations presented in this study, re-epithelisation appears to have taken place between 12 and 24 hours after induction of colitis. Although a substantial infiltrate of neutrophils still remained in both the mucosa and submucosa, this may have been a consequence of using very young rabbits or different TNB. IB4 markedly attenuated neutrophil infiltration into the colon, reduced the extent of epithelial injury as measured by permeability to $^{51}$Cr EDTA, and reduced MPO activity. No other parameters were measured. Treatment with NPC 15669, another inhibitor of neutrophil recruitment, also suppressed neutrophil infiltration in an acute acetic acid-induced colitis in the rat (Noronha-blob et al., 1993). MPO accumulation, inflammatory mediator release (LTB$_4$ and TxB$_2$), and disruption of colonic vascular integrity were also significantly reduced. These alterations were supported by histological findings, and by macroscopic observations showing accelerated healing of colonic ulcers and resolution of tissue inflammation. NPC 15669, however, may have other pharmacological effects, since pretreatment of the same acetic acid-induced rat colitis with an anti-neutrophil antiserum did not attenuate the increases in either colonic mucosal permeability or in colon weight produced by acetic acid administration (Yamada et al., 1991). The anti-neutrophil antiserum treatment reduced both the numbers of circulating neutrophils and the degree of colonic MPO activity to less than 10 per cent of control values. Histological inspection confirmed that anti-neutrophil antiserum treatment was not effective in attenuating the injury to the epithelial barrier. These contrasting data demonstrate that the role of infiltrating neutrophils has not been identified in experimental colitis; the discrepancies may reflect inter-species differences, or different methods of establishing colitis.

To date, no other investigations have been performed to establish the effect of either neutropenia or inhibition of neutrophil adhesion in a chronic model of colitis.

7.5.4 Eicosanoid release in the model

In the saline controls there were very low levels of LTB$_4$ release. Among the eicosanoids studied, in normal colon the release of 6-keto PGF$_{1\alpha}$ was the greatest, followed by that
of PGE$_2$; this observation agrees with previous studies in rabbit colon (Zijlstra et al., 1992).

In the new rabbit model of inflammatory bowel disease, increases in the immunoreactive release of PGE$_2$ and LTB$_4$ were observed. However, there was no change in the release of 6-keto PGF$_{1\alpha}$. The pattern of eicosanoid production in this animal model of intestinal inflammation has striking similarities to that seen in human Crohn's disease. Rectal biopsy specimens in culture and rectal dialysis samples from Crohn's disease patients produce abnormally high amounts of PGE$_2$, LTB$_4$, and thromboxane B$_2$, while 6-keto PGF$_{1\alpha}$ levels are unaffected (Hawkey et al., 1983; Lauritsen et al., 1989). A different profile is obtained from cultured colonic mucosa from patients with active ulcerative colitis; significantly higher amounts of PGE$_2$, 6-keto PGF$_{1\alpha}$, LTB$_4$, and thromboxane B$_2$ are detected compared to normal controls (Boughton-Smith et al., 1983; Eliakim et al., 1992a). Specifically, the colonic mucosa of inflammatory bowel disease patients contains approximately 250ng/g LTB$_4$, more than 50 times the amount found in normal tissue.

In a previous rabbit model, Zipser measured PGE$_2$, LTB$_4$, and LTC$_4$ levels by rectal dialysis in rabbits in which colitis had been induced by injection of immune complexes following formalin enema (Zipser et al., 1986). Here, the colitis was associated with enhanced synthesis of the three compounds studied, LTB$_4$ > PGE$_2$ >> LTC$_4$. There was good correlation between elevated levels of LTB$_4$, the presence of histological inflammation, and diarrhoea production. Comparison of Zipser's results with the present study indicates a reversal in the pattern of leukotriene and prostaglandin synthesis; this may reflect the different method of induction of colitis. Arachidonate metabolism was assayed in another model of intestinal inflammation, acetic acid-induced colitis in the rat. Dilute acetic acid was injected into rat colon and the effects were observed after 24 hours (Sharon & Stenson, 1985). Histological analysis of this model of intestinal inflammation showed the formation of ulcers and extensive neutrophil infiltration. Arachidonic acid metabolism in colonic mucosa from acetic acid-treated rats was compared to that of normal rats. The normal rat mucosa metabolised only a very small portion of the exogenous arachidonic acid, whereas the colonic mucosa from acetic acid-treated rats was converted to that of normal rats. The normal rat mucosa metabolised only a very small portion of the exogenous arachidonic acid, whereas the colonic mucosa from acetic acid-treated rats converted a significant portion of exogenous arachidonate to the lipooxygenase products LTB$_4$, 5-HETE, 12-HETE and 15-HETE. Moreover, when the endogenous mucosal lipids of the normal and acetic acid-treated rats were compared, the acetic acid-treated-mucosa was found to have significant amounts of LTB$_4$, 5-HETE, 12-HETE and 15-HETE. Although other lipooxygenase products were not assayed in the present study, similar increases might be expected. In the rat model of TNB-induced colitis, Boughton-Smith et al. were able to demonstrate increased production of 6-keto
PGF$_{1\alpha}$ and LTB$_4$ two weeks following initiation of colitis in homogenised tissue samples, but do not report the result of the PGE$_2$ assays mentioned in their methodology (Boughton-Smith et al., 1988a). Similar increases in eicosanoid release were found following luminal intracolonic injection of trinitrobenzene sulphonic acid in the rat (Vilaseca et al., 1990). Eicosanoid release was quantified by a different technique in vivo, using a dialysis bag placed into the distal colon. Three days following initiation of colitis, the dialysates showed a highly significant increase in PGE$_2$, 6-keto PGF$_{1\alpha}$, thromboxane B$_2$, and leukotriene B$_4$, compared with levels in controls not subjected to the toxic agent. All the models mentioned display some changes in the release of eicosanoids. Comparison of the results is complicated by the different modes of induction of colitis, the different species used, and the variety of times chosen to study release. However, the similarity of the release observed in the new model to that observed in Crohn’s disease indicates that the model may be a useful tool to investigate the manipulation of these mediators in relation to Crohn’s disease.

Although the present study has confirmed the release of PGE$_2$, LTB$_4$, and 6-keto PGF$_{1\alpha}$ from colonic tissue incubations, the precise source of the eicosanoids released remains open to speculation. The capacity of a number of cell types to produce cyclooxygenase products have been shown in vitro, but this may not represent the contribution in vivo. Cell types able to produce prostacyclin and PGE$_2$ in gut are endothelial cells and macrophages. Fibroblasts produce only PGE$_2$. Prostaglandins are known to be released from the epithelial cells of the gastric mucosa, and so it may be possible that colonic epithelial cells are also producing PGE$_2$. Neutrophils do not produce prostaglandin or prostacyclin; however, neutrophil depletion in an immune arthritis model in the rabbit inhibited the observed rise in PGE$_2$. This may indicate that neutrophils are indirectly responsible for the production of PGE$_2$ by producing factors such as lysosomal phospholipase A$_2$ that may initiate eicosanoid synthesis in resident tissue. However, in the new rabbit model, neutrophils are depleted by methylprednisolone, and in other models (Simmons et al., 1984), the generation of PGE$_2$ and 6-keto PGF$_{1\alpha}$ is independent of neutrophil migration.

