REACTIVE OXYGEN SPECIES AND THE ROLE OF ANTIOXIDANT THERAPY IN INFLAMMATORY BOWEL DISEASE

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ABSTRACT

A proven role for antioxidant therapy in inflammatory bowel disease (IBD) would help establish the pathogenic importance of reactive oxygen species (ROS) and may provide new therapies with low toxicity. The major findings of the work for this thesis were as follows:

• ROS production, as measured by the chemiluminescence response, of colonic biopsies from acetic acid-induced colitis in rats correlated with the macroscopic and microscopic scores of inflammation.

• A method for assessing the antioxidant actions of novel IBD therapy was established by demonstrating that conventional antioxidants, and standard therapies for IBD, alter the chemiluminescence responses of acetic acid-induced colitis biopsies similarly to ulcerative colitis (UC) biopsies (previously published data). The novel compounds, amflutizole and LY231617 were potent antioxidants.

• The chemiluminescence response of UC biopsies correlated with clinical disease activity and sigmoidoscopic scores, and with mucosal neutrophil infiltration.

• Recombinant human recombinant manganese superoxide dismutase (rh-MnSOD) was not an effective antioxidant using acetic acid-induced colitis biopsies. Pretreatment of rats with acetic acid-induced colitis with intraluminal rh-MnSOD did not alter the macroscopic or microscopic scores of inflammation nor chemiluminescence: Rh-MnSOD is probably not, therefore, a suitable agent for topical therapy in IBD.

• The iron chelators, desferrioxamine and 1,10-phenanthroline, reduced ROS production by inflamed biopsies from UC.

• A pilot trial of antioxidant nutrients, selenium, β-carotene, ascorbate, α-tocopherol and methionine, in active UC demonstrated no adverse events, remission in 4/10 patients, and significant improvements in stool frequency, rectal bleeding and sigmoidoscopic score, but not rectal mucosal histology, ROS production, or plasma thiobarbituric acid reactive substances.

This work has shown that the acetic acid-induced colitis model in rats can be used for screening for novel antioxidant therapy, confirms that ROS are likely to be pathogenic in UC, and suggests that antioxidant therapy, using amflutizole, LY231617, iron chelators or antioxidant nutrients, merits controlled therapeutic assessment in IBD.
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α-Tocopherol
Ascorbate
Selenium
Methionine
Advantages of combination therapy
Potential risks with selenium-βCE/methionine therapy

Hypothesis
ABBREVIATIONS

- 1,1,3,3-Tetramethoxypropane (TMP)
- 1-Chloro-2,4-dinitrobenzene (DNCB)
- 2,2'-azo-bisaminopropane (AAPP)
- 2,4-Dinitrochlorobenzene (DNCB)
- 5-Aminosalicylic acid (5-ASA)
- Adenosine triphosphate (ATP)
- Anti-neutrophil cytoplasmic antibodies (ANCA)
- Butyrate hydroxytoluene (BHT)
- C-reactive protein (CRP)
- Coefficient of repeatability (CR)
- Coefficient of variation (CV)
- Colony stimulating factor (CSF)
- Copper zinc superoxide dismutase (CuZnSOD)
- Crohn's disease (CD)
- Cyclic adenosine monophosphate (cAMP)
- Dextran sulphate sodium (DSS)
- Diethylenetriaminopenta-acetic acid (DTPA)
- Dimethyl sulfoxide (DMSO)
- Dulbecco's phosphate buffered saline (D-PBS)
- Endothelial leukocyte adhesion molecule-1 (ELAM-1)
- Erythrocyte sedimentation rate (ESR)
- Ethylenediaminetetra-acetic acid (EDTA)
- Formylmethionine (fMet)
- Glutathione (GSH)
- Gut-associated lymphoid tissue (GALT)
- Heat-inactivated copper zinc superoxide dismutase (HI-CuZnSOD)
- Heat-inactivated recombinant human recombinant manganese superoxide dismutase (HI-rh-MnSOD)
- High performance liquid chromatography (HPLC)
- Hydroxy-6,8,11,14-eicosatetraenoic acid (HETE)
- Inflammatory bowel disease (IBD)
- Inhibitory factor kappa-B (IxB)
- Inter-cellular adhesion molecule-1 (ICAM-1)
- Interleukin (IL)
- Interleukin-1 receptor antagonist (IL-1Ra)
• Interleukin-2 receptor (IL-2R)
• Intraepithelial lymphocytes (IEL)
• Leukotriene (LT)
• Lipopolysaccharide (LPS)
• Major histocompatibility complex (MHC)
• Malondialdehyde (MDA)
• Manganese superoxide dismutase (MnSOD)
• Monocyte chemoattractant protein-1 (MCP-1)
• Myeloperoxidase (MPO)
• N-formyl-methionyl-leucyl-phenylalanine (fMLP)
• Nicotinamide adenine dinucleotide (NAD)
• Nicotinamide adenine dinucleotide phosphate (NADPH)
• Nitric oxide (NO)
• Non-steroidal anti-inflammatory drugs (NSAIDs)
• Nuclear factor kappa-B (NFκB)
• Nω-nitro-D-methyl-arginine methyl ester (D-NAME)
• Nω-nitro-L-methyl-arginine methyl ester (L-NAME)
• Oxidised glutathione (GSSH)
• Para-aminosalicylic (4-ASA)
• Peptidoglycan-polysaccharide polymers (PG-PS)
• Peripheral blood mononuclear cells (PBMC)
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• Phospholipase A2 (PLA2)
• Platelet activating factor (PAF)
• Polymorphonuclear leukocytes (PMN)
• Polyunsaturated fatty acids (PUVA)
• Prostaglandin (PG)
• Reactive oxygen species (ROS)
• Recombinant human recombinant manganese superoxide dismutase (rh-MnSOD)
• Sulphasalazine (SSP)
• Superoxide dismutase (SOD)
• T Helper (TH)
• Thiobarbituric acid (TBA)
• Thiobarbituric acid-reactive substances (TBARS)
• Thromboxane (TX)
• Transforming growth factor-β (TGFβ)
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STATEMENT OF ORIGINALITY

All experimental work presented in this thesis was designed, performed and analysed by the author apart from the following exceptions:

Some of the histological processing and staining was carried out by Selina Blades. Histological processing of biopsy material for the open study of antioxidant nutrients in active ulcerative colitis was undertaken in the routine hospital laboratory.

Scoring of histological material was done in conjunction with Dr Adam Coumbe, Senior Lecturer in Morbid Anatomy, St Bartholomew’s and the Royal London School of Medicine and Dentistry.
Dedicated to all free radicals, especially Theresa, and to my family and friends.
INTRODUCTION

The term inflammatory bowel disease (IBD) defines a group of chronic, relapsing inflammatory disorders of the intestine of unknown aetiology. There have been few changes in the therapy of IBD for two decades and the most frequently used agents, aminosalicylates and glucocorticoids, have non-specific anti-inflammatory actions, giving few clues as to the important pathogenic mediators. There has therefore been much research effort to establish the aetiology and pathogenesis of these diseases with the aim of developing more specific, effective, and less toxic, therapies.

Oxygen free radicals and their associated metabolites, collectively termed reactive oxygen species (ROS), are highly reactive and pro-inflammatory. They are produced in excess by the inflamed mucosa in IBD (Ahnfelt-Ronne et al 1990; Simmonds et al 1992a; Keshavarzian et al 1992b), but their exact role in pathogenesis remains poorly understood. This thesis examines the potential role of ROS and in particular antioxidant therapy, in IBD. A proven role for specific antioxidant therapy would help to establish the pathogenic importance of ROS and potentially lead to novel therapies with low toxicity.

The first part of this introduction presents an overview of the history, epidemiology, aetiology and pathophysiology, clinical features and therapy of inflammatory bowel disease. The second part presents the chemistry and biological origins of ROS. In the third section, evidence for a role for ROS in IBD is examined. The final part sets out the aims of this thesis.

INFLAMMATORY BOWEL DISEASE

The term IBD encompasses ulcerative colitis (UC), Crohn’s disease (CD), collagenous colitis and microscopic colitis. At least 5% of patients cannot be classified into one of these categories. Only UC and CD will be specifically considered further in this thesis.

UC is a chronic, relapsing, inflammatory disease affecting a variable extent of the colonic mucosa in continuum from the rectum. The cardinal clinical feature is bloody diarrhoea. Urgency is a common manifestation and in more severe cases, abdominal pain, which is rarely severe in the absence of colonic perforation. Disease relapses may occur spontaneously or in association with the use of drugs (eg non-
steroidal anti-inflammatory drugs (NSAIDs) and antibiotics), emotional stress and intestinal infection. Patients with pancolitis are at particular risk of developing malignancy late in the course of the disease. Proximal spread occurs in up to 30% of patients with proctitis alone at diagnosis (Ekbom et al 1991). Extraintestinal manifestations are common, including arthropathy, uveitis, pyoderma gangrenosum, erythema nodosum and hepatobiliary disorders.

CD is a chronic, relapsing, inflammatory condition of the intestine which can affect any part of the gastrointestinal tract from the mouth to the anus. The most frequently affected parts are the terminal ileum (30%), ileum and colon (50%), and colon alone (20%) and perineum (Campieri et al 1993). The inflammation is usually transmural and classically produces discrete affected areas ('skip-lesions'). It is characterised by stricture formation and fistulae to other viscera and to skin. Similarities to UC include the range of extraintestinal manifestations and an increased, though less well recognised, risk of colorectal carcinoma (Gillen et al 1994).

Historical Perspective

Idiopathic, extensive ulceration of the colon was first described in the late 19th Century (Wilks et al 1875; Hale-White 1888), and the term ulcerative colitis became established in the early part of this century (Hawkins 1909). Previously described as regional ileitis, the detailed description by Crohn et al (Crohn et al 1932) led to the eponym commonly used today.

Epidemiology

The incidence of IBD varies with geographical location, race, age, and possibly sex (Table 1.1). UC is commoner in developed countries, particularly in Northern Europe, and in urban areas (Ekbom et al 1992). Jews have a higher incidence of IBD (Ekbom et al 1992), which varies according to their geographical origin. In Israel, the incidence is lower in Jews of Asian or African origin than those from America or Europe (Stenson et al 1991). Conversely, IBD is less common in black groups than among Caucasians in both America (Calkins et al 1984) and South Africa (Wright et al 1986).

UC is probably marginally commoner in women; the reported degree of the difference varying between a factor of 30% and equal incidence (Stenson et al 1991). UC is very uncommon before the age of 10, and its highest incidence is at 15-25 years, with a second peak at 55-65. However, UC that presents for the first time in those over 50 is often less severe and has a lower incidence of relapse (Tysk et al 1992b).

The relationship with smoking is one of the main epidemiological dichotomies
between UC and CD, being much less common in UC, and more common in CD, than in the general population (Stenson et al 1991). In addition, UC is more likely to occur after giving up smoking, and patients with UC who continue to smoke experience improvements in symptoms (Tysk et al 1992a). Recently, a controlled clinical trial in active UC has shown some benefit from nicotine patches (Pullan et al 1994). Conversely, CD runs an unfavourable course in those who continue to smoke (Lindberg et al 1992).

Table 1.1 The epidemiology of ulcerative colitis and Crohn's disease.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Ulcerative Colitis</th>
<th>Crohn's Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incidence (per 100,000/year)</td>
<td>2-10</td>
<td>1-6</td>
</tr>
<tr>
<td>Prevalence (per 100,000)</td>
<td>35-100</td>
<td>10-100</td>
</tr>
<tr>
<td>Racial Incidence</td>
<td>whites, ↓ blacks in USA</td>
<td>whites, ↓ blacks in USA</td>
</tr>
<tr>
<td>Ethnic Incidence</td>
<td>Jews</td>
<td>Jews</td>
</tr>
<tr>
<td>Sex</td>
<td>(Probably similar)</td>
<td>(Probably similar)</td>
</tr>
<tr>
<td>Age</td>
<td>15-35*</td>
<td>15-35*</td>
</tr>
<tr>
<td></td>
<td>(55-65)</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
</tbody>
</table>

* Peak at this age does not occur in low-risk populations. ( ) no consensus.

Adapted from (Stenson et al 1991).

Aetiology and Pathophysiology

Although the aetiology of IBD is unknown, a number of pathogenic factors have been proposed.

Genetic Factors

The influence of inheritance in IBD is suggested by the inter-racial differences in disease frequency (Samuels et al 1974), the increased frequency in monozygotic compared with dizygotic twins (Tysk et al 1988) and the occurrence of disease aggregation within families (Orholm et al 1991). CD shows greater concordance rates for monozygotic twins (53%) than UC (6%) (Tysk et al 1988). For first degree relatives of patients with IBD, there is a 15-fold risk for UC and 3.5-fold for CD (Langman 1979). In addition, genetic diseases, such as Turner's syndrome (Weinrieb et al 1976) and Hermansky-Pudlak syndrome (Schinella et al 1980), are associated with IBD.

There have been conflicting results from studies of HLA linkage in IBD, with associations demonstrated for DR2, B35, Bw52 and DRB1*1502, though these vary between different ethnic groups in UC (Asakura et al 1995).
families in which several members had CD, two loci on chromosome 16 were linked to disease susceptibility (Hugot et al 1996). It has been suggested that genetic factors may affect immunological control (Yang et al 1992).

**Infectious Agents**

The colonic lumen contains a vast reservoir of potentially pathogenic microorganisms. Abnormal host responses to commensal bacteria may be responsible for IBD (Phillips 1988), as suggested by the increased adhesion of *E. coli* to the mucosa in UC (Burke et al 1988). Alternatively, induction of an autoimmune response via molecular mimicry with commensal organisms may lead to persistent inflammation.

It has been suggested that exogenous agents such as Mycobacteria (Wall et al 1993), the measles virus (Levin et al 1995), and elementary forms of bacteria (L-forms) (Belsheim et al 1983; Ibbotson et al 1987), may be directly responsible for IBD. However, these studies largely rely on demonstrating the putative agent within the gut wall and could be explained by increased mucosal permeability (Wyatt et al 1993). The evidence for an infectious aetiology for IBD thus remains weak, especially given the clinical response to potent immunosuppressive agents.

An alternative possibility is that structural components of microbes, rather than the viable organism, might cause mucosal inflammation. This is suggested by the presence of circulating antibodies to lipopolysaccharide (LPS) in IBD (Kruis et al 1984) and the correlation of endotoxin plasma levels with disease activity in CD (Wellman et al 1986). Animal models in which colitis is induced by exposure of the intestinal wall to bacterial cell wall components, such as peptidoglycan-polysaccharide (PG-PS) polymers and N-formyl-methionyl-leucyl-phenylalanine (fMLP) (Chester et al 1985; Sartor et al 1985), lends further support to this concept.

**Mucin Abnormalities**

Abnormal gastrointestinal mucus may reduce mucosal protection from the potentially deleterious luminal contents and thus provoke inflammation. A number of abnormalities have been described including the loss of goblet cell mucin in UC, possibly due to abnormal secretion (Cope et al 1988), structural abnormalities of the mucin layer (Podolsky et al 1984), decreased mucus sulphation (Raouf et al 1992) or acetylation of glucosamine (Burton et al 1983).

**Vascular Abnormalities**

It has been proposed that the microvascular changes found in CD (Wakefield et al 1989) and UC (Fairburn 1973) may cause ischaemic injury to the mucosa (Knutson et al 1968; Geller et al 1983; Wakefield et al 1991). Paradoxically, however, mucosal and sub-mucosal blood flow is increased in IBD (Hulten et al 1977). There is radiological evidence of widened arteries and increased venous return
(Bousen et al 1966) and preliminary evidence that the length of the colonic marginal artery in UC matches the extent of well-demarcated disease in UC (Hamilton et al 1995). It remains unclear, however, whether these abnormalities are of aetiological importance or are secondary to the release of vasoactive mediators. The vasoconstrictor, endothelin-1, is found in the submucosa in CD (Murch et al 1992) and nitric oxide, a potent vasodilator, is produced in excess by the mucosa in UC (Boughton-Smith et al 1993; Middleton et al 1993). The hypothesis that these changes are secondary to inflammation is supported by evidence from animal studies in which abnormal mesenteric blood flow accompanies induction of inflammation in both dinitrochlorobenzene- and immune-complex-mediated colitis in rabbits (Hauser et al 1988).

**Platelets and Thromboembolism**

There is good evidence that platelets may have a pathogenic role in IBD. IBD is a risk factor for thromboembolism (Talbot et al 1986; Novotny et al 1992), exacerbations of IBD are associated with thrombocytosis (Harris et al 1983) and platelet thrombi are seen in the microvasculature in CD (Wakefield et al 1989). Furthermore, platelets circulate in an activated state in both UC and CD (Collins et al 1994). The release of inflammatory mediators, such as IL-8 (Kaplanski et al 1993), 12-hydroxy eicosatetraenoic acid, platelet factor 4 (Page 1989) and ROS (Amieson et al 1985) by activated platelets suggests that they are pro-inflammatory. Reports of a beneficial therapeutic response to heparin in UC provide further supporting evidence for the pathogenic importance of thromboembolism (Gaffney et al 1991; Dwarakanath et al 1995; Gaffney et al 1995).

**Mucosal Permeability**

Luminal pathogens and pro-inflammatory molecules may gain access to the mucosa if mucosal permeability is increased. Permeability to polyethylene glycol (PEG) is increased in the colon in active UC (Almer et al 1993) and permeability to PEG and 99mTc-labelled diethylenetriaminopenta-acetic acid (DTPA) is increased in the small bowel in CD (Casallas et al 1986; Teahon et al 1992).

Some workers have shown that permeability to lactulose and mannitol is only abnormal in CD (Casallas et al 1986) and others, using PEG, lactulose and mannitol, have found no differences between IBD and controls (Ruttenberg et al 1992; Munkholm et al 1994). Alterations in permeability are most likely to be secondary to mucosal inflammation, though a possible aetiological significance is suggested by increased intestinal permeability to PEG (Hollander et al 1986), lactulose and mannitol in the uninflamed bowel (May et al 1993; D’Inca et al 1995), and first degree relatives, of patients with CD (Peeters et al 1994). Furthermore, increased
permeability to lactulose and mannitol in quiescent CD predicts subsequent relapse (Wyatt et al 1993).