Neutrophils produce large amounts of LTB$_4$; significant levels have been observed in inflammatory exudates in experimental models (Simmons et al., 1984). However, macrophages may also have the capacity produce LTB$_4$. This may account for the unchanged level of LTB$_4$ release observed in glucocorticosteroid-treated animals, despite a reduction in the number of neutrophils present in the ulcerated tissue. In human tissue, alveolar macrophages have been found to produce LTB$_4$ (Fels et al., 1982).
7.5.5 Effect of glucocorticosteroids on eicosanoid release in the model

This experiments reported in this chapter show that methylprednisolone does not modify the immunoreactive release of LTB4, 6-keto PGF1α, or PGE2. This may reflect poor inducibility of lipocortin, the likely in vivo phospholipase A2 inhibitor. Flower studied the distribution of lipocortin in the rat using a labelled antibody and a solid-phase assay system (Flower, 1988); lipocortin was widely distributed, and could be detected under resting conditions in every organ tested. However, stimulation with dexamethasone was able to increase the immunoreactivity of all organ extracts except some gastrointestinal tissues (stomach, ileum). Similar results were also obtained in guinea pigs. Although corresponding localisation experiments have not been performed with rabbit tissue, the inability of colonic tissue to produce lipocortin in response to glucocorticosteroid may underlie the present results.

An alternative explanation may rest in the known diverse nature of the phospholipase A2 enzymes (Peers & Flower, 1992). Different optimum pH levels and different preferred substrates have been characterised in the phospholipase A2 enzymes. Different inhibitory effects with respect to lipocortin may also exist. It is possible that the phospholipase A2 activity responsible for eicosanoid production in rabbit colon is insensitive to methylprednisolone inhibition.

In humans, various experiments to determine the effect of glucocorticosteroids on eicosanoid production have yielded conflicting results, although the majority of reports suggest that they have an inhibitory effect. The inhibitory effect of glucocorticosteroids is fairly reproducible in various in vitro systems using human tissue; however, under some experimental conditions exceptions have been found (Lauritsen et al., 1989). In vitro synthesis of LTB4 was evaluated in colorectal biopsy specimens and resection tissue from patients with inflammatory bowel disease by Hawthorne et al., 1992, who found that the formation of LTB4 from biopsy tissues stimulated with calcium ionophore A23187 correlated with the degree of mucosal inflammation. Biopsy specimens from patients taking prednisolone formed less LTB4 than those from patients not taking prednisolone, despite comparable levels of inflammation seen at sigmoidoscopy. Other studies in vivo have demonstrated that orally-administered prednisolone decreases concentrations of both PGE2 and LTB4 in rectal dialysates (Lauritsen et al., 1987).

In summary, the effects of the administration of a glucocorticosteroid to the new rabbit model of chronic colitis do not appear to be mediated by a reduction in the release of the arachidonic acid metabolites, a result contrary to generally accepted belief concerning the action of glucocorticosteroids.
7.5.6 Effect of glucocorticosteroids on eicosanoid release in other animal models

In a rabbit model of colitis (induced by injection of immune complexes followed by formalin enema), therapy with methylprednisolone was a poor inhibitor of leukotriene and prostaglandin release (Zipser et al., 1987). The 10mg/kg dose of methylprednisolone used in those experiments was much higher than the human equivalent dose of methylprednisolone (0.5mg/kg) that was used in the present study of the rabbit TNB model of chronic colitis. In the experiments of Zipser and co-workers, 10mg/kg significantly inhibited release of PGE$_2$, but not LTB$_4$. This inhibition may reflect a reduction in total protein synthesis rather than specific enzyme inhibition. Basal prostaglandin production *in vivo* has been reported to be unaffected by pharmacological doses of dexamethasone in rabbits (Naray-Fejes-Toth et al., 1983). In the TNB model of chronic colitis in the rat (Boughton-Smith et al., 1988a), anti-inflammatory drugs were administered 2 weeks after TNB, when there was substantial colonic damage, and continued for a week. 0.5mg/kg prednisolone had no significant effect on the generation of LTB$_4$, but did decrease 6-keto PGF$_{1\alpha}$ release by 56 per cent. In the same study, the experimental drug BW755C, which inhibits cyclooxygenase and 5-lipoxygenase, inhibited the increased formation of both 6-keto-PGF$_{1\alpha}$ and LTB$_4$ by the inflamed colon. The glucocorticosteroid did not have the same effect, which suggests that the indirect action of glucocorticosteroids is more complex.

7.5.7 Inhibition of cyclooxygenase

The increased PGE$_2$ release observed in the new model, which did not respond to glucocorticosteroid administration, might respond to the selective inhibition of cyclooxygenase. In the present experiments *ex vivo* inhibition of PGE$_2$ and 6-keto PGF$_{1\alpha}$ release was achieved by co-incubation with the non-steroidal anti-inflammatory cyclooxygenase inhibitor indomethacin. *In vivo*, in both the TNB-induced rat model (Boughton-Smith et al., 1988a) and the immune complex mediated rabbit model (Schumert et al., 1988), indomethacin has markedly inhibited prostanoid formation in both inflamed and control colon, but does not reduce leukotriene production or inflammation. In another study on the rat model of TNB-induced chronic colitis the parenteral administration of non-steroidal anti-inflammatory drugs resulted in significant exacerbation of experimental colitis in the rat (Wallace et al., 1992). It was clear from the results, however, that ‘shunting’ of arachidonate through the 5-lipoxygenase pathway was not a contributing factor, despite significant PGE$_2$ inhibition, indicating that prostaglandins also have a protective effect.

In the upper gastrointestinal tract the ulcerogenic properties of non-steroidal anti-inflammatory drugs correlate well with their ability to inhibit gastric prostaglandin
synthesis (Fries et al., 1989), but the mechanism through which these substances produce gastropathy remains poorly understood. Recently, the administration of indomethacin alone in relatively young rats was shown to cause ulcers predominantly in the small intestine and the caecum, which are frequently the sites involved in human Crohn's disease; accordingly, this has been proposed as a model for inflammatory bowel disease (Banerjee & Peters, 1990; Matsumoto et al., 1993).

The most likely explanation for the effect of the non-steroidal anti-inflammatory drugs is that endogenous prostaglandins play an important role in maintaining cellular viability as well as in modulating the acute inflammatory response to injury.