Psychological Factors

Psychological stress is associated with disease activity in IBD (Porcelli et al 1994) and a link has been shown between exacerbations of disease and prior psychological stress (Drossman 1988). The stress of captivity appears to increase the incidence of colitis in cotton-top tamarin monkeys (Kirkwood et al 1986). These observations may be explained by the influence of the brain-gut axis on the release of pro-inflammatory neurotransmitters in the intestine (Foreman 1987). Naturally, the psychosocial status of patients with IBD must be taken into account in their management (Ramchandani et al 1994).

The Immune Response in Inflammatory Bowel Disease

CD is characterised by transmural inflammation with a predominantly chronic inflammatory infiltrate and granuloma formation, whereas in UC there is a mixed neutrophil and lymphoid infiltrate confined to the mucosa. The inflammatory cells comprise lymphocytes of the gut-associated lymphoid tissue (GALT) together with neutrophils, lymphocytes and macrophages recruited from the mesenteric vasculature. Immune protection is also provided by the physico-chemical barrier of intestinal mucus, the inter-cellular tight junctions, epithelial cells and intra-epithelial lymphocytes, which are predominantly T cells (Hirata et al 1986). Collections of mucosal lymphoid cells comprise Peyer's patches, which lie below specialised epithelial cells (M-cells) and are intimately involved in the uptake and processing of luminal antigens. A subset of B-cells enter the systemic circulation and later return to the GALT, thus exchanging immunological information with the rest of the immune system (Mayer 1992). Several abnormalities of the immune defence system in IBD have been described.

B-lymphocytes and Immunoglobulin Production

IBD is associated with hypergammaglobulinaemia (Hodgson et al 1978a), and increases in immunoglobulin G (IgG) subtypes have a disease-specific tendency. Serum levels of IgG1 predominate in UC and IgG2 in CD (MacDermott et al 1989).

Antibodies to Baker's yeast (Saccharomyces cerevisiae) have been described in CD (Giaffer et al 1992; Lindberg et al 1992), although not in UC (McKenzie et al 1990). However, it is not currently thought that antibodies to exogenous antigens are important in the pathogenesis of IBD.

Lymphocytes are markedly increased in the lamina propria in IBD and these intra-mucosal lymphocytes differ phenotypically from circulating forms (James et al 1986; Peters et al 1989). The normal predominance of IgA-secreting lymphocytes in
the lamina propria is reduced by a concomitant increase in the population of IgG-secreting cells, particularly in CD, where they concentrate near Peyer's patches and granulomata (Baklien et al. 1975; Baklien et al. 1976). IgG production by endoscopic biopsies is disease specific, as it is in the serum (see above); IgG2 secretion predominates in CD and IgG1 in UC (Ruthlein et al. 1992).

Intra-mucosal lymphocytes in IBD also secrete an increased amount of IgM (Jewell et al. 1990), and IgA, particularly IgA1 (Kett et al. 1987). These findings may impair the mucosal immune response as, unlike IgA2, IgA1 is susceptible to bacterial proteases, and the resulting Fab and Fc fragments are unable to induce bacterial agglutination or prevent bacterial adherence (Plaut 1983). Further evidence for qualitative defects of immunoglobulin secretion in IBD is the overall decrease in J-chain production and binding capacity of the cytoplasmic secretory component by mucosal lymphocytes (Brandtzaeg et al. 1984), and the production of abnormal light chains by peripheral blood lymphocytes (Ginsberg et al. 1981).

**Influence of Autoantibodies**

The pathophysiological similarity of IBD to other autoimmune diseases suggests a role for autoantibodies. Autoantibodies directed against colonic antigens (Das et al. 1978) have been described in UC, although their target epitope(s) and role in pathogenicity remain to be established.

Anti-neutrophil cytoplasmic antibodies (ANCA), may also be involved in IBD, given their pathogenicity in the vasculitis of Wegener's granulomatosis. ANCA are found in 30-80% of patients with UC, and 2-25% of CD (Saxon et al. 1990; Cambridge et al. 1992; Seibold et al. 1992). They have neither the cytoplasmic staining (cANCA) pattern of Wegener's granulomatosis, nor the perinuclear pattern (pANCA) typical of polyarteritis nodosa, but have a mixed diffuse and peri-nuclear pattern. Serum levels of cANCA are a marker of disease activity in Wegener's granulomatosis (Lai et al. 1990), but not in UC (Deusch et al. 1993; Lee et al. 1995), and persist after colectomy (Saxon et al. 1990), though others have shown reduced titres with long-term steroid therapy and after total colectomy (Rump et al. 1993). Relatives of patients with IBD have increased levels of ANCA (Shanahan et al. 1992a), and thus ANCA may be useful as a genetic marker of disease susceptibility.

**Presence of Immune Complexes**

Circulating immune complexes are raised in IBD (Doe et al. 1973; Jewell et al. 1973) and correlate with disease activity (Nielson et al. 1978), though this has been disputed by other workers using alternative methods of detection (Lurhuma et al. 1976); their role in IBD thus remains uncertain.
Complement Activation

Complement is activated by many of the immune mediators that are of proposed pathogenicity in IBD. Circulating levels of complement components are normal, though there is some evidence for activation of the classical pathway (Jewell et al 1990). Tissue deposition of the terminal complement complex has been found in submucosal vessels in both UC and CD (Halstensen et al 1989) and in the epithelium in UC (Halstensen et al 1990). Furthermore, complement components are released into the lumen in CD (Ahrenstedt et al 1990) and it has been suggested that there is defective degradation of the C3b fragment in IBD (MacDermott et al 1988). Others, however, have failed to detect complement deposition in the inflamed mucosa (Keren et al 1984) and thus the pathogenic importance of complement activation in IBD remains to be established.

T-lymphocytes and Cell-Mediated Immunity

There is good evidence that there is marked mucosal T-cell activation in both UC and CD (Shanahan et al 1992b), and activation of peripheral blood lymphocytes in CD (Pallone et al 1987). In contrast to normal bowel, epithelial immune activation in CD is associated with a predominance of T-helper (CD4+) cells over T-suppressor (CD8+) cells (Mayer et al 1990) and suppressor cell activity is suppressed in CD (Goodacre et al 1982). However, the proportion of CD4+ to CD8+ cells in UC is not different to controls (Neil et al 1994). It has been suggested that these alterations might reflect a primary defect of feedback inhibition of T-cell activation, leading to persistent release of pro-inflammatory mediators, such as cytokines, eicosanoids and superoxide (Chang et al 1990). Isolated lamina propria T-cell lymphocytes demonstrate a polyclonal response in IBD, as assessed by analysis of the T-cell receptor genes (Kaulfersch et al 1988), suggesting that multiple antigens are involved in immune stimulation in IBD.

HLA class II antigens are over-expressed on the epithelial cell surface of both diseased, and non-diseased intestine, in CD (Mayer et al 1991), probably under the influence of interferon-γ (Salomon et al 1991), indicating a role for antigen-presentation in the inflamed bowel.

Neutrophils

Mucosal infiltration by neutrophils is a cardinal feature of IBD. There are at least 50 cytopathic toxins (divided into those localised to the cell membrane, and those confined to intracellular granules) which are released by the activated neutrophil (Weiss 1989), as well as other pro-inflammatory mediators, such as elastase, collagenase and gelatinase.

The importance of the cell membrane-based enzyme nicotinamide adenosine
dinucleotide phosphate (NADPH) oxidase, and the release of the intra-cellular enzyme, myeloperoxidase, in the extracellular release of ROS, is discussed in more detail below. Neutrophil-mediated tissue destruction normally halts when the initiating antigen is destroyed (Clark 1983), but one might hypothesise that a defect in this autoregulation would result in the persistent inflammation seen in IBD. The role of neutrophil-mediated release of ROS and their role in inflammation will be discussed in more detail below.

**Monocytes and Macrophages**

Macrophages, derived from circulating monocytes, migrate into the gastrointestinal wall in IBD and produce greater amounts of interleukin-1 (IL-1) and colony-stimulating factor (CSF) than those from control tissue (Pullman et al 1992). Monocytes and macrophages are involved in antigen presentation in IBD (Mahida et al 1989b), and there is evidence of increased numbers of a sub-population of macrophages, ‘veiled cells’, which are particularly active in antigen presentation (Wilders et al 1984).

**Eosinophils, Basophils and Mast Cells**

Mast cells are often seen in the inflamed mucosa in IBD (Dvorak et al 1980; Bakazs et al 1989) and secrete pro-inflammatory mediators. This may explain the therapeutic benefit of mast cell stabilisers, such as sodium cromoglycate, in some patients with UC (Grace et al 1987).

**Adhesion Molecules**

Adhesion molecules play a critical role in the migration, activation and differentiation of lymphoid cells, and in the adhesion of leukocytes to the vascular endothelium and diapedesis through it. Their importance in IBD is demonstrated by the marked increase in the expression of, for example, endothelial leukocyte adhesion molecule-1 (ELAM-1) (Koizumi et al 1992) and inter-cellular adhesion molecule-1 (ICAM-1) by the colonic vascular endothelium (Malizia et al 1991), and the release of E-selectin by intestinal macrophages in IBD (Pooley et al 1995). However, expression of vascular cell adhesion molecule-1 (VCAM-1) is normal in IBD.

**Eicosanoids**

Eicosanoids are a group of lipid mediators derived from essential fatty acids, of which the most important is arachidonic acid. Arachidonic acid is released from cellular phospholipids and metabolised to leukotrienes via the lipoxygenase pathway, and prostaglandins and thromboxanes via the cyclo-oxygenase pathway (Table 1.2). Platelet activating factor (PAF) is formed by the action of acetyl transferase. Phospholipase A2 (PLA2), the key enzyme in the release of arachidonic acid from
membrane phospholipid, is increased in the mucosa in IBD (Minami et al 1994). Eicosanoids are predominantly produced by inflammatory cells in response to inflammatory stimuli. Arachidonic metabolism is both stimulated by ROS and induces ROS production (Chakraborti et al 1989; Riendeau et al 1989; Harris et al 1992). Prostaglandins are important in the maintenance of mucosal integrity, and leukotrienes, particularly leukotriene-B4 (LTB₄), mediate increased microvascular permeability and the recruitment of polymorphonuclear leukocytes.

Table 1.2  Eicosanoids in inflammatory bowel disease.

<table>
<thead>
<tr>
<th>Eicosanoid</th>
<th>Ulcerative Colitis</th>
<th>Crohn’s Disease</th>
<th>Main Pathophysiological Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>S</td>
<td>M</td>
</tr>
<tr>
<td>Leukotrienes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LTB₄</td>
<td>↑*</td>
<td>↑</td>
<td>↑*</td>
</tr>
<tr>
<td>LTC₄/D₄</td>
<td>↑</td>
<td>–</td>
<td>↑</td>
</tr>
<tr>
<td>5-, 12-, 15-HETE</td>
<td>↑</td>
<td>–</td>
<td>↑</td>
</tr>
<tr>
<td>Prostaglandins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGE₂</td>
<td>↑*</td>
<td>N</td>
<td>↑*</td>
</tr>
<tr>
<td>PGI₂</td>
<td>↑</td>
<td>–</td>
<td>↑</td>
</tr>
<tr>
<td>PGF₂α</td>
<td>↑</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Others</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PAF</td>
<td>↑</td>
<td>–</td>
<td>↑</td>
</tr>
<tr>
<td>TXB₂</td>
<td>↑*</td>
<td>N</td>
<td>↑</td>
</tr>
</tbody>
</table>

M - Mucosal/Rectal dialysis, S - Serum levels or production by peripheral blood cells. N = Normal or conflicting data, *Correlates with disease activity, †Present in inactive disease. – Not known.
LT-n - leukotriene-n, n-HETE - n-Hydroxy-6,8,11,14-eicosatetraenoic acid, PG - prostaglandin, PAF - platelet activating factor, TX - thromboxane.

Eicosanoids are released in excess by colorectal biopsies from patients with IBD (Sharon et al 1978; Sharon et al 1984), and in vivo production is suggested by raised levels of eicosanoids in rectal dialysates of patients with active disease.
(Lauritsen et al 1988) (Table 1.2). Similar increases in eicosanoids have been found in acetic acid-induced colitis (Sharon et al 1984; Eliakim et al 1992) and trinitrobenzene sulphonic acid (TNBS)-induced colitis (Rachmilewitz et al 1989). However, specific LTB₄ antagonists have only limited efficacy in UC (Rask-Madsen et al 1992). Furthermore, prostaglandin synthesis inhibition by NSAIDs, which inhibit cyclo-oxygenase, is associated with disease relapse (Rampton et al 1981).

**Cytokines**

Cytokines are glycosylated polypeptides, with a molecular weight ranging from 10,000 to 30,000kDa, that modulate inflammation by intercellular communication (Fiocchi 1992) and act as the hormones of the immune system (Brynskov et al 1994). The role of cytokines in IBD is summarised in Table 1.3.

Mucosal and serum levels of the pro-inflammatory cytokines IL-1β (Ligumsky et al 1990) and IL-8 (Mahida et al 1992), and mucosal levels of IL-6 (Isaacs et al 1992), are raised in IBD. Circulating levels of IL-6 are raised in CD, but rarely in UC (Mahida et al 1991b). Serum levels of IL-2 are decreased in IBD (Fiocchi et al 1984), though the IL-2 receptor (IL-2R) is increased (Matsuura et al 1993), which may explain the efficacy of the IL-2 antagonist, cyclosporin A in active UC (Lichtiger et al 1994). Conversely IL-4, which normally down-regulates the production of IL-1, fails to do so in IBD (Schreiber et al 1995), and levels of another regulatory cytokine, IL-10, are increased in both the serum (Kucharzik et al 1995) and mucosa in IBD (Niessner et al 1995). Further evidence of the importance of interleukins in colonic inflammation is the development of spontaneous colitis in IL-2 and IL-10 knockout mice (Kuhn et al 1993; Sadlack et al 1993).

Tumour necrosis factor-α (TNFα) is increased in the mucosa (MacDonald et al 1990), serum (Murch et al 1991) and stool (Braegger et al 1992; Nicholls et al 1993) of children with IBD, and may contribute to persistent inflammation by a local effect on the mucosa, though mucosal levels in adults are normal (Stevens et al 1992). Preliminary data suggest that a single parenteral dose of a monoclonal antibody against TNFα antibody is of therapeutic benefit in CD (van Dullemen et al 1995).

**Neuropeptides**

The gut neurogenic peptides, vasointestinal peptide (VIP), substance P and somatostatin may mediate inflammation by altering immune function (Foreman 1987; Shanahan et al 1988). Increased levels of VIP are found in the inflamed mucosa and submucosa in CD (Bishop et al 1980), and in the inflamed mucosa in UC (O’Morain et al 1984), though others have disputed these findings (Kubota et al 1992). Plasma levels of VIP increase in parallel with the degree of inflammation in both IBD (Duffy et al 1989), and experimental colitis (Kishimoto et al 1992), and may originate, in
part, from activated neutrophils (O'Dorisio et al 1980).

Mucosal levels of somatostatin, which inhibits T-cell proliferation, but increases immunoglobulin production, are decreased in IBD (Watanabe et al 1992). Levels of substance P are increased in UC (Koch et al 1987; Goldin et al 1989), and increased numbers of receptors for substance P are found in CD (Mantyh et al 1995); substance P may contribute to the vasodilatation and increased mucosal permeability of IBD (Mazumdar et al 1992).

Table 1.3  Cytokines in inflammatory bowel disease.

<table>
<thead>
<tr>
<th>Inflammatory Mediator</th>
<th>Ulcerative Colitis</th>
<th>Crohn's Disease</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>S</td>
<td>M</td>
</tr>
<tr>
<td>Interleukins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1</td>
<td>↑</td>
<td>↑*</td>
<td>↑</td>
</tr>
<tr>
<td>IL-2</td>
<td>↓</td>
<td>N/↓</td>
<td>↓/↑*</td>
</tr>
<tr>
<td>IL-2 receptor</td>
<td>↑/N</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>IL-3</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>IL-4</td>
<td>-</td>
<td>↑</td>
<td>-</td>
</tr>
<tr>
<td>IL-6</td>
<td>↑*</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>IL-8</td>
<td>↑*</td>
<td>N</td>
<td>↑</td>
</tr>
<tr>
<td>IL-10</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Other Cytokines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td>N</td>
<td>N/↓</td>
<td>N/↑</td>
</tr>
<tr>
<td>TGFβ</td>
<td>N</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>MCP-1</td>
<td>-</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td>CSF</td>
<td>↑</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Interferon-γ</td>
<td>N/↓</td>
<td>N/↓</td>
<td>-</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>↑</td>
<td>↑</td>
<td>-</td>
</tr>
</tbody>
</table>

M - Mucosal/Rectal dialysis, S - Serum levels or production by peripheral blood cells.  N = Normal or conflicting data, *Correlates with disease activity, †Present in inactive disease, ‡ mRNA only.  – Not known or no data.

IL-n - interleukin-n, TNFα - tumour necrosis factor-α, TGFβ - transforming growth factor-β, MCP-1 - monocyte chemoattractant protein-1, CSF - colony stimulating factor, IL-1Ra - interleukin-1 receptor antagonist, MHC - major histocompatibility complex, TH - T Helper cell
Management of Inflammatory Bowel Disease

The management of IBD comprises drugs, surgery, nutritional manipulation and attention to the patients' psychological needs. Consideration of non-pharmacological intervention in IBD is beyond the scope of this thesis and will not be considered further.

Pharmacological Therapy

Sulphasalazine is sulphapyridine linked to 5-aminosalicylic acid (5-ASA) by an azo bond which is split by colonic bacteria to release 5-ASA, the active compound. The sulphapyridine moiety is associated with allergic reactions (rash, fever), haematological effects (haemolysis, neutropenia, agranulocytosis, folate deficiency) and hypersensitivity reactions (alveolitis, hepatitis, pancreatitis and, very rarely, neuropathy) in addition to dose-dependent nausea, headaches and dyspepsia and reduced spermatogenesis, though severe reactions are uncommon.

Table 1.4  Mechanisms of action of sulphasalazine (SSP) and 5-ASA.