7.5.8 Inhibition of 5-lipoxygenase

Despite limited inhibition of leukotriene biosynthesis by the glucocorticosteroids and sulphasalazine, there has been much interest recently in the possible therapeutic use of a specific 5-lipoxygenase inhibitor in inflammatory bowel disease. Inhibition of the membrane translocation of 5-lipoxygenase by 3-[1-(p-chlorobenzyl)-5-(isopropyl)-3-tert-butylthioindol-2-yl]-2,2-dimethyl propanoic acid (MK886) gives potent and specific inhibition of leukotriene biosynthesis in vivo in intact cells (Rouzer et al., 1990). The rabbit model proposed in the present study exhibited increased LTB₄ production and may benefit from treatment with specific 5-lipoxygenase inhibitor. It has been shown in the present study that MK886 can reverse leukotriene synthesis ex vivo by reducing LTB₄ release to control levels. MK886 has previously been used in vivo to determine the role of leukotriene biosynthesis in the rat-TNB model of chronic colitis (Wallace & Keenan, 1990). Colonic LTB₄ synthesis was measured in vitro after incubation of tissue samples, and in vivo by equilibrium dialysis. Daily treatment with 10mg/kg MK886 demonstrated that this orally-active inhibitor of leukotriene biosynthesis can significantly accelerate healing in the rat TNB-induced model of colitis when the treatment is given during the early phase of the inflammatory response. Surprisingly, the inhibition of leukotriene biosynthesis during the first 24 hours after induction of colitis did not alter the extent of infiltration of neutrophils into the colon, despite the proposed chemoattractant properties of LTB₄ (Ford-Hutchinson et al., 1984). The same group found that daily intracolonic treatment with a direct 5-lipoxygenase inhibitor, L651-392, during the first 4 days after initiation of the colitis with TNB, also resulted in significant reductions of LTB₄ synthesis and colonic damage score (Wallace et al., 1989).

A study of the effects of a 5-lipoxygenase inhibitor, A-64077, in eleven patients with mild to moderate active ulcerative colitis revealed a decrease in patient discomfort, and an improvement (8/11) in the gross appearance at sigmoidoscopy (Collawn et al., 1992). In a separate study, the same drug significantly reduced LTB₄ release in a group of ten
patients (Laursen et al., 1990). It is clear that much larger studies are needed to fully assess the effect of 5-lipoxygenase inhibitors in human disease.

7.5.9 The effect of exogenous prostaglandins

The role of increased prostaglandin production, and the effects of exogenous prostaglandins on inflammation of colitis, have been investigated, since prostaglandins appear to be cytoprotective. Administration of intramuscular 16,16′-dimethyl PGE₂ to rabbits with formalin immune-complex colitis reduced LTB₄ production, infiltration of neutrophils, mucosal necrosis, inflammatory exudate, and oedema (Schumert et al., 1988). Using the TNB model of chronic colitis in the rat, another group studied the effect of treatment with 16,16′-dimethyl PGE₂ (Allgayer et al., 1989). Treated animals exhibited decreased inflammation (assessed by gross inspection and measurement of myeloperoxidase) and decreased LTB₄ synthesis. However, one trial in humans established that a synthetic analogue of PGE₂, when administered orally to patients with ulcerative colitis in remission, induced diarrhoea and flare-up (Goldin & Rachmilewitz, 1983). Administration of exogenous prostaglandin prophylactically to this model may be of therapeutic benefit.

7.5.10 Combination of 5-lipoxygenase inhibition with prostaglandins

One might suppose that the combined use of both MK886, to reduce inflammation by selectively inhibiting LTB₄ production, and exogenous PGE₂, to repress mucosal damage induced by TNB, would produce good results in the new rabbit colitis model.

Indomethacin treatment prior to induction of colitis in an acetic acid-induced rat model reduced endogenous mucosal PGE₂ levels, and exacerbated macroscopic ulceration and net fluid absorption. The addition of misoprostol, a PGE₁ analogue, to the indomethacin-exacerbated colitis completely healed colonic macroscopic ulceration and inflammation, but only partially improved fluid absorptive injury. Combining MK886 with misoprostol treatment not only improved macroscopic ulceration and inflammation, but also provided a synergistic effect that maintained net colonic fluid absorption at non-colitic control levels (Empey et al., 1992).

7.5.11 The response of similar models to other modes of therapy

Although in the present study no macroscopic improvement was observed following glucocorticosteroid administration, and although Rachmilewitz argues that it would be ‘unlikely that any drug would be able to prevent the development of the inflammatory response to this severe insult [the rat model of TNB induced chronic colitis], or even modify its propagation’ (Rachmilewitz et al., 1989), some workers have demonstrated
macrosopic improvements with drug therapy that is not aimed at reducing the release of arachidonic acid metabolites. Recently, the effect of epidermal growth factor on the rate of healing was examined in the TNB-rat model of chronic colitis. Systemic administration of epidermal growth factor can accelerate healing of colonic ulceration, and is effective in reducing inflammation in this model of colitis (Luck & Bass, 1993).

Investigation of the effects of a 'mast cell stabiliser' has shown that the damage induced in both TNB and acetic acid-induced colitis in the rat can be reduced by pre-treatment with ketotifen (Eliakim et al., 1992b). The same group also investigated the effect of octreotide, a synthetic analogue of somatostatin, on the modulation of the acetic acid-induced model in rats. Mucosal damage was significantly reduced by octreotide and was accompanied by a significant reduction in platelet activating factor activity, in leukotriene B4, and in vasoactive intestinal peptide concentrations (Eliakim et al., 1993).

7.5.12 Myeloperoxidase activity

Myeloperoxidase activity has been used extensively as a marker of the infiltration of granulocytes into intestinal tissues. For the TNB-initiated chronic colitis in rats, an increase in MPO-cellular infiltration was maintained for up to three weeks (Boughton-Smith et al., 1988b; Rachmilewitz et al., 1989).

However, the results obtained in the present study indicate that in the new TNB/ethanol rabbit model of chronic inflammation, myeloperoxidase activity was not a sensitive indicator of colonic inflammation. The focal nature of the disease induced probably accounts for the lack of sensitivity, because the quantity of tissue required for the assay would also have included a proportion of non-inflamed tissue. Mucosal scrapings rather than full thickness segments might have given more significant results. In addition, further assays could have reduced the large standard error.

Glucocorticosteroids reduced the mean MPO activity, albeit non-significantly. This probably reflected the reduction in neutrophil numbers within the mucosa. A more significant reduction might have been expected, in view of the large reduction in neutrophil infiltration observed in the model. However, neutrophils are not the only cells within the colon to contain MPO.

7.5.13 Matrix metalloproteinase staining

It has been reported that glucocorticosteroids decrease matrix metalloproteinase production by connective tissue cells in culture (Hunter et al., 1984). In the present study, localisation of the matrix metalloproteinases in diseased tissue from group III and group IV animals appeared to be unrelated to administration of 0.5mg/kg
methylprednisolone, despite a reduction in the infiltration of acute inflammatory cells. In addition, no differences in the spatial distribution of the matrix metalloproteinases were observed.

It has also been reported that glucocorticosteroids increase TIMP production by a variety of cells in culture. No evidence of such increased expression of TIMP was observed in the present study. Glucocorticosteroids have been shown to have little effect on matrix metalloproteinase and TIMP expression in human tissue from patients with Crohn's disease (Bailey, 1992), although this may be a consequence of glucocorticosteroid tolerance developed prior to an operation to remove part of the bowel.

7.5.14 Summary

The present study indicates that the mechanism of glucocorticosteroid action may not be as simple or as specific as is believed by many clinicians. Overall, methylprednisolone did not influence the erythrocyte sedimentation rate, the occult blood results, the macroscopic damage, or the arachidonic acid metabolism as expected, but did reduce neutrophil infiltration. Review of the literature suggests that species differences and a variety of different protocols for the induction of colitis may account for some of the disparities between results. However, the results suggest that the large polymorphonuclear neutrophil infiltration in the untreated model is not responsible for the damage observed in this model; indeed, the localisation of polymorphonuclear neutrophils under necrotic tissue may in reality indicate a positive defence response.
8 THE RESPONSE OF THE MODEL TO A SECOND CHALLENGE WITH TRINITROBENZENE SULPHONIC ACID

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THE RESPONSE OF THE MODEL TO A SECOND CHALLENGE WITH TRINITROBENZENE SULPHONIC ACID

8.1 Introduction

A characteristic of the idiopathic inflammatory bowel diseases is a pattern of relapse and remission. Indeed, it has been proposed that relapse is the only reasonably certain criterion of inflammatory bowel disease, since there is no other generally accepted definition (Schmacher et al., 1991). In ulcerative colitis, a pattern of intermittent attacks of the disease is reported in 80 to 85 per cent of patients. Continuous active disease is observed in only about 10 to 15 per cent of all cases, and this percentage decreases with time after onset, as colectomy will often be carried out in this group within the first few years. In a regional prospective study covering a group of 783 patients, a relapse-free course was found in only three per cent of patients after 10 years, and in less than one per cent after 18 years (Hendriksen et al., 1985). The study identified no significant predictors of the subsequent disease course, either by sex, age at onset of disease, initial disease extent and activity, or by any other characteristic. However, the frequency of attack seems to decline steadily with advancing age.

Crohn's disease is also a chronic recurring illness. Although approximately 45 per cent of patients are free of symptoms at any one time, nearly all patients have a recurrence within 10 years of their initial episode of disease. Approximately 30 per cent of patients require surgical intervention within the first year of diagnosis, and the remainder will require surgery at a rate of five per cent per year (Boer de Visser et al., 1990; Wright et al., 1987). Following surgery, recurrence is frequent, and is most common immediately proximal to the site of a previous anastomosis or stoma. The disease often recurs soon after surgery, even if manifestations are delayed.

To date, none of the previously described experimentally induced models of inflammatory bowel disease displays the pattern of relapse and remission observed in human inflammatory bowel disease (see chapter 2). The ability to mimic the human disease by inducing relapse in an experimental model would be a great advantage.

The inflammation and injury induced in experimental animals by TNB has been attributed both to its direct cytotoxic properties and to an as-yet-undefined immune-mediated mechanism. Morris and his colleagues describe some preliminary results concerning relapse in the TNB-induced chronic rat model of colitis in their paper.
characterising the model (Morris et al., 1989). They suggest that the animals become sensitised to the damaging effects of TNB following the initial exposure, and that subsequent re-exposure to the hapten alone several weeks after the initial exposure can produce relapse. Beck and co-workers have also reported that an immune component of the TNB model can be demonstrated by tolerising the animals to the hapten and then producing a reaction of reduced severity upon subsequent exposure (Beck et al., 1988). These data suggest that a cell-mediated tissue injury which may be modulated experimentally is involved in the TNB-induced colitis.

Administration of 40mg TNB in 25 per cent ethanol to rabbits as a single intracolonic dose produces inflammation and ulceration that persists for up to six weeks. Examination of the histological changes associated with the model shows that it has many features in common with human idiopathic inflammatory bowel disease. However, despite the chronicity of the model the lesions eventually heal.
8.2 **Aim of the study**

The aim of this part of the study was to simulate relapse by the re-administration of TNB at six weeks, and to determine whether the macroscopic and microscopic appearance would be different following re-exposure, thus investigating the claim that a hypersensitivity to the hapten may have been induced.

8.3 **Materials and methods**

8.3.1 **Animals**

Sixteen female New Zealand white rabbits (section 5.3.2) were randomised into the following treatment groups:

**Relapse group A**

Six rabbits were anaesthetised (section 5.3.3) and colitis was induced by the introduction, through the feeding tube, of 40mg TNB, dissolved in 0.6cm³ of a permeabilising solution of 25 per cent ethanol in 0.9 per cent saline, which was established in earlier experiments as the optimum dose. The tube was then flushed with 7cm³ of air.

Six weeks later the initial induction procedure was repeated, and a further 40mg of TNB in 25 per cent ethanol was introduced to the colon.

**Relapse group B**

Four rabbits were initially treated in an identical manner to those in relapse group A; however, at six weeks when the induction procedure was repeated only 10mg of TNB in 25 per cent ethanol was introduced to the colon. 10mg had previously been shown to induce only minor transient effects.

**'Colitis at six weeks' control group C**

Six animals were anaesthetised and 0.6cm³ of 0.9 per cent saline was introduced to the colon. Six weeks later, the rabbits were anaesthetised again and colitis was induced with 40mg TNB in 25 per cent ethanol.

Two other controls were also included. One rabbit was anaesthetised and 0.6cm³ of 0.9 per cent saline was introduced to the colon. At 8 weeks the rabbit was killed to examine the comparative histology in a rabbit kept in the same environment as the other rabbits for the same length of time. Another animal was treated in an identical manner to the
relapse group A animals, except that it was killed at 8 weeks \textit{without} a second dose of TNB. This animal was included to determine whether any histological features would still be present after the initial dose of TNB. Six weeks was chosen as the time to re-dose because only very few animals still displayed macroscopically visible lesions in the colon, and histologically the acute inflammatory cell infiltrate had diminished.

8.3.2. Erythrocyte sedimentation rate

2cm$^3$ of blood was collected from each animal at the start of the experiment, before the animals received their respective enemas, and the erythrocyte sedimentation rate was determined (see chapter 7). Subsequently, at 2 weeks, 6 weeks and 8 weeks, the measurements were repeated; 2cm$^3$ of blood was collected and the erythrocyte sedimentation rate was again determined.

8.3.3 Macroscopic scores

All the animals were killed at eight weeks. In each instance the colon was quickly removed, opened, and cleaned with saline. A macroscopic score was assigned to each colon (chapter 5.3.13) and tissue was then selected for histology.

8.3.2 Histology

Segments of colon were selected at autopsy both from obviously inflamed and ulcerated regions and from normal colon, as described in section 5.3.10; the segments were processed for haematoxylin and eosin staining (appendices A&B). The presence or absence of each of a series of histopathological features as shown in table 5.4 was recorded and the groups were compared.
8.4 Results

8.4.1 Erythrocyte sedimentation rate in relapse model

Erythrocyte sedimentation rate measurements made at six and eight weeks, on blood from animals of relapse groups A and B and the colitis group C, were compared using repeated measures analysis of variance.

The two principal questions addressed were whether the erythrocyte sedimentation rate changed between the sixth and eighth weeks, and whether the pattern of change over this period differed according to the different treatments. Firstly, the erythrocyte sedimentation rate was significantly (P=0.037) increased over the intervening two-week period in those animals that received 40mg TNB at 6 weeks (relapse group A and the colitis group C) (see figure 8.1a). Secondly, factor analysis of the results, splitting by relapse group A and the colitis group C, revealed that there was no interaction between the erythrocyte sedimentation rate and the group (P=0.349). There was no significant change in the erythrocyte sedimentation rate (P=0.79) for the relapse group B animals over the two-week period following the administration of 10mg TNB at 6 weeks. These results reveal that the erythrocyte sedimentation rate, following initiation of colitis at six weeks, was not affected by previous exposure to 40mg TNB at the start of the experiments.