<table>
<thead>
<tr>
<th>Mechanism Of Action</th>
<th>5-ASA/SSP</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROS Scavenger* (See also Table 4.1)</td>
<td>5-ASA/SSP</td>
<td>(Miyachi et al 1987; Gionchetti et al 1991; Miyachi 1987; Gionchetti 1991; Allgayer 1994)</td>
</tr>
<tr>
<td>Inhibits of lipid peroxidation</td>
<td>5-ASA/SSP</td>
<td>(Greenfield et al 1991)</td>
</tr>
<tr>
<td>Iron chelator</td>
<td>5-ASA</td>
<td>(Grisham et al 1992)*</td>
</tr>
<tr>
<td>Inhibits neutrophil degranulation</td>
<td>5-ASA/SSP</td>
<td>(Neal et al 1987)</td>
</tr>
<tr>
<td>Inactivates nitric oxide</td>
<td>5-ASA</td>
<td>(Pallapies et al 1995)</td>
</tr>
<tr>
<td>Inhibits 5-lipoxygenase</td>
<td>5-ASA/SSP</td>
<td>(Nielsen et al 1987; Mahida et al 1991a)</td>
</tr>
<tr>
<td>Inhibits prostaglandin synthesis and degradation</td>
<td>5-ASA/SSP</td>
<td>(Sharon et al 1978; Hout et al 1981; Hawkey et al 1985)</td>
</tr>
<tr>
<td>Inhibits PAF</td>
<td>5-ASA/SSP</td>
<td>(Eliakim et al 1988)</td>
</tr>
<tr>
<td>Inhibits IL-1 production</td>
<td>5-ASA/SSP</td>
<td>(Mahida et al 1991a; Rachmilewitz et al 1992)</td>
</tr>
<tr>
<td>Stabilises mast cells</td>
<td>5-ASA</td>
<td>(Fox et al 1991)</td>
</tr>
<tr>
<td>Inhibits interferon-γ binding</td>
<td>5-ASA</td>
<td>(Crotty et al 1992)</td>
</tr>
<tr>
<td>Inhibits stimulated E-selectin expression</td>
<td>SSP</td>
<td>(Pooley et al 1995)</td>
</tr>
<tr>
<td>Inhibits platelet function</td>
<td>SSP</td>
<td>(Stenson et al 1983; Boughton-Smith et al 1986)</td>
</tr>
</tbody>
</table>

*5-ASA may be pro-oxidant if chelated with iron at a ≥1:1 ratio (Grisham et al 1992).
To counteract the high incidence of side-effects due to the sulphapyridine component of sulphasalazine a group of drugs containing 5-ASA alone have been developed. Drugs consisting of 'free' forms of 5-ASA are known generically as mesalazine (mesalamine in the United States). Side-effects of 5-ASA include nausea, diarrhoea, and less commonly, pancreatitis, hepatitis and nephritis. The mechanism of action of sulphasalazine and the aminosalicylates is unknown, as they have a wide range of anti-inflammatory actions (Table 1.4).

Other therapies include glucocorticoids and azathioprine. Side-effects of conventional glucocorticoids include a 'cushingoid' appearance, skin-thinning, bone demineralisation, diabetes mellitus and hypertension. Azathioprine may cause rashes, nausea, vomiting, fever, pancreatitis, bone marrow suppression, hepatitis and a long-term risk of teratogenicity.

The pharmacological treatment of IBD thus relies on agents with multiple actions and side-effects. Elucidating the important pathogenic mediators of IBD may well lead to more specific, and less toxic, therapies.

**REACTIVE OXYGEN SPECIES**

Having discussed the aetiology, pathogenesis and relevant therapy of IBD, a review of the literature is presented concerning the proposed pathogenic mediator, ROS, and the mechanisms of oxidant stress.

**The Chemistry of Free Radicals in Biology**

A free radical is defined as any molecular species capable of independent existence with a single, unpaired electron in the outer shell. Molecular oxygen is technically a di-radical, as it contains two electrons in the outer shell with parallel rather than anti-parallel spin, to maintain thermodynamic stability. Sequential one-electron reduction of molecular oxygen requires four steps and results in the production of ROS, as shown below:

\[
O_2 + e^- \rightarrow O_2^{*}\quad \text{(superoxide radical)}
\]
\[
O_2^{*} + H_2O \rightarrow HO_2^{*} + OH^- \quad \text{(hydroxyperoxyl radical)}
\]
\[
HO_2^{*} + e^- + H \rightarrow H_2O_2 \quad \text{(hydrogen peroxide)}
\]
\[
H_2O_2 + e^- \rightarrow OH^* + OH^- \quad \text{(hydroxyl radical)}
\]

Other molecules derived from these reactions, such as the peroxyl radical and hypochlorous acid, are also susceptible to further reduction, and are thus included in the term ROS. ROS are highly reactive because of the availability of the unpaired...
electron for oxidation and reduction or because of their potent oxidising actions. The main biologically important ROS, and their half lives, are shown in Table 1.5.

<table>
<thead>
<tr>
<th>Reactive Oxygen Species</th>
<th>Symbol</th>
<th>Half-life (seconds at 37°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide</td>
<td>O$_2^-$</td>
<td>10$^{-6}$</td>
</tr>
<tr>
<td>Singlet Oxygen</td>
<td>$^{1}$O$_2$</td>
<td>10$^{-6}$</td>
</tr>
<tr>
<td>Hydroxyl</td>
<td>OH$^*$</td>
<td>10$^{-9}$</td>
</tr>
<tr>
<td>Alkoxyl</td>
<td>RO$^*$</td>
<td>10$^{-6}$</td>
</tr>
<tr>
<td>Peroxyl</td>
<td>ROO$^*$</td>
<td>7</td>
</tr>
<tr>
<td>Lipid Hydroperoxide</td>
<td>ROOH</td>
<td>Stable</td>
</tr>
<tr>
<td>Hydrogen Peroxide</td>
<td>H$_2$O$_2$</td>
<td>Stable</td>
</tr>
<tr>
<td>Hypohalous acid</td>
<td>HOX (X=Cl/Br)</td>
<td>Stable</td>
</tr>
<tr>
<td>Nitric oxide*</td>
<td>NO</td>
<td>0.1-5.6</td>
</tr>
</tbody>
</table>

*Nitrogen-centred radical.

**Superoxide**

Superoxide is the first intermediate in the sequential reduction of oxygen to water (Halliwell et al. 1989c), and is the precursor for all other ROS. In an acidic environment superoxide acts as a Brønsted base shifting the acid-base equilibrium and generating the hydroperoxyl radical, which may then react further with superoxide to generate hydrogen peroxide (see above).

In biological systems with a near neutral pH, and in the absence of iron, this reaction is slow, and therefore the production of significant quantities of hydrogen peroxide from superoxide, in biological systems, depends on the action of superoxide dismutase (Gregory et al. 1973; Fridovich 1975).

**Hydrogen Peroxide**

Hydrogen peroxide is highly lipophilic and more stable than other ROS. These qualities allow hydrogen peroxide to diffuse through cell membranes, resulting in oxidant stress distant from the point of production and increasing the risk of tissue injury (Halliwell et al. 1989c). Hydrogen peroxide is metabolised via the Fenton reaction to the highly reactive hydroxyl ion, and also by myeloperoxidase, to hypochlorous acid. The injurious effects of hydrogen peroxide applied topically to the rectal mucosa in humans is well-recognised and gives rise to a typical macroscopic appearance, ‘the snow white sign’ (Bilota et al. 1989).
The Hydroxyl Radical

Hydroxyl radicals are the shortest-lived of the ROS. Because of their extreme reactivity their diffusion capability is restricted to approximately two molecule diameters (Fee et al 1977). The hydroxyl ion is capable of reacting with almost all biological molecules, often resulting in the formation of further ROS (Halliwell et al 1990). There is no natural defence against hydroxyl ions, and this, combined with their reactivity, makes them potentially highly deleterious. The hydroxyl ion is derived from ROS in the presence of iron, as described below. Non-iron mediated hydroxyl ion production may result from the reaction of superoxide with hypochlorous acid (Ramos et al 1992; Candeias et al 1993), as follows:

\[ \text{O}_2^{-} + \text{HOCl} \rightarrow \text{OH}^{\bullet} + \text{Cl}^{-} + \text{O}_2 \]

Fenton Chemistry and the Haber-Weiss Reaction as Sources of the Hydroxyl Radical

Iron and other transition metals, because of their polyvalency, can catalyse the breakdown of hydrogen peroxide in the Fenton reaction, summarised as follows:

\[ \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{OH}^{\bullet} + \text{OH}^{-} + \text{Fe}^{3+} \]

Superoxide plays a role in this reaction, as it reduces iron from the ferric (Fe^{2+}) to the ferrous (Fe^{3+}) form:

\[ \text{Fe}^{3+} + \text{O}_2^{-} \rightarrow \text{Fe}^{2+} + \text{O}_2 \]

This is referred to as the superoxide-driven or Haber-Weiss reaction, and is of importance as iron is often stored in the ferric (Fe^{3+}) state in biological tissues. The Fenton and Haber-Weiss reactions readily take place at physiological temperatures and pH (Smith et al 1990).

Other biological iron-containing molecules which allow iron to participate in Fenton chemistry include haemoglobin and myoglobin (Sadrzadeh et al 1984; Grisham 1985; Kanner et al 1985). It has been suggested that the increased mucosal blood flow and microvascular injury in IBD may lead to extravascular leakage of haemoglobin, with resulting increases in tissue iron and concomitant promotion of Fenton chemistry (Babbs 1992).

Hypochlorous Acid

Hypochlorous acid is a more potent oxidant than either superoxide or
hydrogen peroxide (Halliwell et al 1989c). It may react with superoxide to generate the hydroxyl radical \textit{in vitro} (Ramos et al 1992; Candeias et al 1993). \textit{In vivo}, it is formed by the action of neutrophil myeloperoxidase (Weiss 1986a), which catalyses the reaction:

\[
\text{MPO} \quad \text{H}_2\text{O}_2 + \text{Cl}^- + \text{H}^+ \rightarrow \text{HOCl} + \text{H}_2\text{O}
\]

Hypochlorous acid plays an essential role in neutrophil-mediated killing of micro-organisms following phagocytosis; however, if released into the intercellular milieu, it may cause oxidative damage to extracellular components (Clark et al 1986). The role of hypochlorous acid in interference with cell metabolism, and inactivation of antioxidant enzymes, is discussed below (p. 31).

**Non-Oxygen-Centred Free Radicals**

Nitric oxide (NO) and its product with superoxide, peroxynitrite (OONO\textsuperscript{*}) are discussed below. Other radicals of biological importance include carbon-centred radicals (lipid radicals, L\textsuperscript{*} and alkoxy radicals, R\textsuperscript{*}), the sulphur centred thiyl radical (R-S\textsuperscript{*}) and the iron centred perferryl radical (Fe\textsuperscript{3+}-O\textsubscript{2}-Fe\textsuperscript{2+}).

**SOURCES OF REACTIVE OXYGEN SPECIES IN BIOLOGICAL SYSTEMS**

There are many potential sources of ROS in biological systems, summarised in Table 1.6. The following discussion concentrates on those of prime importance in IBD.

**Reactive Oxygen Species in Cellular Respiration**

The mitochondrial electron transport chain is the major source of superoxide under physiological conditions (McCord \textit{et al} 1970; Halliwell 1989a), with the steady-state intra-mitochondrial concentration of superoxide approximately 8 x 10\textsuperscript{-12} molar (Tyler 1975).

Aerobic metabolism involves the four-electron reduction of oxygen to water by the mitochondrial respiratory chain. During this process the oxygen molecule remains bound to the ferricytochrome c:oxygen oxidoreductase chain (complex IV). It has been estimated that 1-2\% of the electrons involved in this reduction do not reach complex IV but 'leak' to form superoxide and its dismutation product, hydrogen peroxide (Boveris \textit{et al} 1972). Although some have questioned whether such a rate of leakage actually occurs \textit{in vivo} (Kehrer \textit{et al} 1994), it seems certain that the cell is continually exposed to internal oxidant stresses, thus explaining the evolution of antioxidant protection in even the most primitive life-forms, for instance manganese.
superoxide dismutase (MnSOD) in bacteria.

Other sources of intra-cellular superoxide production include NADPH oxidase (Thomas et al 1985) and NADPH-cytochrome P-450 reductase (Morehouse et al 1984; Ames et al 1993; Kehrer et al 1994). The role of superoxide production from xanthine oxidase in ischaemia-reperfusion injury is discussed below.

Table 1.6 Important sources of ROS in biology.

<table>
<thead>
<tr>
<th>Endogenous Sources</th>
<th>Exogenous Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cellular Respiration</strong></td>
<td>Redox-cycling substances, eg</td>
</tr>
<tr>
<td>Microsomal Electron Transport Chain</td>
<td>Paraquat</td>
</tr>
<tr>
<td>Mitochondrial Electron Transport Chain</td>
<td>Doxyrubicin</td>
</tr>
<tr>
<td><strong>Oxidant Enzymes, eg</strong></td>
<td><strong>Drug oxidation, eg</strong></td>
</tr>
<tr>
<td>Xanthine oxidase</td>
<td>Paracetamol</td>
</tr>
<tr>
<td>Lipoxygenase</td>
<td>Carbon tetrachloride</td>
</tr>
<tr>
<td>Cyclooxygenase</td>
<td>Bleomycin</td>
</tr>
<tr>
<td>Galactose oxidase</td>
<td>Mitomycin C</td>
</tr>
<tr>
<td>Monoamine oxidase</td>
<td>Adriamycin</td>
</tr>
<tr>
<td><strong>Phagocytic Cells</strong></td>
<td><strong>Miscellaneous</strong></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>Cigarette smoke</td>
</tr>
<tr>
<td>Monocytes and macrophages</td>
<td>Ionising Radiation</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>Ultraviolet radiation (eg sunlight)</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>Thermal injury</td>
</tr>
</tbody>
</table>

**Reactive Oxygen Species Production by Inflammatory Cells**

**Superoxide Production by Polymorphonuclear Leukocytes - The 'Respiratory Burst'**

Superoxide release by neutrophils occurs in response to inflammatory stimuli and in conjunction with the extracellular release of cytoplasmic granules and leukotrienes, chemotaxis and adhesion to cell-surface markers (Winterbourne 1990). This release of superoxide is accompanied by the uptake of oxygen in the ‘respiratory burst’. The respiratory burst also occurs in eosinophils (Souness et al 1991) and macrophages (Turner et al 1993), and does not involve conventional cellular respiration as it is resistant to cyanide poisoning (Sbarra et al 1959), but instead utilises NADPH, as follows:

\[
\text{NADPH} + \text{O}_2 \rightarrow \text{NADP}^+ + \text{H}^+ + \text{O}_2^-
\]

(Rossi 1986)
The hexose monophosphate shunt then regenerates NADPH from NADP⁺ (Iyer et al 1961). The main biomolecular inducers of neutrophil superoxide production are shown in Table 1.7.

Neutrophils also undergo respiratory burst activity when in contact with activated human T lymphocytes, and their contact exacerbates the neutrophil response after stimulation with fMLP (Zhang et al 1992). Furthermore, neutrophil superoxide production is enhanced by P-selectin expressed on the surface of activated platelets (Nagata et al 1993).

The control of the respiratory burst is complex, and its molecular basis incompletely understood. Stimulation at the cell membrane leads to sequential activation of phospholipase C, protein kinase C and finally NADPH oxidase, which results in the release of superoxide (Winterboume 1990), but other pathways are also involved (McPhail et al 1983). Cyclic adenosine monophosphate (cAMP) is important in the respiratory burst in neutrophils and eosinophils but not macrophages (Turner et al 1993). The response to unopsonized latex particles, but not the response to fMLP, is inhibited by cytochalasin B (an inhibitor of microtubule function), suggesting that for some stimulants endocytosis is a pre-requisite for superoxide production (Hallett et al 1983).

**Action of Neutrophil Myeloperoxidase**

Activated neutrophils release myeloperoxidase, which catalyses the reaction of chloride ions and hydrogen peroxide into the powerful oxidant hypochlorous acid (HOCl) (Weiss 1986a). Hypochlorous acid, in turn, reacts with primary amines, with the side group -NH₂ to yield chloramines (-NHCl) which may be more or less toxic depending on their lipophilicity (Thomas et al 1983). Neutrophils also produce monochloramine (NH₂Cl) from the reaction of hypochlorous acid and ammonia:

\[
\text{HOCl} + \text{NH}_2 \rightarrow \text{NH}_2\text{Cl} + \text{H}_2\text{O}
\]

Monochloramine is lipophilic and of greater toxicity than HOCl (Grisham et al 1984). Some endogenous protection is provided by the reaction of the amine taurine with hypochlorous acid to produce taurine monochloramine (Dallegri et al 1990), as follows:

\[
\text{HOCl} + \cdot \text{O}_2\text{-CH}_2\text{-CH}_2\text{-NH}_2 \rightarrow \cdot \text{O}_2\text{-CH}_2\text{-CH}_2\text{-NHCl} + \text{H}_2\text{O}
\]

Taurine monochloramine, although a strong oxidant, is highly hydrophilic and thus of lower toxicity than monochloramine, and acts as a natural defence against endogenous HOCl toxicity (Thomas et al 1983). Taurine provides additional protection by scavenging the hydroxyl radical (Green et al 1991).
Table 1.7 Biomolecular inducers of neutrophil superoxide production.

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Pathway Activated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opsonized particles (eg micro-organisms)</td>
<td>Cell membrane receptor</td>
</tr>
<tr>
<td>Immune complexes</td>
<td>Cell membrane receptor</td>
</tr>
<tr>
<td>Complement fragment C5a</td>
<td>Cell membrane receptor</td>
</tr>
<tr>
<td>Lectins</td>
<td>Cell membrane receptor</td>
</tr>
<tr>
<td>N-formylated particles of bacterial origin</td>
<td>Cell membrane receptor</td>
</tr>
<tr>
<td>such as f-Met-Leu-Phe (fMLP)</td>
<td></td>
</tr>
<tr>
<td>Ca-ionophore A23187</td>
<td>Penetrates membrane to stimulate</td>
</tr>
<tr>
<td>Lectins eg concanavalin A</td>
<td>Cell membrane receptor</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>Cell membrane receptor</td>
</tr>
<tr>
<td>Cerebroside sulphate</td>
<td>Not known</td>
</tr>
<tr>
<td>Leukotriene</td>
<td>Cell membrane receptor</td>
</tr>
<tr>
<td>Tumour necrosis factor</td>
<td>Cell membrane receptor</td>
</tr>
<tr>
<td>Platelet activating factor</td>
<td>Cell membrane receptor</td>
</tr>
<tr>
<td>Platelet-derived growth factor</td>
<td>Cell membrane receptor</td>
</tr>
<tr>
<td>Phorbol esters e.g. PMA</td>
<td>Activates Protein kinase C</td>
</tr>
<tr>
<td>Diacylglycerol</td>
<td>Activates Protein kinase C</td>
</tr>
</tbody>
</table>

Adapted from (Winterbourne 1990)

Production of ROS by Other Inflammatory Cells

Macrophages and eosinophils may, under some circumstances, produce larger quantities of ROS than neutrophils. For instance, the granuloma in CD produces 20-30 times the amount of ROS compared with unfused macrophages (Adams et al 1989), and superoxide production by eosinophils is greater than that of neutrophils following stimulation with opsonized zymosan (Tauber et al 1979). The relative contributions of neutrophils, eosinophils and macrophages to oxidant stress in IBD is unknown, but given the relative numbers of neutrophils it is likely that these are of prime importance in ROS production by the inflamed intestinal mucosa.

The Role Of Iron In Biological Tissues

The transition metals have variable valency, and are thus able to act as catalysts in oxidation and reduction, 'redox', reactions. Iron is the most common transition metal in the human body. Although considerable iron is present in biological tissues, most, if not all, is chelated to metal-binding proteins, such as ferritin, caeruloplasmin and transferrin, that prevent involvement in free radical
reactions (Halliwell 1991). The possibility that this defence mechanism is by-passed in IBD is discussed more extensively in Chapter 7.

Ischaemia-Reperfusion Injury and the Role of Xanthine Oxidase

Xanthine dehydrogenase is a molybdenum-iron sulphur flavin hydroxylase which oxidases a variety of purines and other heterocyclic nitrogenous compounds, and is the rate limiting step in nucleic acid degradation (Dupont et al 1992). During episodes of ischaemia the mammalian form of the enzyme is converted from the NAD$^+$-dependent dehydrogenase (D) form to the O$_2$-dependent oxidase (O) form (Dupont et al 1992). This appears to be a consequence of the activation of a protease via a reduction in cytosolic calcium (McCord 1985). Ischaemia also causes the degradation of intra-cellular stores of ATP to the purines, xanthine and hypoxanthine (Dupont et al 1992). During reperfusion superoxide is formed as a by-product of the xanthine oxidase-mediated conversion of hypoxanthine to xanthine and xanthine to urate (McCord 1985), leading to tissue injury (Granger et al 1981). There is now considerable evidence that this occurs in vivo in both animals (Parks et al 1988; Yokoyama et al 1990) and humans (Friedl et al 1990).

Xanthine oxidase is present in many mammalian tissues, with relatively high concentrations found in the small intestine in animals (Battelli et al 1972; Krenitsky et al 1974), and it is readily detected in human endothelial cells (Jarasch et al 1986; Bailey et al 1988).

Ischaemia-reperfusion injury involves ROS-mediated adhesion of leukocytes to the microvascular endothelium (Granger et al 1989) and is inhibited by copper zinc superoxide dismutase (CuZnSOD), MnSOD, catalase and oxypurinol (Suzuki et al 1991). A role for iron is suggested by increased iron uptake following stimulation of intestinal xanthine oxidase (Topham et al 1982), and by the protective effect of desferrioxamine in hypoxanthine/xanthine oxidase-induced damage to cell monolayers (Kvietys et al 1989). Desferrioxamine similarly protects against ischaemia-reperfusion induced neutrophil infiltration in the cat intestine (Hernandez et al 1987). Other inflammatory mediators, such as PAF, are also likely to be involved in ischaemia-reperfusion injury, as superoxide from xanthine oxidase stimulates PAF release (Suzuki et al 1991), and PAF inhibitors are protective in ischaemia-reperfusion injury (Kubes et al 1990).

Arachidonic Acid Metabolism

Free radicals can both result from and initiate membrane-based arachidonic acid metabolism (Chakraborti et al 1989; Riendeau et al 1989; Harris et al 1992). Three main pathways, lipoxygenase, cyclooxygenase and epoxygenase, produce leukotrienes, prostaglandins and epoxies respectively. Fig 1.1 shows these pathways
and the role of free radicals in these reactions.

Fig 1.1 Diagram showing involvement of ROS in the arachidonic acid pathway including the chain propagation of lipid peroxidation - adapted (Harris et al 1992). Lipid radical (L'), Leukotriene (LT), Hydroxy-6,8,11,14-eicosatetraenoic acid (HETE), Prostaglandin (PG), Platelet activating factor (PAF), Thromboxane (TX).

**Nitric Oxide**

Nitric oxide (NO) is a nitrogen-centred radical, which has recently been recognised to be of prime importance in biology. The effects of NO on the gastrointestinal tract are summarised in Table 1.8.

NO is probably the non-adrenergic non-cholinergic neurotransmitter of the intestine, and plays a central role in maintenance of normal vascular, and other
smooth muscle, tone. It is formed by three forms of the enzyme nitric oxide synthase, two constitutive forms, found predominantly in endothelial and neuronal cells, and an inducible form expressed mainly in macrophages (Vallance et al 1994). Both catalyse the reaction:

\[
\text{L-arginine} + 2\text{O}_2 \rightarrow \text{L-citrulline} + 2\text{H}_2\text{O} + \text{NO}
\]

The constitutive enzyme is dependent on activation by calcium and calmodulin and produces picomolar quantities of NO, whereas the inducible enzyme is calcium-independent and produces nanomolar amounts (Knowles et al 1992). NO is released by the target cell and migrates, or is transported, to the target cell, where it stimulates guanylate cyclase-mediated conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) (Moncada et al 1991).

<table>
<thead>
<tr>
<th>Biological Action</th>
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<tbody>
<tr>
<td><strong>Pro- and anti-inflammatory actions</strong></td>
</tr>
<tr>
<td>Combination with superoxide to form peroxynitrite</td>
</tr>
<tr>
<td>Cytotoxic macrophage effector molecule</td>
</tr>
<tr>
<td>Modulates leukocyte adhesion</td>
</tr>
<tr>
<td><strong>Epithelial Permeability</strong></td>
</tr>
<tr>
<td>Maintains epithelial permeability</td>
</tr>
<tr>
<td><strong>Intestinal blood flow</strong></td>
</tr>
<tr>
<td>Mucosal blood flow</td>
</tr>
<tr>
<td>Hyperdynamic circulation in cirrhosis</td>
</tr>
<tr>
<td><strong>Smooth muscle relaxation</strong></td>
</tr>
<tr>
<td>Gastroesophageal, pyloric sphincter and sphincter of Oddi relaxation</td>
</tr>
<tr>
<td>May mediate toxic dilatation</td>
</tr>
<tr>
<td>May mediate opiate effects</td>
</tr>
<tr>
<td><strong>Metabolic effects</strong></td>
</tr>
<tr>
<td>Inhibition of protein synthesis in cirrhosis with sepsis</td>
</tr>
</tbody>
</table>

Table 1.8 The effects of nitric oxide on the gastrointestinal tract.

NO is highly reactive and, in particular, reacts readily with other radical species, particularly superoxide, with which it forms peroxynitrite (OONO*). Peroxynitrite, once protonated, readily decomposes to the hydroxyl radical (Beckman et al 1990; van der Vliet et al 1994), as follows:

\[
\text{NO} + \text{O}_2^{**} \rightarrow \text{OONO}^* \\
\text{OONO}^* + \text{H}^+ \rightarrow \text{OH}^* + \text{NO}_2^*
\]
Peroxynitrite also directly and rapidly oxidises sulfhydryl groups (Radi et al. 1991), and initiates lipid peroxidation in the absence of a transition metal ion (Radi et al. 1991). The concomitant tissue production of superoxide and nitric oxide is potentially tissue damaging, as shown by the colonic inflammation induced by the intra-rectal administration of peroxynitrite in rats (Rachmilewitz et al. 1993).

NO may not always act as a prooxidant. NO binds to ferrous complexes to prevent iron-mediated oxidation of hydrogen peroxide (Kanner et al. 1991), and inhibits neutrophil NADPH oxidase-mediated superoxide production (Clancy et al. 1992). Other anti-inflammatory actions of NO include the maintenance of vascular integrity in endotoxin-induced intestinal damage (Hutcheson et al. 1990) and protection against ethanol-induced mucosal gastric injury (Quintero et al. 1992). The exact role of NO in mediating or protecting against tissue damage thus remains unclear at the present time.

DEFENCES AGAINST DAMAGE FROM REACTIVE OXYGEN SPECIES

Normal cellular metabolism results in the production of ROS within the cell which is also exposed to oxidant stress from external sources (Griffiths et al. 1988), and therefore metabolic strategies have evolved in prokaryotic and eukaryotic organisms to deal with this threat. These have been termed ‘antioxidant defences’ (Davies 1986; Heffner et al. 1989). The primary defences are divided into those functioning as scavenging systems, which prevent oxidant damage by chemical reaction to produce relatively inert and less harmful molecular species, and those that prevent oxidant formation by chelating transition metals, such as iron and copper, thus inhibiting hydroxyl ion formation by Fenton chemistry. Table 1.9 outlines these defences. Secondary defence systems limit damage by repairing oxidant damage to carbohydrates, lipids, proteins and DNA.

Direct ROS Scavengers

These can be subdivided into those in the aqueous phase and those that are predominantly found in the lipid phase. Despite this apparent phase specificity of antioxidant activity there is evidence of inter-dependence between the various components of the primary antioxidant defence (Esterbauer et al. 1989b). The important endogenous ROS scavengers are discussed in more detail below.

Ascorbate (Vitamin C)

Ascorbate is highly water soluble and an essential component of the diet in primates, and a few other mammals, which are unable to synthesise it from glucose. It is important in several processes, particularly collagen repair, in which it acts as cofactor to proline and lysine hydroxylases (Levine 1986). Chemically, it is a potent
electron donor, and thus a reductant. Loss of an electron leads to the semidehydroascorbate radical, which undergoes further reduction to dehydroascorbate or dissociation to ascorbate and dehydroascorbate (Halliwell et al 1989c). Dehydroascorbate itself rapidly degrades to oxalic and L-threonic acids. Oxygen-induced oxidation of ascorbate is catalysed by transition metals, such as iron and copper, to produce hydrogen peroxide and hydroxyl radicals. Thus, under certain circumstances, ascorbate can act as a generator of free radicals and not as an antioxidant. Doses of oral ascorbate over 5g substantially raise faecal ascorbate levels (Staerk-Laursen et al 1990), and this may lead to oxidant damage via iron-mediated oxidation of ascorbate to the ascorbate radical (semidehydroascorbate).

Table 1.9 Primary antioxidant defences in eukaryotic cells.

<table>
<thead>
<tr>
<th>Antioxidant Defence</th>
<th>Mechanism</th>
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</thead>
<tbody>
<tr>
<td><strong>ROS scavengers - aqueous phase</strong></td>
<td></td>
</tr>
<tr>
<td>Ascorbate</td>
<td>Free radical scavenger</td>
</tr>
<tr>
<td>Urate</td>
<td>Free radical scavenger</td>
</tr>
<tr>
<td>Albumin</td>
<td>Free radical scavenger</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>Free radical scavenger</td>
</tr>
<tr>
<td>Oestrogens</td>
<td>Free radical scavenger</td>
</tr>
<tr>
<td>Adenosine</td>
<td>Inhibits neutrophil superoxide production</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>Maintains ATP levels in oxidative stressed cells</td>
</tr>
<tr>
<td>Glutathione (and precursors)</td>
<td>Free radical scavenger and substrate for glutathione peroxidase</td>
</tr>
<tr>
<td>Selenium</td>
<td>Essential for glutathione peroxidase</td>
</tr>
<tr>
<td><strong>ROS scavengers - lipid phase</strong></td>
<td></td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>Free radical scavenger</td>
</tr>
<tr>
<td>β-carotene</td>
<td>Free radical scavenger</td>
</tr>
<tr>
<td><strong>Enzymatic defences</strong></td>
<td></td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>Catalyses superoxide dismutation</td>
</tr>
<tr>
<td>Glutathione peroxidase</td>
<td>Metabolises hydrogen peroxide</td>
</tr>
<tr>
<td>Catalase</td>
<td>Metabolises hydrogen peroxide</td>
</tr>
<tr>
<td><strong>Transition Metal Chelation</strong></td>
<td></td>
</tr>
<tr>
<td>Ferritin</td>
<td>Chelates iron</td>
</tr>
<tr>
<td>Transferrin</td>
<td>Chelates iron</td>
</tr>
<tr>
<td>Caeruloplasmin</td>
<td>Chelates copper and oxidises ferrous to ferric ions (ferroxidase activity)</td>
</tr>
</tbody>
</table>
Ascorbate reacts with many ROS, including peroxides, superoxide (Nishikimi 1975), the hydroxyl radical, singlet oxygen (Niki 1991), thyl radicals and hypochlorous acid (Bendich et al 1987). Concentrations of ascorbate are 25 times higher in the neutrophil than in the plasma (Evans et al 1982), probably to provide protection against neutrophil-derived hypochlorous acid.

**α-Tocopherol**

Vitamin E consists of four tocopherols α, β, γ and δ, of which α-tocopherol is the most biologically important. Deficiency leads to severe neurological damage and sterility in a variety of animal species (Bieri et al 1983). α-Tocopherol reacts with the hydroxyl ion, quenches singlet oxygen, reduces superoxide production by neutrophils (Engle et al 1988) and terminates lipid peroxidation chain reactions (Halliwell et al 1989c) resulting from the reaction of ROS with unsaturated lipids. α-Tocopherol binds preferentially with lipid radicals, as follows:

$$\text{LOO}^* + \text{tocopherol-OH} \rightarrow \text{LOOH} + \text{tocopherol-O}^*$$

α-Tocopherol is thus termed a 'chain-breaking' antioxidant. The poorly reactive tocopherol radical is converted back to α-tocopherol by reaction with the water-soluble ascorbate at the lipid-cytosol border (Esterbauer et al 1989b).

**β-Carotene**

This lipid soluble precursor of vitamin A (retinol) is an important antioxidant in plants and may have antioxidant activity in humans. Carotenoids are powerful quenchers of singlet oxygen (though there is no evidence for the presence of singlet oxygen in IBD), and inhibit lipid peroxidation (Krinsky 1989).

**Glutathione**

Glutathione (GSH) is a substrate for the enzyme glutathione peroxidase which catalyses the breakdown of hydrogen peroxide (Ross 1988). It is also a scavenger for the hydroxyl radical and singlet oxygen (Halliwell et al 1989c). In both reactions glutathione is converted to glutathione disulphide (GSSH), which is reconverted to glutathione by glutathione reductase, using NADPH as the energy source (Weiss et al 1982). The role of glutathione peroxidase in antioxidant defence is discussed below. GSH may also be involved in the reduction of the ascorbate radical in a NADPH-driven cycle, though this mechanism has not yet been demonstrated in mammalian cells (Halliwell et al 1989c). The superoxide-induced inactivation of glutathione peroxidase is reversed by glutathione in vitro (Blum et al 1985).

Depletion of hepatic glutathione by ingestion of paracetamol is thought to
mediate liver damage by limiting the defence against endogenous exposure to hydrogen peroxide and, possibly, the hydroxyl radical, and this liver damage can be ameliorated by the administration of glutathione precursors, such as N-acetylcysteine (Smilkstein et al. 1988).

**Selenium**

Selenium is an essential co-enzyme for mammalian glutathione peroxidase, and its deficiency causes Keshan disease and Kaschin-Beck disease in areas where dietary selenium is low, such as China (Yang et al. 1995), and muscle pains in patients receiving long-term, selenium-deficient, total parenteral nutrition (Litov et al. 1991). Low levels are found in diseases with a possible ROS-mediated pathogenesis such as rheumatoid arthritis (Tarp 1994) and myotonic dystrophy (Ihara et al. 1995). Administration of doses of selenium above those required for maximal growth causes small to moderate increases in glutathione peroxidase activity (Tarp 1994).

**Other Endogenous Non-Enzymatic Antioxidants**

Plasma antioxidant protection is provided by albumin, which binds copper (Halliwell et al. 1989c) and serves to protect biological molecules against HOCl (Hu et al. 1993). Although uric acid is a powerful antioxidant in the plasma and scavenges a variety of ROS (Ames et al. 1981), as well as ozone and nitrogen dioxide in the respiratory tract (Peden et al. 1993), it also stimulates neutrophils to release superoxide (Thomas 1992), and ferritin to release iron (Biemond et al. 1986); thus its antioxidant role remains unclear.

**Enzymatic Defences**

**Superoxide Dismutase (SOD).**

The catalytic function of this enzyme was first described by McCord and Fridovich in 1969 (McCord et al. 1969). The importance of superoxide dismutase (SOD) in biological systems can be judged by its presence in virtually all aerobic organisms. At pH 7.4 superoxide dismutates at a slow rate, approximately $5 \times 10^5 \text{M}^{-1}\text{s}^{-1}$ (Halliwell et al. 1989c), as follows:

$$2\text{H}^+ + \text{O}_2^{*-} + \text{O}_2^{*-} \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$$

SOD speeds up this reaction to $1.6 \times 10^9 \text{M}^{-1}\text{s}^{-1}$ (Halliwell et al. 1989c). There are three main types of SOD. CuZnSOD is the predominant cytosolic form of the enzyme and is a dimer of 32,000kD; MnSOD is found mostly in mitochondria and is a tetramer. A third type, extra-cellular SOD (EC-SOD) has been described which is
attached to carbohydrate and has a larger molecular mass (135,000kD) and is bound to endothelium, apparently providing a layer of antioxidant defence (Marklund 1990).