When the erythrocyte sedimentation rate was compared to the macroscopic scores assigned at endoscopy at seven weeks and on killing the animals at eight weeks, there was a significant correlation (from Spearman's rho, P=0.003) between the erythrocyte sedimentation rate and the macroscopic score as an indication of the severity of the disease (figure 8.1b).
Figure 8.1 a and b: Erythrocyte sedimentation rate results from the relapse experiments. a: Interaction line plot showing the mean erythrocyte sedimentation rate for groups A, B and C at six weeks and at eight weeks immediately prior to killing the animals. There was a significant increase in erythrocyte sedimentation rate in groups A and C (P=0.037). However, previous administration of 40mg TNB at t0 did not effect the erythrocyte sedimentation rate. b: Scattergram showing the relationship of erythrocyte sedimentation rate with the macroscopic score assigned. A significant Spearman rank correlation coefficient was obtained (P=0.003).

8.4.2 Macroscopic scores

A Fisher’s exact test, covering relapse group A and colitis group C was performed to compare those animals that had visible macroscopic ulceration at 8 weeks (score 2 or 3) with those that exhibited no ulceration (scores 0 or 1). Previous administration of 40mg TNB in 25 per cent ethanol did not produce a significant change (P=0.356) in the outcome of the macroscopic scores for animals given 40mg TNB in 25 per cent ethanol at 6 weeks (table 8.1).
In case the effect of prior dosing in group A was masked by administration of 40mg TNB, which would in any case be expected to produce severe inflammation and ulceration, the four animals in group B received 10mg in 25 per cent ethanol at six weeks. Previous experiments had shown that little visible damage was present after 2 weeks in the colon of animals that had received 10mg TNB in 25 per cent ethanol with no prior dose. One animal killed at eight weeks, in the present study, exhibited no damage; three of the four other colons were still inflamed.

No damage was observed in either the saline control animal or the rabbit that was killed at eight weeks after a single dose of 40mg TNB/ethanol.

<table>
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<th>Score</th>
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Table 8.1: Macroscopic scores at 8 weeks. Table displaying the frequencies of the macroscopic scores of the damage in the distal colon of rabbits killed at eight weeks in treatment groups A, B and C.

8.4.3 Comparison of the histology

In the colitis animal killed at 8 weeks without a second dose of TNB there was slightly more branching and bifurcation, an indicator of previous injury, than was present in the mucosa from the saline control animal also killed at 8 weeks. However, no other abnormalities were observed.

Microscopically the degree of damage was identical in both relapse group A and colitis group C animals. The damage was characterised by the presence of crypt abscesses, a mixed inflammatory cell infiltrate in both the mucosa and submucosa, and ulceration of the mucosa. There appeared qualitatively to be more fibrosis in the colitis group C animals than in the relapse group A animals. The colons of both groups exhibited oedema and regenerative hyperplasia, which was more common in the group A animals (4/6) than in the group C 'colitis at six weeks' animals (2/6).

The animals in group B (which received 10mg of TNB at six weeks) displayed a small increase, compared to the saline control animal, in the number of acute and chronic
inflammatory cells in the lamina propria. However, there were no areas of ulceration on any of the sections examined.

8.4.4 Summary of results

In summary, both the macroscopic and the microscopic results indicate that there is no morphological difference in pathology between the relapse model and the single dose model of chronic colitis.
8.5 Discussion

8.5.1 Dosage

The objective of the experiments presented in this chapter was to investigate the effect of re-administering the hapten trinitrobenzene sulphonic acid six weeks after the initial exposure. Two alternative doses of TNB were chosen for re-initiation: (1) a repeat dose of 40mg TNB in 25 per cent ethanol, or (2) a dose of 10mg TNB in 25 per cent ethanol (which had previously been shown not to induce ulceration at 2 weeks in the original study characterising this model). The optimum dose was used again to determine whether the rabbits were still able to mount at least the same response to TNB, whereas the lower dose was chosen to show clearly whether an increase in the reactivity to TNB had developed.

Those animals that received the lower, 10mg, dose of TNB at six weeks displayed slightly more acute inflammatory cells in the mucosa than would normally be expected. However, the presence of inflammatory cells in the lamina propria is not unusual, and the normal gut is often described as being in a state of physiological inflammation. Thus, the slight increase in inflammatory cells observed may not be significant. Cell counting was not considered feasible owing to time constraints.

8.5.2 Erythrocyte sedimentation rate

The results of the erythrocyte sedimentation rate experiments revealed a significant increase in both groups that received 40mg of TNB at six weeks. The erythrocyte sedimentation rates were also found to be significantly correlated with the independently-assigned macroscopic scores. The inflammation observed in both groups as assessed by erythrocyte sedimentation rate was, therefore, unaffected by previous exposure of the animals to TNB. However, the erythrocyte sedimentation rate measurement is neither specific nor highly sensitive. Subtle differences between the groups would not be expected to be identified by the erythrocyte sedimentation rate technique in samples of this small size.

8.5.3 Pathology

No significant difference in the macroscopic scores was observed between the animals that received 40mg of TNB at six weeks only and those that received 40mg of TNB at six weeks after being previously exposed to TNB at the start of the experiment. In addition, no difference was found when the histopathologies of these two groups were compared, except for a small increase in the amount of fibrosis in those animals that had
received a second dose. Fibrosis and architectural abnormalities were still typical of the histopathology of the animals killed at six weeks. Complete resolution of these features might be expected to take much longer.

8.5.4 Mechanisms

Some of the mechanisms by which TNB is thought to induce colitis have already been discussed in chapter 5. The most popularly held belief is that TNB induces a cell-mediated immunity that recognises and degrades TNB modified (altered-self) cells. A typical feature in cell-mediated immune reactions is the formation of granulomas when foreign antigenic material cannot be degraded. The presence of granulomas is a feature of the single-dose model of chronic inflammatory bowel disease in the rabbit. When a hapten induces a cell-mediated immunity there is a chance that the host will become sensitised to it. Dinitrochlorobenzene has been used to induce chronic colitis in guinea pigs and rabbits using a technique to induce hypersensitivity (Bicks & Rosenberg, 1964; Rabin & Rogers, 1978). Morris and his colleagues suggest that rats given TNB in ethanol intracolonically become hypersensitive to the hapten (Morris et al., 1989). The results of the present study strongly reject the hypothesis that the soluble hapten TNB may sensitisze New Zealand white rabbits to subsequent exposure to the TNB at six weeks. Type IV contact hypersensitivity was not observed in the colon of any animal that received a second dose of TNB at six weeks; there was no difference between the damaged observed in the re-exposure groups and the damage observed on first exposure in the control group C.

8.5.5 Limitations

In the light of the results obtained, the methodology may be criticised for examining the effect of re-exposure to TNB at only one time point (two weeks after re-exposure at six weeks). Further experiments might have revealed differences between the groups at earlier or later time points. However, the small amount of previous data available suggested that macroscopic differences between the groups could be expected at two weeks. Beck found that tolerised animals had significantly lower lesion scores at two weeks (Beck et al., 1988). Contact hypersensitivity reactions are maximal between 48 and 72 hours after exposure. However, at these early times the amount of damage in this model is generally severe, and the superficial necrosis that is often observed would obscure results. Preliminary experiments performed in the single-dose model of colitis clearly showed that the damage produced at two weeks was consistent, and the effects of the ethanol vehicle had been completely resolved. If there had been any difference in the severity of the inflammation, it would have been expected to still be manifest at two weeks. However, further time points need to be investigated. Species differences may
account for the disparities between the results obtained in these experiments and those described by Morris and co-workers (1989). It has been shown previously that even the strain of an animal species may be important in producing TNB-induced chronic colonic inflammation (Beagly et al., 1991). Different batches of TNB are also known to give variable results.

8.5.6 Summary

In conclusion, these results do not support the claim that a single colonic administration of TNB sensitises animals to subsequent exposure, indicating that a type IV hypersensitivity is not the immunological mechanism underlying the action of TNB. Further experiments might be performed to determine if the lesions are more persistent in the relapse model. However, the similarity of the responses at two weeks in the single-dose model and at eight weeks following re-exposure suggest that this model might be useful for investigating potential therapeutic agents where each animal could act as its own control, thereby reducing some of the inherent biological variation between animals.
OVERVIEW AND FUTURE DIRECTIONS

Crohn's disease and ulcerative colitis are chronic inflammatory and ulcerative diseases, which are characterised by periods of relapse followed by remission. Their aetiology is unknown; it seems likely that the cause is multifactorial, comprising a genetic element and possibly environmental factors (see Chapter 2). Since the cause of the inflammatory bowel disease is unknown, the treatment is non-specific, and glucocorticosteroids are employed as the mainstay of therapy, especially for Crohn's disease. However, the currently available therapies are of limited use, and are associated with a high incidence of side effects.

The identification of an ideal model for Crohn's disease and ulcerative colitis as defined by Kim and Berstad (1992) requires the cause to be known, and is, therefore, impossible to achieve at present. However, with this limitation in mind, animal models of chronic gastrointestinal inflammation and ulceration are necessary for investigation of inflammatory processes in the gut, which may differ from those in other tissues. In addition, models of inflammatory bowel disease are required to investigate modulation of the system that could assist in improving therapies.

As discussed in Chapter 5, a variety of approaches has been employed to initiate inflammatory bowel diseases in several species of animals. All the models described so far have their limitations, such as a short-lived duration or a complex initiation regime or limited pathological similarities to Crohn's disease and ulcerative colitis. Of the models available, the trinitrobenzene sulphonic acid and ethanol-induced model in the rat is the most satisfactory (Morris et al., 1989). This model is easy to induce, is relatively persistent, and has several of the features characteristic of Crohn's disease or ulcerative colitis. However, there are several reasons for having a rabbit model as well as a rat model. For example, the rabbit's colon, although anatomically different from human colon, has greater physiological and pharmacological similarities to human colon than does that of the rat; and the rabbit's larger size allows endoscopy and antisera to some potentially interesting antigens to be used (e.g., antisera to the matrix metalloproteinases and anti-CD18 antiserum are currently available only for the rabbit).

In this thesis, a rabbit trinitrobenzene sulphonic acid based model for inflammatory bowel disease was characterised (Chapter 5). An optimum dose of 40mg
trinitrobenzene sulphonic acid in a 25 per cent ethanol solution was established which consistently produced discontinuous lesions with a 'cobblestone' appearance, strictures, and bowel wall thickening in all rabbits. Other histological features frequently observed were crypt architectural distortion, ulceration, crypt abscesses, fissuring ulceration, pseudopolyps and granulomas. All of these features are seen in ulcerative colitis and/or Crohn's disease. There was a bias to more Crohn's disease like features, but the changes produced could not be strictly classified as pertaining to one or the other. The histological abnormalities were found only in the distal colon extending from approximately 3cm to 20cm proximally from the anus, and persisted for the duration of the study (six weeks). No histological abnormalities were observed in the colons of control animals or in the ileum, liver, lung, or kidney of any of the animals. The rabbit model fulfilled the aims set out at the beginning of the study. The simplicity of induction of this model, its persistence, and the histopathological similarity are all features that make it attractive for study of both the pathophysiology and the treatment of intestinal inflammation. This study has also demonstrated that endoscopy may be used to evaluate the effects of possible new therapies. Until the aetiologies of Crohn's disease and ulcerative colitis are better understood there will be no perfect model for inflammatory bowel disease. However, this trinitrobenzene sulphonic acid-induced model in the rabbit is a practical study tool for assessing hypotheses of the pathogenesis of inflammation in the colon.

Once the model had been characterised, it was used for other studies (see Chapters 6, 7 and 8).

The matrix metalloproteinases were observed only in areas of damage, and never in microscopically normal tissue. The limited distribution suggests that these proteinases play an important role in the connective tissue breakdown and remodelling which occurs in colitis. The rapid onset of the matrix metalloproteinases in the absence of their inhibitor implies that matrix metalloproteinase-mediated tissue degradation is taking place. However, the antisera used detect the pro-enzyme, the active form and the enzyme-TIMP complex, so that it is not known whether the extracellular enzyme seen is active or not. At six hours post-induction, extensive collagenase is observed on the matrix with no TIMP; breakdown of the matrix must be occurring at this time, because only active collagenase binds to the matrix. Therefore, in the acute phases of the disease an inhibitor of matrix metalloproteinases may be of therapeutic benefit. The natural inhibitors of the metalloproteinases have already been shown to be powerful anti-invasive agents when added to various model systems, both in vitro and in vivo (Schultz et al., 1988).
Several synthetic inhibitors have been described, based on the chelation of the active-site zinc atom of the metalloproteinase enzymes by moieties carried on peptide mimetics that reflect the substrate specificities of the enzymes (Lelievre et al., 1990). Other low-molecular-weight inhibitors have been described that are efficacious in vitro, but fail to have in vivo effects. Recently, a novel generalised inhibitor of the matrix metalloproteinases, BB-94, has been described which appears to improve the condition of nude mice with human ovarian carcinoma xenografts (Davies et al., 1993). Manipulation of the activity of the matrix metalloproteinases would determine whether these enzymes have a central role in the development of ulceration and the remodelling required, and thus whether BB-94 might be of therapeutic benefit in this model.

When considering the possible benefits of matrix metalloproteinase inhibition in inflammatory bowel disease, the potential side effects of such a therapy must also be taken into account. Remodelling of the collagenous extracellular matrices of tissues is a normal homeostatic activity and the continuous administration of matrix metalloproteinase inhibitors may induce severe side effects. The distribution of the matrix metalloproteinases from one week onwards, restricted to the boundary of the ulcer and normal tissue, suggests a remodelling process in which damaged bowel is removed and replaced by new tissue. The matrix metalloproteinases are required for the remodelling process and interference with this process may impair healing. Thus, in inflammatory bowel disease the use of an inhibitor for a short period, or the use of a specific collagenase inhibitor, which would allow gelatinase and stromelysin to continue to act, might be necessary.

Identification of the cell type secreting the matrix metalloproteinases would be of value, since this may provide a further therapeutic target. Experiments in which the antisera to both the matrix metalloproteinases and to specific cell types are used simultaneously would allow identification of the cells secreting metalloproteinases. The antisera to the metalloproteinases are polyclonals raised in sheep, and so any antibodies used to identify the cell types involved need to be raised in other species. These antibodies may then be immunolocalised with different fluorochromes (e.g. tetra methyl rhodamine isothiocyanate).