SOD protects prokaryotes and eukaryotes against oxidant stress. Examples are SOD-deficient mutant yeast, which are more prone to oxidant stress than wild type variants with normal levels of the enzyme (Gregory et al 1973). Furthermore, over-expression of SOD in Drosophila (fruit fly) mutants protects against paraquat toxicity (Reveillaud et al 1991). Encoding for CuZnSOD (chromosome 21q) has recently been shown to be abnormal in amyotrophic lateral sclerosis (Rosen et al 1993).

Catalase
Catalase consists of four Fe$^{3+}$-containing protophorphyrin subunits, specifically metabolises hydrogen peroxide, and is present in most aerobic organisms. Metabolism of hydrogen peroxide to water is accompanied by formation of an intermediate compound (compound I), as follows:

\[ \text{catalase-Fe}^{3+} + 2\text{H}_2\text{O}_2 \rightarrow \text{compound I} \rightarrow \text{catalase-Fe}^{3+} + 2\text{H}_2\text{O} + \text{O}_2 \]

Glutathione Peroxidase
Glutathione peroxidase is a selenium-containing enzyme which also metabolises hydrogen peroxide. Glutathione is the substrate for this reaction, in which two glutathione molecules are converted to oxidised glutathione. Glutathione is a tri-peptide (Gly-Cys-Glu; GSH); oxidised glutathione consists of two glutathione molecules bound by a disulphide bridge at the cysteine residue (GSSG). The oxidation of glutathione proceeds at the expense of hydrogen peroxide:

\[ 2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2\text{H}_2\text{O} \]

GSSG is then reconverted to GSH by glutathione reductase using NADPH as the energy source.

Transition Metal Chelation
The chemistry of Fenton chemistry is described above, and the potential for such a mechanism in IBD will be discussed in detail in Chapter 6. Free iron will catalyse the Fenton reaction, but chelation does not always remove this potential; for instance, iron chelates of ethylenediaminetetra-acetic acid (EDTA) are potent catalysts of the Fenton reaction (Smith et al 1990). However, most biological chelates do not support Fenton chemistry, and thus iron-binding by transferrin, ferritin and lactoferrin and copper-binding by caeruloplasmin and albumin forms part of the endogenous antioxidant defence (Halliwell et al 1989c).
PRO-INFLAMMATORY CONSEQUENCES OF EXCESS REACTIVE OXYGEN METABOLITE PRODUCTION

ROS may mediate the inflammatory response in a variety of ways. These are shown schematically in Fig 1.2, and discussed in more detail below.

Fig 1.2  The potential pro-inflammatory effects of ROS in inflammatory bowel disease.

Lipid Peroxidation

ROS can react with polyunsaturated fatty acids (PUVA) to form lipid peroxides that propagate free radical reactions by chain reaction. There are three phases to these reactions, initiation, propagation and termination (Pryor 1976); a PUVA is represented by LH:

**Initiation**

\[ \text{LH} + \text{oxidant (e.g. OH*)} \rightarrow \text{L}^* \]

**Propagation**

\[ \text{L}^* + \text{O}_2 \rightarrow \text{LOO}^* \]
\[ \text{LOO}^* + \text{LH} \rightarrow \text{LOOH} + \text{L}^* \]
\[ 2\text{LOO}^* + \text{H}^+ \rightarrow \text{LOH} + \text{LO}^* + \text{1O}_2 \]
Termination

\[ L^* + L^* \rightarrow \text{non-radical species} \]
\[ L^* + \text{LOO}^* \rightarrow \text{non-radical species} \]
\[ \text{LOO}^* + \text{LOO}^* \rightarrow \text{non-radical species} \]

Peroxy radicals may damage other cellular components, including proteins, carbohydrates, sulphhydryl side-chains and cause haemolysis of erythrocytes (Sandhu et al 1992).

Increased Mucosal Permeability

ROS-induced damage to the gastrointestinal mucosa increases permeability. Perfusion of the rat ileum with neutrophil-derived oxidants, hydrogen peroxide, hypochlorous acid and monochloramine, produced a dose-dependent increase in mucosal permeability to EDTA (Grisham et al 1990b). In the latter experiments, hypochlorous acid and monochloramine were more potent than hydrogen peroxide and, in vitro, were cytotoxic to cultured intestinal epithelial cells, whereas taurine monochloramine and hydrogen peroxide, were not (Grisham et al 1990b).

Permeability to \(^{51}\text{Cr}\)-labelled EDTA increased in isolated rat ileal loops perfused with fMLP, an effect shown to be due to ROS, as it was ameliorated by dimethylsulphoxide (DMSO), which scavenges the hydroxyl ion, and the iron chelator, desferrioxamine (von Ritter et al 1989). Ischaemia-reperfusion-induced increased permeability in the rat ileum can be ameliorated by SOD administered in conjunction with either allopurinol (Otamiri 1989), or catalase (Kanwar et al 1994). Intestinal permeability to EDTA was increased in rats with colitis induced by the free radical generating compound, mitomycin C (Keshavarzian et al 1992c). These effects do not appear to be purely due to ROS-induced cytotoxicity, as increased cell permeability to inulin occurs after exposure of cultured epithelial cells (Caco-2) to ROS at concentrations which do not alter cell viability (Baker et al 1995).

Increased Intestinal Secretion

The effects of ROS, including superoxide, hypochlorous acid, monochloramine and high concentrations of nitric oxide on increases of electrogenic chloride secretion in the rat intestine and in isolated intestinal epithelial cell monolayers, has recently been reviewed (Gaginella et al 1995). The secretory response is biphasic and is mediated by the enteric nervous system and prostaglandins (Bern et al 1988). Interestingly, the maximal response to monochloramine occurs at a concentration of 50\(\mu\)mol/l (Tamai et al 1990), equivalent to levels in the inflamed mucosa (Grisham et al 1990b). Nitric oxide promotes absorption under physiological conditions but increases secretion in pathophysiological states. The nitric oxide
synthase inhibitor, N⁰-nitro-L-methyl-arginine methyl ester (L-NAME), increases secretion in the normal rat ileum (Mailman 1994), but reverses luminal fluid accumulation in guinea pigs with ileitis (Miller et al 1993).

The effects of ROS on fluid secretion in the intestine must be taken in context with evidence that luminal fluid accumulation in the inflamed intestine is more likely to reflect decreased absorption than over-secretion, as secretory mechanisms are suppressed in the inflamed mucosa (Goldhill et al 1993; Bell et al 1995).

**Neutrophil Adhesion and Up-regulation of Adhesion Molecules**

ROS induce neutrophil binding to endothelial (intracellular adhesion molecule-1) ICAM-1, expressed on human umbilical cord vein endothelial cells, but do not alter its expression (Sellak et al 1994). The antioxidants dithiocarbamate and N-acetylcysteine inhibit the expression of VCAM-1, though not ICAM-1 on human umbilical cord vein endothelial cells (Marui et al 1993). Other evidence shows that ICAM-1 expression is up-regulated by hydrogen peroxide via activation of the transcription factor, AP-1; TNFα, in contrast, upregulates ICAM-1 via the transcription factor, nuclear factor-kappa B (NFκB) activation (Roebuck et al 1995).

**Interference with Cell Metabolism**

Disturbances of cellular function may also result from the reaction of ROS with carbohydrates, proteins and DNA. Reaction with proteins activates collagenase, inhibits anti-proteases (Weiss 1986a) and it has been suggested that an important action of HOCl may be inactivation of antiproteinas that limit the actions of the neutrophil-derived enzymes involved in tissue destruction (Weiss 1989). For instance, neutrophil elastase is released at concentrations that allow solubilization of the subendothelial matrix (Weiss et al 1986b), but normally has a very short half-life (3msec) (Travis et al 1983), due to inactivation by α₁-proteinase inhibitor (Wasil et al 1987b). Inactivation of α₁-proteinase inhibitor by HOCl may thus readily exacerbate tissue damage by extending the activity of elastase.

The role of ROS in promoting carcinogenesis via DNA damage has been extensively researched (Cerutti 1985; Ames 1989), and by-products of oxidative DNA damage are excreted in human urine (Ames 1989). It is possible that the increased risk of colorectal cancer in IBD is related to the chronic mucosal exposure to ROS.

**Activation of the Complement Pathway**

The hydroxyl ion can activate the alternative complement pathway, releasing C5a, which has a role in neutrophil activation, and causes erythrocyte lysis (Vogt et al 1987).
Release of Pro-inflammatory Mediators

Arachidonic acid metabolism can result in lipid peroxidation, and free radical reactions can, in turn, initiate arachidonic acid metabolism resulting in the activation of phospholipase A2 (Chakraborti et al 1989) and 5-lipoxygenase (Riendeau et al 1989) (as above). In addition ROS appear to directly promote neutrophil chemotaxis (Zimmerman et al 1990). Hydrogen peroxide induces histamine release from mast cells (Ohmori et al 1978), stimulates the synthesis of PAF by the bovine pulmonary artery endothelium, induces neutrophil adhesion to human umbilical vein endothelial cells (Lewis et al 1988) and induces prostaglandin release by the rat colon in vitro (Murthy et al 1990). ROS also act as second messengers for the expression of the monocyte chemoattractant protein-1 (MCP-1), as TNFα-induced stimulation of this cytokine was inhibited by dimethylthiourea, and was stimulated by superoxide, though not hydrogen peroxide (Satriano et al 1993).

Inactivation of Antioxidant Defence Mechanisms

There is good evidence that ROS interfere with enzymatic and non-enzymatic endogenous antioxidant defence; such a mechanism may be of importance in understanding the persistence of inflammation in IBD.

Superoxide is capable of inactivating a number of important enzymes, including catalase (Kono et al 1982) and glutathione peroxidase (Blum et al 1985). Hypochlorous acid is also capable of inactivating catalase (Mashino et al 1988; Beyer et al 1990) and glutathione peroxidase (Aruoma et al 1987a), and depletes ascorbic acid (Halliwell et al 1987). Peroxides inactivate pyruvate dehydrogenase (Vlessis et al 1991), part of the mechanism of oxygen detoxification in eukaryotic cells, and thus its inactivation by ROS may promote further oxidant damage. Prolonged release of hydrogen peroxide by stimulated neutrophils overpowers natural defences, such as taurine, leading to neutrophil-induced tissue damage (Nathan 1988). HOCl may exert an additional oxidant effect by release of iron from haem-containing proteins (Rosen et al 1985).

In vivo studies confirm that antioxidant depletion occurs in circumstances of oxidant stress. Cardiothoracic surgery (Ballmer et al 1994) and smoking (Tribble et al 1993) reduce vitamin C, though not vitamin E levels, in humans. Possible free radical-mediated inflammatory disorders, such as rheumatoid arthritis, are also associated with depletion of antioxidants (Lunec et al 1985; Honkanen et al 1989). Antioxidant defence in IBD is dealt with below (p 39).

Activation of Nuclear Transcription Factors

Cytokines, such as IL-1 and TNFα, render the endothelium more susceptible to ROS-induced injury and also promote ROS release from neutrophils (Ward et al
In turn, ROS activate a number of transcription factors including NFκB (Schreck et al 1991) and c-fos (Amstad et al 1992). Of these, NFκB is particularly interesting, as it is involved in the induction of many genes with pro-inflammatory gene products including, IL-1, IL-6, IL-8, IL-2 receptor, E-selectin and adhesion cell molecules (Baeuerle et al 1991), which will stimulate further tissue oxidant stress by neutrophil recruitment, thus setting up a potential positive-feedback loop. NFκB exists in the cytosol bound to an inhibitor, IκB. Activation results in release of the p65 component which migrates into the nucleus (Fig 1.3). The activation of NFκB by diverse stimuli is inhibited by antioxidants suggesting that there may be a common pathway of activation involving ROS (Schreck et al 1991).

Glucocorticoids appear to act by increasing transcription of IκB (Marx 1995) and salicylates inhibit NFκB mobilisation (Kopp et al 1994; Weber et al 1995) and IL-1 mediated activation of NFκB (Barve et al 1995). It is interesting to hypothesise that inflammatory disorders, such as IBD, might result from inherited disorders of NFκB regulation. This pathway may prove to be of vital importance in understanding the mechanisms for ROS-induced tissue damage.

![Diagram of NFκB activation](image)

Fig 1.3 The role of NFκB in the ROS-mediated activation of cytokine production. (Redrawn from Baeuerle et al (1991).)
BENEFICIAL EFFECTS OF FREE RADICALS IN BIOLOGICAL SYSTEMS

Antimicrobial Defence

Hydrogen peroxide, superoxide and hypochlorous acid are part of the neutrophil and macrophage microbial defence system. Individuals with chronic granulomatous disease, who have deficiency of the oxidases responsible for production of superoxide, suffer persistent and recurrent bacterial infections. Hypochlorous acid appears of lesser importance for microbial killing as clinically important infections are rather uncommon in hereditary myeloperoxidase deficiency, though neutrophil killing in vitro is impaired (Parry et al 1981).

Maintenance of Vascular Tone

NO has an important role in maintenance of vascular tone via reduction of vascular smooth muscle contractility by cGMP; this is inhibited by superoxide, which thus has a vasoconstrictive effect, possibly via the formation of peroxynitrite.

REACTIVE OXYGEN SPECIES IN INFLAMMATORY BOWEL DISEASE

A predominant feature of IBD is the infiltration of the lamina propria with neutrophils which, when activated, produce large quantities of ROS (Klebanoff 1992) which, in addition to episodes of ischaemia-reperfusion (Grisham et al 1988), are likely to be the main sources for the observed excess of ROS in IBD (Fig 1.4).

Fig 1.4 The main sources of ROS in IBD, showing the important molecules and enzymes (italics) involved in ROS production and antioxidant defence.
Systemic Reactive Oxygen Species in Inflammatory Bowel Disease

Peripheral Blood Neutrophils

Many studies have examined the oxidative metabolism of circulating neutrophils in IBD, with conflicting results (studies with UC shown in Table 1.10). In children with IBD, circulating neutrophils produce increased luminol-amplified chemiluminescence compared to controls both in the resting state and in response to opsonized zymosan (Faden et al 1985). In adult UC, the results vary with the methods used for neutrophil stimulation and detection of ROS activity, but the results in general either indicate defective oxidative neutrophil metabolism in active UC or show no difference from controls (Table 1.10).

The results are similarly variable for CD. There is a reduction in the oxidation of ferricytochrome c by neutrophils stimulated by phorbol-12-myristate-13-acetate (PMA) (Verspaget et al 1984; Curran et al 1991; Solis-Herruzo et al 1993). Conversely, nitroblue tetrazolium reduction (Ward et al 1976) and chemiluminescence response are increased in response to neutrophil stimulation with fMLP (Anton et al 1989), though others have found a decrease in the latter (Solis-Herruzo et al 1993), and ferricytochrome c reduction is reduced after stimulation with PMA (Gionchetti et al 1994). Circulating neutrophil hydrogen peroxide production is negatively correlated with disease activity in CD (Verspaget et al 1984) and UC (Verspaget et al 1986). These disparities may reflect the variable nature of the disease in extent and severity, and the nature of therapy used; the method of cell separation used may also influence the results (Kelleher et al 1990). In a recent study the oxidative metabolism of peripheral blood neutrophils was compared in patients with inactive and active UC, who were not taking any medication, and with controls. Stimulation with fMLP and tetradecanoylphorbol acetate was used to stimulate ROS production which was detected by chemiluminescence. There were no differences between the groups in peak chemiluminescence, area-under-the-curve or time to peak chemiluminescence, though 27% of the patients with active UC had increased chemiluminescence to stimuli (Haydek et al 1995).

Other studies of neutrophil metabolism in IBD are similarly inconsistent. Whereas neutrophil elastase activity was no different from controls in one study (Gionchetti et al 1994), other studies have demonstrated an increase in CD and UC in active compared to inactive disease (Andus et al 1993).

Recent evidence has shown that sera from patients with IBD, and particularly active UC, stimulate normal neutrophils to produce more superoxide than control sera (Griga et al 1995). It is therefore possible that impaired neutrophil ROS production in IBD reflects an autoregulatory process as a result of hyperstimulation by circulating inflammatory mediators, such as cytokines and eicosanoids.
In this context, peripheral neutrophil responses to colonic inflammation have recently been studied in TNBS-induced colitis in rats (Carter et al 1995). Phagocytosis was persistently enhanced, and chemotaxis depressed, for at least 2 weeks; however, although LTB₄ production transiently decreased, and superoxide production transiently increased, both returned to normal by seven days. Interestingly, LTB₄ production was inversely related to the peripheral blood neutrophil count, suggesting an autoregulatory process.