One of the modes of actions of the glucocorticosteroids is thought to be inhibition of the matrix metalloproteinase activity both in vivo and in vitro (McGuire et al., 1983; Pelletier et al., 1987). However, glucocorticosteroids also have a large number of other effects in vivo, and it is likely that matrix metalloproteinase inhibition is a secondary effect. In the investigations reported in Chapter 7 no change in the pattern of matrix
metalloproteinase or TIMP expression was observed following a week of methylprednisolone administration.

The effect of intramuscular methylprednisolone on a number of parameters was investigated in the rabbit model; the only difference observed was a decrease in the infiltration of neutrophils, but this did not appear to lessen the damage. Surprisingly, the levels of the inflammatory mediators PGE$_2$ and LTB$_4$ were unaffected by the administration of methylprednisolone. The dose used was equivalent to a high human dose (~70mg) and the reduction in neutrophil infiltration suggests that this was a therapeutic dose. Review of the literature indicates that species differences and different protocols for the induction of colitis may account for some of the disparities between results. The use of a different glucocorticosteroid, administration by another route, and study of other time-points after induction might all produce different findings. However, the results suggest that the large polymorphonuclear neutrophil infiltration in the untreated model is not responsible for the damage observed in this model, and that their localisation under necrotic tissue may, in reality, indicate a positive defence response. The importance of neutrophils in the initiation and development of ulceration could be determined by preventing their infiltration using an anti-CD18 antibody, which inhibits neutrophil adhesion to endothelial cells, and determining the effect on tissue damage, inflammatory mediators and matrix metalloproteinase production.

As explained above, there is evidence suggesting that neutrophils are not responsible for the colonic damage induced by trinitrobenzene sulphonic acid; a hypersensitivity reaction also seems unlikely, as the tissue changes produced on re-challenge with trinitrobenzene sulphonic acid were no different from those occurring with the first dose. Considering the results obtained, the methodology might be criticised for examining the effect of re-exposure to trinitrobenzene sulphonic acid only at one time point (two weeks following re-initiation). Further experiments may reveal difference between the groups at earlier or later time points. Species differences may account for the disparities between the results obtained in these experiments and the results implied by Wallace (1989).

The results do not support the claim that a single colonic administration of trinitrobenzene sulphonic acid sensitises animals to subsequent exposure. Further experiments need to be performed to determine whether the lesions are more persistent in the relapse model. Despite these negative results, the similarities of the responses at two weeks and at eight weeks following re-exposure suggest that this model might be useful for investigating potential therapeutic agents where each animal
could act as its own control. Paired observations could reduce some biological variation observed when groups are compared.

In conclusion, the studies presented in this thesis describe an animal model that has sufficient similarity to the human inflammatory bowel diseases to be useful for research on their pathophysiology and their treatment. The model has allowed the pattern of matrix metalloproteinase expression with time to be established in an ulcerative disease of the colon, which is not feasible with human tissue. In addition, it has been demonstrated that neutrophils are not of primary importance in this type of model, and that animals exposed to trinitrobenzene sulphonic acid do not appear to become sensitised to it.
### Appendix A

**Procedure for the manual embedding of tissue in paraffin wax**

<table>
<thead>
<tr>
<th>Step</th>
<th>Fluid</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Neutral buffered formaldehyde^a</td>
<td>overnight</td>
</tr>
<tr>
<td>2.</td>
<td>70% ethanol</td>
<td>2 hours</td>
</tr>
<tr>
<td>3.</td>
<td>90% ethanol</td>
<td>2 hours</td>
</tr>
<tr>
<td>4.</td>
<td>Absolute ethanol</td>
<td>1.5 hours</td>
</tr>
<tr>
<td>5.</td>
<td>Absolute ethanol</td>
<td>1.5 hours</td>
</tr>
<tr>
<td>6.</td>
<td>Absolute ethanol</td>
<td>1.5 hours</td>
</tr>
<tr>
<td>7.</td>
<td>Absolute ethanol</td>
<td>overnight</td>
</tr>
<tr>
<td>8.</td>
<td>CNP30^b</td>
<td>1.5 hours</td>
</tr>
<tr>
<td>9.</td>
<td>CNP30^b</td>
<td>2 hours</td>
</tr>
<tr>
<td>10.</td>
<td>Paramat paraffin wax</td>
<td>2.5 hours</td>
</tr>
<tr>
<td>11.</td>
<td>Paramat paraffin wax</td>
<td>overnight</td>
</tr>
</tbody>
</table>

a) Neutral buffered formaldehyde pH 7.0 (also known as 10% buffered formalin)

- 32.5g Disodium hydrogen phosphate anhydrous ($\text{Na}_2\text{HPO}_4$)
- 26.0g Sodium dihydrogen phosphate dihydrate ($\text{NaH}_2\text{PO}_4\cdot2\text{H}_2\text{O}$)
- 500ml Formalin (40% w/v formaldehyde)
- 4500ml Distilled water

b) CNP30

CNP30 is a trade name for a solution that contains a stabilised mixture of 1,1,1 trichloroethane and tetrachloroethane, supplied by Merck.
Appendix B

Procedure for haematoxylin and eosin staining

Before beginning procedure place slides in 60°C oven for 10 minutes.

<table>
<thead>
<tr>
<th>Step</th>
<th>Fluid</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dewax sections in CNP30</td>
<td>1 minute</td>
</tr>
<tr>
<td>2</td>
<td>Dewax sections in CNP30</td>
<td>1 minute</td>
</tr>
<tr>
<td>3</td>
<td>Absolute ethanol</td>
<td>1 minute</td>
</tr>
<tr>
<td>4</td>
<td>Absolute ethanol</td>
<td>1 minute</td>
</tr>
<tr>
<td>5</td>
<td>70% ethanol</td>
<td>1 minute</td>
</tr>
<tr>
<td>6</td>
<td>Tap water</td>
<td>1 minute</td>
</tr>
<tr>
<td>7</td>
<td>Stain in Harris's haematoxylin</td>
<td>5 minutes</td>
</tr>
<tr>
<td>8</td>
<td>Wash well in running tap water</td>
<td>30 seconds</td>
</tr>
<tr>
<td>9</td>
<td>Differentiate in 1% acid alcohol</td>
<td>-6 seconds</td>
</tr>
<tr>
<td>10</td>
<td>Wash well in running tap water</td>
<td>30 seconds</td>
</tr>
<tr>
<td>11</td>
<td>'Blue' sections in Scott's tap water</td>
<td>-2 minutes</td>
</tr>
<tr>
<td>12</td>
<td>Wash briefly</td>
<td>30 seconds</td>
</tr>
<tr>
<td>13</td>
<td>1% eosin yellowish</td>
<td>5 minutes</td>
</tr>
<tr>
<td>14</td>
<td>Wash in running tap water</td>
<td>3 minutes</td>
</tr>
<tr>
<td>15</td>
<td>70% ethanol</td>
<td>15 seconds</td>
</tr>
<tr>
<td>16</td>
<td>Absolute ethanol</td>
<td>30 seconds</td>
</tr>
<tr>
<td>17</td>
<td>Absolute ethanol</td>
<td>30 seconds</td>
</tr>
<tr>
<td>18</td>
<td>CNP30</td>
<td>1 minute</td>
</tr>
<tr>
<td>19</td>
<td>CNP30</td>
<td>1 minute</td>
</tr>
<tr>
<td>20</td>
<td>Mount slides using DPX straight from CNP30</td>
<td></td>
</tr>
</tbody>
</table>
Appendix C

Tissue culture

Hank’s Balanced Salt Solution with N-2-hydroxyethylpiperazine N´-2-ethanesulphonic acid (HEPES) and antibiotics.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>grams/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8.0</td>
</tr>
<tr>
<td>KCl</td>
<td>0.4</td>
</tr>
<tr>
<td>CaCl$_2$.2H$_2$O</td>
<td>0.185</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>0.1</td>
</tr>
<tr>
<td>MgCl$_6$.H$_2$O</td>
<td>0.1</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$ anhydrous</td>
<td>0.048</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.06</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>0.35</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.0</td>
</tr>
<tr>
<td>HEPES</td>
<td>4.766</td>
</tr>
<tr>
<td>penicillin 1x</td>
<td>0.06</td>
</tr>
<tr>
<td>penicillin 10x</td>
<td>0.6</td>
</tr>
<tr>
<td>streptomycin 1x</td>
<td>0.1</td>
</tr>
<tr>
<td>streptomycin 10x</td>
<td>1.0</td>
</tr>
<tr>
<td>amphotericin 1x</td>
<td>0.0025</td>
</tr>
<tr>
<td>amphotericin 10x</td>
<td>0.025</td>
</tr>
</tbody>
</table>

Additives to medium used for tissue culture

The following supplements to Dulbecco’s Modification of Eagle’s Medium (DMEM) containing HEPES (ICN Flow, High Wycombe, UK) were used:

- L-glutamine (ICN Flow, High Wycombe, UK) 292.0mg/l
- Sodium bicarbonate (ICN Flow, High Wycombe, UK) 500.0mg/l
- Benzyl penicillin (ICN Flow, High Wycombe, UK) 60.0mg/l
- Streptomyccin (ICN Flow, High Wycombe, UK) 100.0mg/l
- Amphotericin B (ICN Flow, High Wycombe, UK) 2.5mg/l
- Monensin (Sigma chemicals, Poole, Dorset, UK) 5.0μM
All supplements were added to the medium through millipore filters to ensure sterility. Penicillin, streptomycin and amphotericin B were added to the medium to prevent the growth of any remaining contaminant micro-organisms.
### Appendix D

**Immunohistochemistry**

**Phosphate buffered saline (PBS) (5x concentration)**

<table>
<thead>
<tr>
<th>Salt</th>
<th>grams/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>42.5</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$.2H$_2$O</td>
<td>16.08</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$.2H$_2$O</td>
<td>0.78</td>
</tr>
</tbody>
</table>

Dilute to 1x when required and adjust pH to 7.35.

**Gelatin**

7% in normal saline (0.9%) with 0.02% azide. For 100ml, 0.9g NaCl, 7g gelatin, 0.02g NaN$_3$ were dissolved in distilled water at 60°C.

**Paraformaldehyde**

4g of paraformaldehyde were added to 50ml PBS + 4 to 5 drops 5M NaOH. Heated until dissolved. Cooled and neutralized with acetic acid to pH 7.4. The volume was adjusted to 100ml with PBS.

**4-chloro-1-naphthol (Sigma, Poole, Dorset)**

162mg was dissolved in 54ml methanol of AnalAr quality. The chloronaphthol had to be completely dissolved before adding to 270ml PBS. Immediately before use 90μl H$_2$O$_2$ was added. The solution was prepared fresh each day.
## Appendix E

**Composition of the Krebs**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>grams/litre</th>
<th>mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>6.92</td>
<td>118.4</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>2.10</td>
<td>25.0</td>
</tr>
<tr>
<td>KCl</td>
<td>0.35</td>
<td>4.7</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.15</td>
<td>0.6</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.16</td>
<td>1.2</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>2.00</td>
<td>11.1</td>
</tr>
<tr>
<td>CaCl₂ (1M solution)</td>
<td>(1.3ml/l)</td>
<td>1.3</td>
</tr>
</tbody>
</table>
Appendix F

ELISA Buffers

Buffers were prepared using deionized water free of trace organic contaminants.

A. **EIA Buffer**
   
   1.0 M potassium phosphate buffer, pH 7.4 (see below) 100ml  
   Sodium azide 100mg  
   Sodium chloride 23.4g  
   Ethylenediaminetetraacetic acid (tetrasodium) (EDTA) 370mg  
   Bovine serum albumin 1.0g  
   
   Diluted with deionized water to 1.0 litre.

For the 1.0 M potassium phosphate buffer, pH 7.4:

A 1.0 M solution of dipotassium hydrogen phosphate (K$_2$HPO$_4$) and a 1.0 M solution of potassium dihydrogen phosphate (KH$_2$PO$_4$) were prepared. While stirring constantly the KH$_2$PO$_4$ solution was added to the K$_2$HPO$_4$ solution until a pH of 7.4 was obtained.

B. **Saturation Buffer**

An additional 200 mg of sodium azide and an additional 2 g of bovine serum albumin was added to 1.0 litre of EIA buffer.

C. **Wash Buffer**

1.0 M potassium phosphate buffer, pH 7.4 10 ml  
Tween 20 0.5 ml  

Diluted with deionized water to 1.0 litre.

(All buffers were stored at 4 °C.)
Appendix G

Radioimmunoassay solutions

The radioimmunoassay buffer:

- 0.1M Phosphate buffer at pH 7.4
- 0.15M NaCl
- 0.1% NaN3
- 0.1% Bovine serum albumin

Dextran-coated charcoal

1.0g of Norit A activated charcoal was added to 0.1g Dextran T.70 and dissolved in 100ml of RIA buffer.
Appendix H

Myeloperoxidase extraction and assay solutions

Preparation of 0.05M sodium phosphate buffer, pH6.0.

- 61.5ml 0.1M Na₂HPO₄
- 438.5ml 0.1M NaH₂PO₄

Made up to 1.0 litre with distilled water and adjusted to pH6.0 and stored at 4°C.


References


References


References


References


Cambray, G. J., Murphy, G., Page, T., & Reynolds, J. J. (1981a). The production in culture of metalloproteinases and an inhibitor by joint tissues from normal rabbits, and from rabbits with a model arthritis. *Rheumatology International*, 1, 11-16.


References


References


References


References


Hall, R., & Malia, R. G. (1990). Medical Laboratory Haematology (Second edn.).


References


References


Hunter, J. (1794). A treatise on the blood, inflammation, and gunshot wounds (pp. 221-277). London: printed by J. Richardson, for G. Nicol.


References


References


References


References


References


References


Rampart, M., & Williams, T. J. (1986). Polymorphonuclear leukocyte-dependent plasma leakage in the rabbit skin is enhanced or inhibited by prostacyclin, depending on the route of administration. *American Journal of Pathology, 124*, 66-73.


References


References


References


References


References


References