Table 1.10 Oxidative metabolism of neutrophils in ulcerative colitis.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Assay</th>
<th>Active UC</th>
<th>Inactive UC</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>fMLP</td>
<td>Chemiluminescence</td>
<td>(\Leftrightarrow)</td>
<td>(\Leftrightarrow)</td>
<td>(Anton et al 1989)</td>
</tr>
<tr>
<td></td>
<td>Chemiluminescence</td>
<td>(\downarrow)</td>
<td>(\downarrow)</td>
<td>(Williams 1990)</td>
</tr>
<tr>
<td></td>
<td>Chemiluminescence</td>
<td>(\Leftrightarrow)</td>
<td>(\Leftrightarrow)</td>
<td>(Haydek et al 1995)</td>
</tr>
<tr>
<td>fMLP-cyt E</td>
<td>Ferricytochrome c</td>
<td>(\Leftrightarrow)</td>
<td>(\Leftrightarrow)</td>
<td>(Verspaget et al 1986)</td>
</tr>
<tr>
<td></td>
<td>Homovanillic acid</td>
<td>(\Leftrightarrow)</td>
<td></td>
<td>(Verspaget et al 1986)</td>
</tr>
<tr>
<td>PMA</td>
<td>Chemiluminescence</td>
<td>(\Leftrightarrow)</td>
<td>(\uparrow)</td>
<td>(Suematsu et al 1987)</td>
</tr>
<tr>
<td></td>
<td>Ferricytochrome c</td>
<td>(\downarrow)</td>
<td></td>
<td>(Verspaget et al 1986)</td>
</tr>
<tr>
<td></td>
<td>Ferricytochrome c</td>
<td>(\downarrow)</td>
<td></td>
<td>(Gionchetti et al 1994)</td>
</tr>
<tr>
<td></td>
<td>Homovanillic acid</td>
<td>(\Leftrightarrow)</td>
<td></td>
<td>(Verspaget et al 1986)</td>
</tr>
<tr>
<td></td>
<td>oxidation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nitroblue tetrazolium</td>
<td>(\Leftrightarrow)</td>
<td></td>
<td>(Williams 1990)</td>
</tr>
<tr>
<td>PMA-cyt E</td>
<td>Ferricytochrome c</td>
<td>(\downarrow)</td>
<td></td>
<td>(Verspaget et al 1986)</td>
</tr>
<tr>
<td>Zymosan</td>
<td>Homovanillic acid</td>
<td>(\Leftrightarrow)</td>
<td></td>
<td>(Verspaget et al 1986)</td>
</tr>
<tr>
<td></td>
<td>oxidation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Opsonized</td>
<td>Ferricytochrome c</td>
<td>(\downarrow)</td>
<td></td>
<td>(Verspaget et al 1986)</td>
</tr>
<tr>
<td>zymosan</td>
<td>Homovanillic acid</td>
<td>(\Leftrightarrow)</td>
<td></td>
<td>(Verspaget et al 1986)</td>
</tr>
<tr>
<td>Latex</td>
<td>Chemiluminescence</td>
<td>(\Leftrightarrow)</td>
<td></td>
<td>(Williams 1990)</td>
</tr>
<tr>
<td>Opsonized</td>
<td>Nitroblue tetrazolium</td>
<td>(\uparrow)</td>
<td></td>
<td>(Wandall et al 1982)</td>
</tr>
<tr>
<td>lipopolysaccharide</td>
<td>reduction</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

fMLP-cyt E = formyl-methionyl-leucyl-phenylalanine in combination with cytochalasin E.
PMA-cyt E = phorbyl-12-myristate-13-acetate in combination with cytochalasin E.
Peripheral Blood Monocytes

In contrast to studies with neutrophils, there is more consistent data on ROS production by peripheral blood monocytes from patients with both UC and CD, with several studies demonstrating increases in ROS production by peripheral blood monocytes in response to a variety of stimuli, such as fMLP (Williams 1990), PMA (Suematsu et al 1987; Kitahora et al 1988), opsonized zymosan (Okabe et al 1986; Kitahora et al 1988) and latex (Williams 1990).

Reactive Oxygen Metabolites in the Gastrointestinal Tract in Inflammatory Bowel Disease

Isolated Intestinal Phagocytes

Studies of the oxidative metabolism of phagocytes isolated from the inflamed mucosa of patients with CD, UC and appendicitis, consistently show increases in ROS production, as measured by luminol-amplified chemiluminescence when stimulated by PMA, fMLP and latex beads as compared with those isolated from non-inflamed or control mucosa (Williams 1990). However, in these studies no differences were found in the basal levels of chemiluminescence between phagocytes isolated from inflamed and uninflamed tissue. These results are confirmed using techniques other than chemiluminescence; reduction of nitroblue tetrazolium (a measure of superoxide production) under the influence of PMA and zymosan is greater in IBD than in controls (Mahida et al 1989a), and reduction of cytochrome c is greater in intestinal than circulating monocytes (Verspaget et al 1985). Rugtveit et al, confirmed increased superoxide production by intestinal macrophages stimulated by interferon-γ and LPS. Furthermore, he showed that superoxide production of macrophages isolated from the inflamed mucosa, but depleted of CD14+ cells, was equivalent to that of macrophages isolated from uninflamed intestine (Rugtveit et al 1995); suggesting that the increased respiratory burst activity in macrophages isolated from inflamed bowel is largely due to recently recruited macrophages, rather than to stimulation of the pre-existing resident population.

Mucosal Tissue in vitro

ROS production by colorectal biopsies from patients with IBD is significantly greater in biopsies from IBD than in controls, is proportional to the degree of inflammation (Simmonds et al 1992a; Keshavarzian et al 1992b), and is thought to be largely due to increased numbers of activated macrophages and neutrophils recruited to the inflamed mucosa of patients with active IBD (Simmonds et al 1992a; Keshavarzian et al 1992b). Reduction of nitro-blue tetrazolium, as a marker of superoxide production locates both to inflammatory cells, and vascular endothelium
and epithelium in UC (Oshitani et al 1993). Neither SOD nor catalase inhibited this reduction, an anomaly attributed to poor tissue penetration by these enzymes.

Lipid peroxidation is also increased in the mucosa in IBD (Ahnfelt-Ronne et al 1990), and is further suggested by increased levels of breath pentane, an alkane generated by peroxidation of cellular fatty acids, which correlated with indium-labelled leukocyte scanning (Kokoszka et al 1993). The resulting oxidant stress may overwhelm the endogenous defences which regulate ROS production during normal metabolism, particularly if there are relatively low tissue levels of endogenous antioxidants, as in the colonic mucosa (Grisham et al 1990c).

As with experiments using isolated neutrophils, measurement of ROS production by mucosal biopsies in IBD must take into account the possible effects of mechanical stimulation involved in harvesting and processing the mucosal specimens. It is therefore difficult to know if the noted differences between inflamed, non-inflamed and control mucosa (Simmonds et al 1992a) reflect a true excess of ROS production in vivo or merely a potential for excess ROS production.

**ROS in the intestinal lumen**

There are few data on this, though oxidative products of 5-ASA metabolism are found in the faeces of patients with IBD that are distinct from those due to auto-oxidation (Ahnfelt-Ronne et al 1990; Jensen et al 1993).

**Nitric Oxide in Inflammatory Bowel Disease**

NO is produced in excess by the inflamed mucosa (Boughton-Smith et al 1993; Middleton et al 1993; Rachmilewitz et al 1995) and is detected in the lumen (Lundberg et al 1994) in UC. The potential sources include the infiltrating monocytes, macrophages, neutrophils, submucosal blood vessels and epithelium (Table 1.11).

<table>
<thead>
<tr>
<th>Source of nitric oxide</th>
<th>Enzyme type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endothelium</td>
<td>Constitutive</td>
</tr>
<tr>
<td>Non-adrenergic, non-cholinergic enteric nerves</td>
<td>Constitutive</td>
</tr>
<tr>
<td>Epithelium</td>
<td>Inducible</td>
</tr>
<tr>
<td>Macrophages/Monocytes</td>
<td>Inducible</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>Inducible</td>
</tr>
<tr>
<td>Mast cells</td>
<td>Inducible</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>Inducible</td>
</tr>
</tbody>
</table>

NO protects the gastrointestinal mucosa by maintaining perfusion, inhibiting
neutrophil and platelet adhesion, and preventing mast cell activation in TNBS-induced colitis in rats (Saltman 1995). However at the high concentrations which result from production by the inducible isoform of NO synthase, disruption of cellular tight junctions and increased endothelial permeability ensue (Saltman 1995). NO thus has both beneficial and pro-inflammatory effects. Inhibition of NO synthase is protective in TNBS-induced colitis (Miller et al 1993; Hogaboam et al 1995; Rachmilewitz et al 1995), acetic acid/casein-induced colitis (Rachmilewitz et al 1995) and peptidoglycan/polysaccharide (PG-PS)-induced colitis (Grisham et al 1994b). However others have shown that high doses of the NO synthase inhibitor L-NAME are proinflammatory in the rat (Pfeiffer et al 1995) and guinea pig intestine (Miller et al 1994). The relative benefits of NO inhibition or stimulation as therapy in IBD remain uncertain, though specific inhibitors of inducible NO synthase would seem the most promising avenue for future research. Recently, it has been shown that TNBS-induced colitis is ameliorated by selective inhibition of inducible NO synthase by aminoguanidine (Miller et al 1995).

**Antioxidant Defence in Inflammatory Bowel Disease**

The endogenous antioxidant defence mechanisms in the mucosa in IBD are shown in Fig 1.5
Patients suffering acute attacks of IBD have been reported to have low serum levels of the antioxidant vitamins, β-carotene, α-tocopherol, (Abad-Lucruz et al 1988; Fernandez-Banares et al 1989; Fernandez-Banares et al 1990), and ascorbate (Pettit et al 1989; Fernandez-Banares et al 1991). However, other workers have suggested that the reduced serum levels of tocopherol in CD correlate more with low lipid levels than with the activity or extent of the disease (Kuroki et al 1994).

In CD levels of reduced glutathione are decreased, and oxidised glutathione increased, in both the inflamed and uninflamed mucosa (Iantomasi et al 1994). The function of glutathione peroxidase may be further impaired by low levels of the co-enzyme, selenium, in IBD (Penny et al 1983; Rannem et al 1992). In addition, levels of the antioxidant enzymes, glutathione peroxidase, SOD and catalase, are low in the intestinal mucosa of normal human colon, the activity being 8, 4, and 40%, respectively, of that in the liver (Grisham et al 1990c), suggesting that the intestine may be more susceptible to oxidant damage than other tissue. Furthermore levels of mucosal CuZnSOD levels are reduced in IBD compared to controls (Verspaget et al 1988; Mulder et al 1991).

**Antioxidant Activity of Drug Therapy in Inflammatory Bowel Disease**

If ROS are of pathogenic importance in IBD then current therapies would be expected to have antioxidant activity. Indeed, both 5-ASA and sulphasalazine are potent antioxidants (Craven et al 1987; Miyachi et al 1987; Ahnfelt-Ronne et al 1990). 5-ASA scavenges superoxide (IC$_{50}$ 10-20µM) (Yamada et al 1990) and the hydroxyl radical, though not lipid radicals (Allgayer et al 1992). However, 5-ASA does inhibit lipid peroxidation *in vitro* (Greenfield et al 1991). 5-ASA also scavenges hypochlorous acid (Aruoma et al 1987a; Williams et al 1989).

5-ASA also acts as an iron chelator (IC$_{50}$ 300µM) (Yamada et al 1990), which may reduce the production of ROS via the Haber-Weiss and Fenton reactions, and inhibits myeloperoxidase (IC$_{50}$ 20µM) (Yamada et al 1990). However, when chelated with iron at a ratio of ≥1:1, 5-ASA acts as a pro-oxidant (Grisham et al 1992).

5-ASA (0.3mM) also reduces PMA-, fMLP- and, to a lesser extent, sodium fluoride-induced superoxide production by isolated, human neutrophils (Allgayer et al 1994), which are thought to be the prime source of ROS in IBD (Simmonds et al 1992a; Keshavarzian et al 1992b).

The concentrations required for the main rival potential anti-inflammatory actions of 5-ASA, inhibition of cyclo-oxygenase, lipoxygenase and fMLP binding to neutrophils, are much higher (IC$_{50}$ 10, 6, 5mM, respectively) than those required for its *in vitro* antioxidant activity (Horn et al 1991), which are closer to plasma levels (10-15µM) (Yamada et al 1990). However the concentration of 5-ASA required to
reduce LTB₄ production by biopsies of normal colorectal mucosa (median, 3.5mM) (Peskar et al 1987) is fairly similar to those required to inhibit ROS production by inflamed colorectal biopsies from patients with active IBD (Simmonds et al 1992a). Thus, the question as to the exact mode of action of 5-ASA remains unanswered. Mucosal biopsies, from patients with active UC, exposed to equivalent concentrations to those found in the bowel lumen (5-10mM) (Staerk-Laursen et al 1990), demonstrate a substantial reduction of ROS production in vitro (Simmonds et al 1992c). Furthermore, mucosal lipid peroxidation is reduced in vivo in IBD patients taking aminosalicylates (Ahnfelt-Ronne et al 1990).

There is no adequate data on the mucosal concentration of 5-ASA in humans. Estimated mucosal concentrations of 5-ASA in cats by assay of the local venous drainage following luminal perfusion, were approximately 650µM and 160µM in the terminal ileum and colon, respectively (Grisham et al 1989).

Hydrocortisone and other glucocorticoids are highly effective in the treatment of active IBD, and although they do not directly scavenge ROS (Simmonds et al 1992c) they do inhibit the function of neutrophils (Davies et al 1984; Baltch et al 1986). One study has demonstrated disturbed function of NADPH oxidase, and thus superoxide generation, by neutrophils treated with steroids (Umeki et al 1992).

There are very limited data on the use of specific antioxidant therapy in IBD. In steroid-resistant CD, a combination of SOD and desferrioxamine was reportedly effective in an uncontrolled study (Emerit et al 1991), which, if substantiated, would suggest that superoxide, and/or iron, is pathogenic. Allopurinol has been used successfully in another uncontrolled study in acute and chronic pouchitis (Levin et al 1992), suggesting that the disease may be mediated by superoxide produced by xanthine oxidase.

Excess ROS may be pathogenic in experimental models of intestinal inflammation (Keshavarzian et al 1990; Grisham et al 1991) and antioxidants have successfully been used to treat this inflammation (Yoshikawa et al 1992; Keshavarzian et al 1992a). This subject is discussed in more detail in Chapter 3. In Chapter 8 the possible use of specific antioxidant nutrients in treating active UC is explored.
AIMS OF THIS THESIS

The broad aim of this thesis is to develop methodologies for the assessment of the effects of antioxidant therapy in IBD in vitro and in vivo. Demonstration of the efficacy of antioxidant therapy could establish the importance of ROS in the pathogenesis of IBD.

Chapter 2 - Analytical Methods and Materials

The main experimental and statistical methods for experiments used in later chapters are described.

Chapter 3 - Establishment of the Acetic Acid-Induced Model of Colitis

Recent findings from this laboratory have shown that ROS production by rectal biopsies from patients with UC and CD can be measured in vitro using a chemiluminescence technique, and that addition of conventional antioxidants reduces the measurable ROS (Simmonds et al 1992a). Application of human tissue for screening for the potential efficacy of new drugs is hampered by the limited availability of biopsy material from patients with active UC. Acetic acid-induced colitis in the rat has been widely used as a model for inflammatory bowel disease, having similarities to UC in its histological appearances, lipid mediator release and response to therapy, as discussed in Chapter 3, p. 67. It has the added advantages of being simple, rapid and inexpensive to induce. The aim of this initial study was to establish a reliable method of inducing colitis with provision of adequate quantities of material for in vitro studies and to compare the chemiluminescence responses of the biopsy material with the macroscopic and microscopic scores of the inflammation.

Chapter 4 - Evaluation of Antioxidant Compounds Using Colonic Biopsies from the Acetic Acid-Induced Model of Colitis in Vitro

The aim of this study was to develop a method for screening compounds for antioxidant effects using colorectal biopsies from acetic acid-induced colitis in rats. The technique was validated by demonstrating comparable chemiluminescence responses using biopsies from acetic acid-induced colitis with previous data using biopsies from patients with active UC (Simmonds et al 1992a; Simmonds 1992b; Simmonds et al 1992c) to a range of conventional antioxidants (sodium azide, catalase, taurine, reduced glutathione, CuZnSOD, DMSO, N-acetylcysteine, ascorbate) and standard therapies for IBD (5-ASA, hydrocortisone). In addition, the effects of potential new antioxidant approaches in the form of two novel antioxidants, LY231617 and amflutizole, were studied.
Chapter 5 - Chemiluminescence of Human Colorectal Mucosal Biopsies

Luminol-amplified chemiluminescence correlates with the macroscopic and microscopic scores of colorectal mucosal inflammation in UC (Simmonds et al 1992a). However, a correlation with clinical disease activity has not been shown. The aim of this study was to extend the studies previously performed in this laboratory (Simmonds et al 1992a) to examine the relationship between mucosal ROS production and clinical and laboratory measures of disease activity (modified Harvey-Bradshaw Index (Stevens et al 1992).

Chapter 6 - The Antioxidant and Anti-inflammatory Efficacy of Human Recombinant Manganese Superoxide Dismutase

Human recombinant manganese superoxide dismutase (rh-MnSOD) was made available by Bender & Co, Ges mbH, Austria. It has potential advantages over CuZnSOD in having a long plasma half-life and being less susceptible to oxidant damage. Its human origin makes it less likely, if given in man, to induce antigenic reactions. The antioxidant potential of rh-MnSOD in cell-free systems, using colonic biopsies from acetic acid-induced colitis in vitro, and in acetic acid-induced colitis in vivo was explored.

Chapter 7 - The Role of Iron Chelation as Antioxidant Therapy in Inflammatory Bowel Disease

To explore the role of iron-mediated ROS production in the inflamed mucosa, the iron chelators desferrioxamine, which chelates ferrous ions, and 1,10-phenanthroline, which chelates ferric ions, and the addition of exogenous iron, were assessed for their ability to alter ROS production by inflamed colorectal biopsies from acetic acid-induced colitis and UC.

Chapter 8 - Antioxidant Nutrient Therapy in Active Ulcerative Colitis

To determine the importance of reactive oxygen species in active UC a pilot study was carried out to examine the therapeutic response to specific antioxidant therapy in patients with active left-sided or distal colitis. Patients received antioxidant nutrient vitamins, in addition to their usual therapy, for two to eight weeks. The prime aim of this small pilot study was to ensure the safety of antioxidant nutrient therapy in active ulcerative colitis. The effects of antioxidant nutrient therapy on clinical and laboratory measures of disease activity and on ROS production in the plasma and mucosa was also assessed.

Chapter 9 - General Discussion

The main findings of this thesis are summarised and discussed, particularly with respect to future studies.
METHODS AND MATERIALS

The statistical and experimental methods described in this chapter are those used in subsequent chapters. Modifications in the statistics and assays for individual experiments are described in the relevant chapters.

STATISTICAL ANALYSIS

Computer-Aided Analysis

Analyses were performed using the computer software programmes Statview™ and Instat™ and were carried out on an Apple Macintosh™ IIci computer.

Graphing Methods

For display of data in this thesis, the preferred method is the 'Box and Whiskers' plot in which the median is represented as a heavy line, the 75th centiles as a box around this line, the 95th centile as the 'whiskers' and the outliers shown as individual points. Where appropriate the data is displayed as a scattergram or as a line chart showing the mean and standard deviation (SD).

Comparison of Unpaired Groups with Non-Parametric Data

The data in this thesis is predominantly non-parametric. Therefore comparisons of groups were carried out using the Mann-Whitney U test (two-tailed); a P value of less than 0.05 was taken as significant. For multiple comparisons an initial analysis was carried out using the Kruskal-Wallis test, followed by the Mann-Whitney U test with the Bonferroni correction for multiple comparisons. The Bonferroni method for comparing multiple groups relies on multiplying the derived P value from individual comparisons by the total number of comparisons made (Godfrey 1985).

Comparison of Paired Groups with Non-Parametric Data

Comparisons between groups of paired data were made using the Wilcoxon-signed rank test (two-tailed). The Bonferroni correction was made for multiple comparisons.
Variability of Assays

The coefficient of variation (CV) was used to assess the variability of assays within groups. The CV was therefore used to describe, for instance the variability of the chemiluminescence response of colonic biopsies from rats with acetic acid colitis. The CV is calculated as follows: \( CV = 100 \times \frac{SD}{\text{mean}} \) (Snedecor 1956). Using the example of the chemiluminescence response by colonic biopsies from acetic acid-induced colitis, the CV for the chemiluminescence response of a number of biopsies from the same animal is calculated. To assess the CV in groups of animals the median (IQR) of the CV between similarly prepared animals is then calculated. For calculation of the CV where only one measure is available from each individual, the CV is calculated as 100 x SD/mean of the group.

Calculation of the Intra- and Inter-Observer Repeatability of Scoring Systems and Intra- and Inter-Assay Repeatability

To assess the repeatability of assays and scoring systems, the intra- and inter-observer variability and intra- and inter-assay variability was calculated using the coefficient of repeatability (CR) (Bland et al 1986). The CR is defined as the twice the standard deviation of the differences between the two measurements, and is valid if 95% of the differences fall within this figure, as defined by the British Standards Institution (British Standards Institution 1979).

Correlation Between Assay Measurements and Scoring

Correlations between the assays were assessed using Spearman’s rank correlation test. A P value of < 0.05 was taken as indicating a significant relationship between the two test variables. For graphical representation the regression line is demonstrated and its descriptive equation included in the legend.

Multiple Linear Regression Analysis

Multiple linear regression analysis was performed to determine which factor of the histological scoring systems for both acetic acid-induced colitis and UC most closely correlated with luminol-amplified chemiluminescence.

ACETIC ACID-INDUCED COLITIS

The stepwise development of the acetic acid-induced model of experimental colitis is described in detail in Chapter 3. The final protocol followed for subsequent experiments is described below.
**Induction Of Acetic Acid-Induced Colitis in Rats**

Acetic acid-induced colitis was induced in male Wistar rats (150–200g, Charles River, UK) using a modification of the method described by MacPherson and Pfeiffer (MacPherson 1977). The animals were housed in light/dark controlled conditions and fed a standard chow diet. Prior to induction of colitis animals and controls were fasted for 16 hours with access to water ad libitum. Each rat was sedated by brief respiration of 20–25% CO$_2$ followed by anaesthesia with 35mg/kg intraperitoneal pentobarbital. An infant feeding tube (Pennine Healthcare FT-1608/40, OD 2mm (Plate 2.1) was inserted into the colon to 8cm and 2 ml of acetic acid (3% v/v in 0.9% saline) or saline alone (control animals) infused into the colon. The acetic acid/saline was retained in the colon for 30sec, after which fluid was withdrawn. The rats were killed at 24 hours by CO$_2$ asphyxiation.

**HISTOLOGY**

The methods for processing of histological material common to both UC and acetic acid-induced colitis are described below.

**Light Microscopy**

Colonic biopsies from rats with acetic acid-induced colitis, those given 0.9% saline alone (saline controls) and absolute controls, and colorectal biopsies from patients with IBD and normal controls, were transferred to 10% formol saline. Biopsies were taken from macroscopically normal mucosa at least 10cm distant from any lesion. Human control colorectal biopsies were from patients undergoing sigmoidoscopy or colonoscopy for suspected colonic carcinoma or polyps. The biopsies were wax-embedded and sectioned using a Leitz Wetzlar Microtome, Germany.

**Haematoxylin and Eosin Staining Procedure**

Sections were transferred to glass slides and dried overnight at 60°C. The slides were de-waxed in xylene, dehydrated in 100% alcohol, and re-hydrated in running tap water. Staining was carried out using Mayers haematoxylin for 10min, then washed in running tap water for 5min, stained in 1% aqueous eosin for 5min, washed in running tap water for 3min, dehydrated in industrial methylated spirit, then cleared in xylene and mounted with DPX mountant (BDH laboratory supplies, UK).

**MEASUREMENT OF REACTIVE OXYGEN SPECIES IN VITRO**

Because of their high reactivity, and short-half life, the measurement of most ROS in biological systems relies on identification of the presence of ROS using detector molecules. Such detection molecules may be endogenous, so-called
“footprints” of oxidative damage, such as the measurement of 8-hydroxyguanine residues (metabolite of free radical attack on guanine residues of DNA (Cheng et al 1992), or exogenous detectors, such as chemiluminescence amplifiers, electron spin resonance (ESR) traps, and thiobarbituric acid reactive substances (TBARS). Methods which rely on the detection of footprints of oxidative stress may be inaccurate if non-ROS-mediated reaction products are detected, and use of exogenous compounds may cause inaccuracies by altering the balance between radical consumption and production (Nahum et al 1989). The main methods used for detection of ROS are shown in Table 2.1; those used in this thesis are described in more detail below.

CHEMILUMINESCENCE TECHNIQUES

Detection of ROS by amplified chemiluminescence requires a photo-multiplier tube, linked to a sensitive photon detector and an amplifier/discriminator which converts the pulses into a digital read out. A dedicated chemiluminometer or scintillation counter, adapted for chemiluminescence, is appropriate for this purpose (Plate 2.1).

Liberation of energy as light is well-recognised in oxidation reactions (Cadenas 1986), an example being the respiratory burst of neutrophils, during which light is emitted with a wavelength of 380–460nm, resulting from the decay of excited carbonyl-combinations, the dismutation of superoxide and from singlet oxygen (Weber 1990). The light production by these reactions is, however, extremely low, and although it is detectable by highly sensitive detectors in several biological systems, including experimental colitis (Keshavarzian et al 1992b), quantitative analysis is difficult. Accordingly, fluorescent dyes have been developed to act as amplifiers, the most commonly used being luminol and lucigenin. These react with ROS to liberate energy as photons with a relatively high quantum yield.

Luminol- and Lucigenin-Amplified Chemiluminescence

Luminol and lucigenin react with oxidants, such as ROS, to form 3-aminophthalate and N-methylacridone, respectively. Electrons in the luminol and lucigenin are elevated to higher energy levels during the reaction with oxidants (Fig 2.1). On reverting to the ground state, energy is released in the form of photons which are detected by the photomultiplier tubes and photon detectors which comprise the scintillation counter (Allen 1982) (Fig 2.2).

Luminol (5-amino-2,3-dihydrophthalalzindion-1,4) reacts with ROS with a quantum yield of between 1 and 2%, and emits light at 450nm (Weber 1990). This reaction can be used to study the formation kinetics of ROS by biological tissues, in

Table 2.1 Some of the techniques for detection of ROS in biological systems.

<table>
<thead>
<tr>
<th>Assay</th>
<th>ROS detected</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Direct, Non-specific Methods</strong></td>
<td></td>
</tr>
<tr>
<td>Ultra-weak chemiluminescence</td>
<td>$O_2^-$, $^{1}O_2$, RO$^-$</td>
</tr>
<tr>
<td>Amplified chemiluminescence</td>
<td>$O_2^-$, $H_2O_2$, HOCl, $^{1}O_2$, ROO$^*$, H$^+$</td>
</tr>
<tr>
<td>Electron Spin Resonance and Spin-</td>
<td></td>
</tr>
<tr>
<td>trapping</td>
<td></td>
</tr>
<tr>
<td>Phycoerythrin fluorescence</td>
<td>ROO$^<em>$, OH$^</em>$</td>
</tr>
<tr>
<td>UV-spectrophotometer</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Direct, Specific Methods</strong></td>
<td></td>
</tr>
<tr>
<td>Nitroblue tetrazolium reduction</td>
<td>$O_2^-$</td>
</tr>
<tr>
<td>Reduction of ferricytochrome c</td>
<td>$O_2^-$</td>
</tr>
<tr>
<td>Nitric oxide gas electrodes</td>
<td>NO</td>
</tr>
<tr>
<td>Scopoletin fluorescence</td>
<td>Extracellular $H_2O_2$</td>
</tr>
<tr>
<td>Homovanillic acid fluorescence</td>
<td>Extracellular $H_2O_2$</td>
</tr>
<tr>
<td>Monitoring catalase-$H_2O_2$ (compound I)</td>
<td>Intracellular $H_2O_2$</td>
</tr>
<tr>
<td>Flow cytometry</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Indirect, Non-specific Methods</strong></td>
<td></td>
</tr>
<tr>
<td>Thiobarbituric acid reactive substances</td>
<td>lipid peroxidation</td>
</tr>
<tr>
<td>Diene conjugates</td>
<td>lipid peroxidation</td>
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<tr>
<td>Deoxyribonucleic acid degradation</td>
<td>non-specific/ DNA damage</td>
</tr>
<tr>
<td><strong>Indirect, Specific Methods</strong></td>
<td></td>
</tr>
<tr>
<td>Dimethyl sulphoxide degradation</td>
<td>OH$^*$</td>
</tr>
<tr>
<td>Deoxyribose degradation</td>
<td>OH$^*$</td>
</tr>
<tr>
<td>Bleomycin assay</td>
<td>OH$^*$</td>
</tr>
<tr>
<td>Anthracene trapping</td>
<td>$O_2^-$</td>
</tr>
<tr>
<td>Nitrate, nitrite production</td>
<td>NO</td>
</tr>
<tr>
<td>Conversion of arginine$^{14}$ to citrulline$^{14}$</td>
<td>NO</td>
</tr>
<tr>
<td><strong>In vivo measurements</strong></td>
<td></td>
</tr>
<tr>
<td>Consumption of antioxidants</td>
<td>ascorbate, tocopherol</td>
</tr>
<tr>
<td>Breath ethane/pentane production</td>
<td>lipid peroxidation</td>
</tr>
</tbody>
</table>

Adapted from (Weber 1990).
Plate 2.1 Infant feeding tube (Pennine Healthcare FT-1608/40, OD 2mm), shown with syringe attached, used to instil acetic acid or saline into the colon of rats.

Plate 2.2 Scintillation counter used for chemiluminescence experiments.
Fig 2.1  The reaction of luminol with ROS to produce light.

Luminol  ROS  α-Aminophthalate

\[ \text{Luminol} + \text{ROS} \rightarrow \text{α-Aminophthalate} \]

Fig 2.2  Schematic representation of a colorectal biopsy placed in a solution containing the chemiluminescence amplifier (luminol or lucigenin) which react with ROS to release light detected by the photomultiplier and photon detector comprising the Packard Tri Carb 1600 CA liquid scintillation counter analyser operated in ‘the out-of-coincidence’ mode thus acting as a chemiluminometer.

Luminol penetrates cells and has thus been widely used to probe cellular events, particularly in stimulated neutrophils (Falck 1986; Dahlgren 1987; Vilim et al 1989). The technique is highly sensitive for ROS production, but is fairly non-specific. Chemiluminescence can result from reactions with hydrogen peroxide, hypochlorous acid (Aniansson et al 1984; Wymann et al 1987; Lock et al 1988) and superoxide (Misra et al 1982). Studies using isolated neutrophils have demonstrated that luminol-amplified chemiluminescence is dependent on myeloperoxidase activity, as there was little response from neutrophils from patients with myeloperoxidase deficiency, and superoxide dismutase had no inhibitory effect (De Chatelet et al 1982).

Lucigenin responds more specifically to superoxide than luminol, though with a lower quantum yield (Halliwell 1989a), and also reacts, to lesser extent, with hydrogen peroxide and singlet oxygen, though not with the hydroxyl radical (Wang et al 1989).
Other luminescence amplifiers, which have higher specificity for individual ROS but are less sensitive than luminol and lucigenin, include a synthetic analogue of Cypridina luciferin, 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]pyrazin-3-one, which detects superoxide (Nishida et al 1986), and trans-1-(2'-methoxyvinyl)pyrene as a probe for singlet oxygen (Posner et al 1984).

The Effect of Physical Conditions on Amplified-Chemiluminescence

Hydrogen peroxide-stimulated luminol-amplified chemiluminescence is highly pH sensitive, with a rise of approximately 100-fold in the chemiluminescence response between pH 7 and pH 8 (Samuni et al 1991). In these studies it was also noted that the yield from luminol-amplified chemiluminescence was temperature-sensitive but with concomitant increases in the signal-to-noise ratio, thus decreasing the sensitivity of the assay. The experiments described in this thesis were carried out at ambient temperature as temperature-controlled equipment was not available.

Although there is a 50% rise in chemiluminescence between an oxygen saturation of 2% and 20%, there is almost no rise above concentrations of 20% (Samuni et al 1991). Thus, for in vitro experiments, tissue viability can be maintained in an oxygen rich environment without substantially altering the chemiluminescence response. The experiments described in this thesis were thus carried out after saturation of solutions with 95% O₂, 5% CO₂ to maintain tissue viability.

Choice of Chemiluminescence to Measure ROS in the Mucosa

Chemiluminescence was used to detect ROS production in gut mucosa because the technique was well established in this laboratory and thus results of some assays could be directly compared to those previously described. This was particularly important for validation of the assay of the antioxidant effects of compounds using colonic biopsies from acetic acid-colitis. The chemiluminescence technique is highly sensitive and allows quantitative analyses of ROS production to be carried out. It suffers from being non-specific in relation to the radicals detected, at least using luminol as the amplifier, and in some situations lacks reproducibility (see below).

Reagents

All chemical reagents were of analytical grade and were obtained from Sigma Chemical Co, Poole, Dorset, unless otherwise stated. Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) was kept as stock solution (50mg in 1 ml of dimethylsulphoxide (DMSO)) for up to one month. Luminol and lucigenin (N,N'-Dimethyl-9,9'-biacridinium dinitrate) were made up freshly on the day of the experiment in Dulbecco’s phosphate buffered saline (D-PBS) with added calcium (1.13mM) and glucose (5mM), oxygenated for 10min with 95% O₂, 5% CO₂ to
maintain tissue viability, and the pH adjusted to 7.4 with 1M NaOH.

**Cell-Free Systems**

Cell-free systems are employed to produce ROS in the absence of a biological source; these experiments used the production of superoxide from the reaction of xanthine with xanthine oxidase. Lucigenin (300μM) and xanthine (50μM) were dissolved in D-PBS. To 900μl of this solution was added 100μl of the test solution at ten times the final concentration. 990μl of this solution was placed in chemiluminescence vials and 10μl xanthine oxidase at 0.1U/ml (final concentration 0.001U/ml) was added just prior to placing the vial in the chemiluminometer and starting the count. The final concentrations of lucigenin and xanthine were thus 267μM and 45μM, respectively. The time from addition of xanthine oxidase to the start of the count was kept at a constant 10sec. All vials were vortexed for 2sec prior to counting to ensure mixing of the xanthine oxidase in the vial. Each vial was then counted for 1.33min in Packard Tri Carb 1600 CA liquid scintillation counter analyser operated in ‘the out-of-coincidence’ mode.

**Chemiluminescence Using Colonic Biopsies from Acetic Acid-Induced Colitis**

Full thickness biopsies were taken by cutting cross-sectional strips of the dissected, inflamed colon (macroscopic score 2 or greater). Biopsies were placed in pre-oxygenated (95% O₂, 5% CO₂ for 10min) D-PBS, with added calcium (1.13mM) and glucose (5mM) at ambient temperature. To assess ROS activity by chemiluminescence, biopsies were placed in 300μM luminol or lucigenin and chemiluminescence measured in a Packard Tri Carb 1600 CA liquid scintillation counter analyser operated in ‘the out-of-coincidence’ mode for 2min (Fig 2.3). The time course of the chemiluminescence counts for cell-free systems and colonic biopsies from acetic acid-induced colitis were chosen as initial studies showed stability of the count over this time and were equivalent to previous studies (Simmonds et al 1992a; Simmonds 1992b).

**Choice of Luminol and Lucigenin Concentration**

Preliminary studies were carried out to select the optimal concentration of luminol and lucigenin for detection of amplified chemiluminescence by inflamed biopsies from acetic acid-induced colitis. The chemiluminescence response to different concentrations of luminol was recorded in four biopsies from two rats with acetic acid-induced colitis with a macroscopic score of 2 or greater (Fig 2.4): the maximal chemiluminescence response was obtained at 100-500μM luminol. 300μM luminol was used for subsequent experiments because a 10% reduction in its
concentration on addition of test compound or vehicle to the vial containing luminol and the biopsy would have little effect on the subsequent chemiluminescence recording, and because the same concentration had been used in earlier studies using human biopsies (Simmonds et al 1992a).

![Diagram of method for assessing the amplified chemiluminescence response produced by inflamed colorectal biopsies from rats and human subjects.](image)

**RATS**

- Excised rat colon opened longitudinally and pinned out on card with mucosal surface uppermost
- Biopsies are cut from the dissected rat or human colon and placed in Dulbecco's PBS with 1.13mM CaCl$_2$ and 5mM glucose, pre-oxygenated with 95% oxygen, 5% CO$_2$ for 10min
- The biopsy is transferred to a scintillation vial containing 1.8ml luminol/lucligenin (300|µ|M) and counted in the scintillation counter for 2 min
- The biopsy is blotted dry and weighed

**HUMANS**

- Colon/sigmoidoscopy
- Biopsies are cut from the dissected rat or human colon and placed in Dulbecco's PBS with 1.13mM CaCl$_2$ and 5mM glucose, pre-oxygenated with 95% oxygen, 5% CO$_2$ for 10min
- The biopsy is transferred to a scintillation vial containing 1.8ml luminol/lucligenin (300|µ|M) and counted in the scintillation counter for 2 min
- The biopsy is blotted dry and weighed

Fig 2.3 Method for assessing the amplified chemiluminescence response produced by inflamed colorectal biopsies from rats and human subjects. The figure for the chemiluminescence response is calculated as the chemiluminescence response in photons/min divided by the wet weight of the biopsy.

For lucigenin, chemiluminescence was recorded in five biopsies from three rats with acetic acid-induced colitis with a macroscopic score of 2 or greater (Fig 2.4).
Although the plateau occurs between 300 and 600μM lucigenin there is only a small change between 200 and 300μM confirming previous data that the quantum yield of lucigenin resembles that of luminol (Allen 1982): accordingly 300μM lucigenin was used for subsequent studies.

![Graph showing percentage increase in chemiluminescence with increasing concentrations of luminol (four biopsies from two rats) and lucigenin (five biopsies from three rats) in acetic acid-induced colitis. The first point on each graph is the percentage increase between 1 and 10μM amplifier.](image)

**Fig 2.4** Percentage increase in chemiluminescence with increasing concentrations of luminol (four biopsies from two rats) and lucigenin (five biopsies from three rats) in acetic acid-induced colitis. The first point on each graph is the percentage increase between 1 and 10μM amplifier.

**Chemiluminescence Using Colorectal Biopsies from Humans**

Colorectal biopsies were obtained during sigmoidoscopy or colonoscopy. Control biopsies were taken from patients undergoing investigation or follow-up for polyps or carcinoma. Each patient gave informed consent and ethical approval was given by the Tower Hamlets Health Authority Ethics Committee.

Biopsies were placed in pre-oxygenated (95% O₂, 5% CO₂ for 10min) D PBS, with added calcium (1.13mM) and glucose (5mM) at ambient temperature. To assess ROS activity by chemiluminescence, biopsies were placed in 300μM luminol or lucigenin and chemiluminescence measured in a Packard Tri Carb 1600 CA liquid scintillation counter analyser operated in 'the out-of-coincidence' mode for 2min (Fig 2.3).

**Chemiluminescence Assay Validation**

Validation of these assays for acetic acid-induced colitis is described in Chapter 3, p. 91-97 and for UC in Chapter 5, pp. 120-127.
THIOBARBITURIC ACID REACTIVE SUBSTANCES

The assay for TBARS is a widely used, non-specific assay for lipid peroxidation products in biological fluids and tissues. The test relies on the detection of the reaction product of thiobarbituric acid (TBA) with peroxides in the sample. A major product assayed is malondialdehyde (MDA); one mole of MDA reacting with two of TBA (Lunec 1989) (Fig 2.5).

![Chemical structure](image)

**Fig 2.5** The reaction of malondialdehyde and thiobarbituric acid to give a chromogen which absorbs light at 532nm.

The chromogen absorbs light at 532nm (Nair et al 1984) and is thus detectable by spectrophotometry. Other compounds which may contribute to the formation of the chromogen include water-soluble substances such as urea, sucrose and proteins (Marshall et al 1985). Confusion with water-soluble substances reacting with thiobarbituric acid is avoided by extraction into butanol, which will dissolve lipid products, leaving water soluble substances in the aqueous fraction. The level of TBARS in controls is inversely proportional to the adequacy of the separation of malondialdehyde from other reaction products of TBA, particularly water-soluble substances which give a detectable chromogen on reaction with TBA, such as bilirubin (Yagi 1982). Thus, failure to isolate the butanol-soluble reaction product results in higher estimates of TBARS, in the order of 35μmol/l (Santos et al 1980). The protocol for the TBARS assay was therefore chosen to include butanol extraction. The assay in this thesis was carried out according to the protocol of (Rowley et al 1984).

**Normal levels of Thiobarbituric Acid Reactive Substances**

The published levels of TBARS in normal adults differ according to the methodology used and the nature of the sample, there being apparent differences between levels measured in plasma and serum. Previous studies have described a varying range of plasma levels of TBARS, from a mean (number studied) of 0.14μMol/l (n=12) (Muindi et al 1994), 1.1μmol/l (n=50) (Brown et al 1994), 1.6μmol/l (n=93) (Weiland et al 1995), 3.3μmol/l (n=22) (Hunter et al 1986), 5.0μmol/l (n=10) (Blom et al 1995), 6.6μmol/l (n=23) (Jankowski et al 1991), to
8.1 μmol/l (n=38) (McMurray et al 1992).

Reported levels of serum TBARS are lower than plasma levels in healthy adults with mean (number studied) level ranging from 0.22 μmol/l in (n=34) (Kalra et al 1991), 0.46 μmol/l (n=15) (Young et al 1994), 1.01 μmol/l (n=47) (Wasowicz et al 1993), to 1.3 μmol/l (n=18) (Kalavacherla et al 1994).

It is likely that the increased levels of TBARS measure in plasma samples are artificially high, since use of a more accurate method of assessing MDA levels (high performance liquid chromatography (HPLC)) gives serum and plasma levels that are closer to that of serum samples assessed in conventional assays (mean 0.04 μmol/l in healthy controls) (Carbonneau et al 1991). HPLC is now recommended as the optimal method of assessing circulating MDA levels (Knight et al 1988). Access to HPLC was not available for the current studies and therefore the assay was carried out conventionally using serum samples.

Adjustment by Serum Lipids

Other peroxides may be produced from the oxidation of polyunsaturated fatty acids (Pryor et al 1975). To avoid the potential confounding factor of alterations in TBARS by alterations in serum lipids (Staprans et al 1993), which might occur as result of diet (P. Winyard, personal communication), the serum TBARS levels were divided by the total serum lipid (cholesterol + triglyceride). The total lipid was calculated from the sum of the cholesterol and triglyceride levels as measured in the routine hospital laboratory. However, in a recent study in which a similar correction factor was made, the total serum lipid did not appear to affect the results (Grieshmacher et al 1995). In this thesis (Chapter 8, pp. 178-179), serum TBARS concentrations are expressed both as raw data, and corrected for total lipid levels.

Systemic TBARS in Inflammatory Disorders

TBARS have shown to be raised in inflammatory disorders such as rheumatoid arthritis (Kalavacherla et al 1994), systemic sclerosis (Blann et al 1993) and peptic ulcer disease (Jankowski et al 1991). Malondialdehyde levels, measured by HPLC, are raised in acute and chronic pancreatitis (Schoenberg et al 1995), and correlate with disease activity in rheumatoid arthritis (Muus et al 1979). Levels of TBARS are raised in the mucosa of IBD (Ahnfelt-Ronne et al 1990), but no studies have been carried out on circulating levels of TBARS in active UC.

Choice of TBARS to Assess Circulating ROS

The assay for TBARS was chosen because it is a widely used assay for lipid peroxidation in the blood. Although non-specific, it was felt to be the most appropriate assay for the detection of systemic changes in lipid peroxidation in
patients with UC treated with antioxidant therapy, as described in Chapter 8. It has the additional advantage of being relatively simple and was a pragmatic choice as other investigators in the laboratory (Bone and Joint Research Unit) were also using this assay.

**Reagents**

Thiobarbituric acid, sodium hydroxide, potassium phthalate, hydrochloric acid, butan-1-ol and 1,1,3,3-tetramethoxypropane (TMP) were obtained from BDH Chemicals Ltd, UK.

**Solutions**

The following solutions were prepared in advance; stock solutions were used for one month:
- TBA 1% (w/v) in 50mM NaOH (stock)
- Potassium phthalate (500μM) was dissolved in HPLC grade water and the pH adjusted with 0.1M HCl to 3.5 prior to the experiment (acid buffer)
- TMP standard: 0, 0.05, 0.1, 0.2, 0.4, 0.6 and 1.0μM
- Rodamine B (0.3μM)

**Samples**

10 ml of venous blood was collected into sterile glass tubes, supplied by the hospital clinical chemistry department (Vacutainer®) containing SST® gel and clot activator. Samples were centrifuged within 2 hours of collection at 1500rpm for 10min and serum aliquotted into 1.8 ml Nunc Cryotubes, stored at -80°C and analysed within 8 weeks.

**Assay procedure**

For each assay two aliquots from each sample was assessed and a standard curve to TMP drawn from a range of concentrations of the standard. The following solutions were combined in polyethylene centrifuge tubes:
- 30μl of TMP standards or 30μl sample
- 270μl double distilled H₂O
- 200μl 1% TBA Reagent
- 400μl acid buffer

The tubes were briefly vortexed, tightly sealed to prevent moisture loss and heated at 100°C for one hour in a boiling water bath and then cooled on ice. 100μl concentrated HCl and 1.5 ml butan-1-ol were then added and the samples vortex mixed for 3min, centrifuged at 3000rpm for 10min and then stored in ice until assayed.
Measurement of Fluorescence

An SFM 25 spectrophotometer (Kontron Instruments, Zurich) was used for all experiments. Rodamine (0.3μM) was used to calibrate at 480nm excitation and 580nm emission. The excitation and emission were then set at 532nm and 553nm, respectively and butan-1-ol used as the zero reference. The butanol layer of the samples was then removed by pipette into quartz cuvettes, and read at 532nm excitation and 553nm emission.

Calculation of the Standard Curve

Fig 2.6 shows a typical concentration gradient used as the reference for assessing the malondialdehyde concentration in the serum from patients with IBD and controls.

![Fluorescence against Rodamine (0.3μM)](image)

Fig 2.6 Concentration gradient of TMP fluorescence against rodamine to assess malondialdehyde levels in serum from patients with IBD and controls. Each concentration was tested twice, one before and one after the measurement of the samples. Malondialdehyde levels in the samples were calculated from the average of the two.

Variability of the Thiobarbituric Acid Reactive Substances Assay

The intra-assay repeatability (Bland *et al* 1986) was assessed in a random selection of 25 samples which had been divided and separately analysed. Table 2.2 shows the results and the corresponding coefficient of repeatability of 16.1. One sample falls out of this range, thus representing 4% of the sample; nevertheless >95%
fell within the required range. No adjustment for the standards or the total serum lipid has been made as this would have been the same for both samples.

Table 2.2  Coefficient of repeatability of fluorescence readings from the same sample of serum.

<table>
<thead>
<tr>
<th>TBARS Fluorescence 1st Score</th>
<th>TBARS Fluorescence 2nd Score</th>
<th>Differences between 1st and 2nd</th>
</tr>
</thead>
<tbody>
<tr>
<td>83.8</td>
<td>74.5</td>
<td>-9.3</td>
</tr>
<tr>
<td>76.8</td>
<td>81.3</td>
<td>4.5</td>
</tr>
<tr>
<td>57.1</td>
<td>55.5</td>
<td>-1.6</td>
</tr>
<tr>
<td>50.0</td>
<td>50.7</td>
<td>0.7</td>
</tr>
<tr>
<td>45.0</td>
<td>47.1</td>
<td>2.1</td>
</tr>
<tr>
<td>44.4</td>
<td>45.8</td>
<td>1.4</td>
</tr>
<tr>
<td>130.8</td>
<td>95.9</td>
<td>-34.9</td>
</tr>
<tr>
<td>84.0</td>
<td>76.8</td>
<td>-7.2</td>
</tr>
<tr>
<td>45.7</td>
<td>50.5</td>
<td>4.8</td>
</tr>
<tr>
<td>55.4</td>
<td>53.4</td>
<td>-2.0</td>
</tr>
<tr>
<td>69.4</td>
<td>69.4</td>
<td>0.0</td>
</tr>
<tr>
<td>58.0</td>
<td>56.6</td>
<td>-1.4</td>
</tr>
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<td>67.4</td>
<td>60.9</td>
<td>-6.5</td>
</tr>
<tr>
<td>106.3</td>
<td>102.1</td>
<td>-4.2</td>
</tr>
<tr>
<td>77.5</td>
<td>73.0</td>
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</tr>
<tr>
<td>60.2</td>
<td>53.4</td>
<td>-6.8</td>
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<td>57.8</td>
<td>-3.2</td>
</tr>
<tr>
<td>56.3</td>
<td>43.9</td>
<td>-12.4</td>
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<td>56.7</td>
<td>54.4</td>
<td>-2.3</td>
</tr>
<tr>
<td>42.5</td>
<td>45.2</td>
<td>2.7</td>
</tr>
<tr>
<td>46.2</td>
<td>51.6</td>
<td>5.4</td>
</tr>
<tr>
<td>57.2</td>
<td>57.8</td>
<td>0.6</td>
</tr>
<tr>
<td>66.1</td>
<td>56.9</td>
<td>-9.2</td>
</tr>
<tr>
<td>57.3</td>
<td>57.7</td>
<td>0.4</td>
</tr>
</tbody>
</table>

| Mean                           | -3.4                           |
| Standard Deviation             | 8.03                           |
| **Coefficient of Repeatability** | **16.06**                     |

The inter-assay variability was similarly assessed using 14 samples which had been assessed twice in separate experiments. As the figure of final interest is that corrected by the total serum lipid, the results are assessed after this adjustment. These results and the corresponding coefficient of variability are shown in Table 2.3. With 14 samples and 13 of the 14 (93%) falling inside the 95% limit for acceptability, the coefficient of repeatability just failed to reach an acceptable level.
Table 2.3  Inter-assay coefficient of repeatability of TBARS (nmol/mmol lipid/litre), measured in separate aliquots from the same sample.

<table>
<thead>
<tr>
<th>TBARS nmol/mmol lipid/litre 1st Score</th>
<th>TBARS nmol/mmol lipid/litre 2nd Score</th>
<th>Differences between 1st and 2nd</th>
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<td>0.31</td>
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<td>0.03</td>
</tr>
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<td>0.29</td>
<td>0.35</td>
<td>0.06</td>
</tr>
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<td>0.53</td>
<td>0.62</td>
<td>0.09</td>
</tr>
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<td>0.25</td>
<td>0.28</td>
<td>0.02</td>
</tr>
<tr>
<td>0.34</td>
<td>0.41</td>
<td>0.08</td>
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</tr>
<tr>
<td>0.87</td>
<td>0.98</td>
<td>0.10</td>
</tr>
<tr>
<td>0.53</td>
<td>0.53</td>
<td>0.00</td>
</tr>
<tr>
<td>0.16</td>
<td>0.35</td>
<td>0.19</td>
</tr>
<tr>
<td>0.27</td>
<td>0.49</td>
<td>0.22</td>
</tr>
<tr>
<td>0.40</td>
<td>0.26</td>
<td>-0.14</td>
</tr>
<tr>
<td>0.54</td>
<td>0.25</td>
<td>-0.29</td>
</tr>
<tr>
<td>0.25</td>
<td>0.32</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Mean 0.04  
Standard Deviation 0.13  
Coefficient of Repeatability 0.26
3
THE ACETIC ACID-INDUCED MODEL OF COLITIS

INTRODUCTION

Study of gastrointestinal inflammation in animals is useful in understanding the pathophysiology of IBD and in the development of new treatments. Because of the rarity of spontaneous colitis in animals (Table 3.1), many models of experimental colitis have been developed (Table 3.2). The aetiology of the majority of these models is probably dissimilar to IBD; however the cellular pattern of the inflammatory process in both spontaneous and experimental colitis often resembles human disease, probably because the gastrointestinal tract has a fairly limited range of inflammatory responses to injury. Thus, most models of IBD are better considered as models of gastrointestinal inflammation than IBD (Rhodes 1990). More appropriate models will have to await a better understanding of the aetiology of IBD.

Selection of an animal model for experimental work largely depends on the nature of the intended studies. The main aim of this study was to develop a model of colitis suitable for in vitro and in vivo testing of novel antioxidant therapy for IBD. The commonest examples of spontaneous enteritis and colitis and the important experimental models of colitis are presented below, along with evidence of a role for ROS in their pathogenesis. The choice of acetic acid-induced colitis as the appropriate experimental model for these experiments is discussed.

Spontaneous Colitis in Animals

Well-researched examples of experimental colitis include the cotton-top tamarin (Sanguinus oedipus) and Boxer dogs. The cotton-top tamarin is arguably the closest animal equivalent to human UC. For instance, spontaneous colitis develops in a higher proportion of animals in the potentially stressful conditions of captivity than in the wild (Kirkwood et al 1986). Furthermore, there is a high incidence of colonic carcinoma in chronic disease, developing in 25–40% of affected animals after 2–5 years (Rhodes 1990).

Colitis in Boxer dogs has similarities to UC, in that it is ameliorated by sulphasalazine, although histologically it more closely resembles Whipple's disease (Kennedy et al 1966). Table 3.1 lists other species which develop spontaneous colitis
or enteritis, a substantial proportion of which have a known, or suspected, infective aetiology.

Research into novel therapies for IBD using spontaneous colitis or enteritis in animals is rarely practicable because of difficulties with husbandry, the rarity and value of the animals, or the unpredictable nature of the inflammation. Some of these difficulties may be overcome by newer models in which selective breeding, or gene knockout techniques, are used to produce spontaneous colitis in commonly used laboratory animals; some of these models are discussed below.

Table 3.1 Spontaneous enterocolitis in animals*.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Distribution</th>
<th>Organism</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Idiopathic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cotton-top tamarin</td>
<td>colitis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>monkeys</td>
<td></td>
<td>Cotton-top tamarin monkeys</td>
<td></td>
</tr>
<tr>
<td>Boxer dogs</td>
<td>granulomatous</td>
<td>? Chlamydia</td>
<td>Responds to sulphasalazine</td>
</tr>
<tr>
<td>colitis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rats</td>
<td>cecal inflammation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Horses</td>
<td>granulomatous</td>
<td>? Clostridium difficile</td>
<td>'Colitis X'</td>
</tr>
<tr>
<td>enterocolitis</td>
<td></td>
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</tr>
<tr>
<td>Texel sheep</td>
<td>terminal ileitis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pigs</td>
<td>regional enteritis</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Infected aetiology</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Swiss-Webster mice</td>
<td>colitis</td>
<td>Citrobacter freundii</td>
<td>High mortality</td>
</tr>
<tr>
<td>Infant pigs</td>
<td>colitis</td>
<td>Escherichia coli</td>
<td></td>
</tr>
<tr>
<td>Cattle</td>
<td>regional enteritis</td>
<td>Mycobacteria johnnei</td>
<td>Johne's disease</td>
</tr>
<tr>
<td>Cattle</td>
<td>terminal ileitis</td>
<td>Chlamydia Cw 613</td>
<td></td>
</tr>
<tr>
<td>Pigs</td>
<td>colitis</td>
<td>Serpulia hyodysenteriae</td>
<td>Swine dysentery</td>
</tr>
<tr>
<td>Pigs</td>
<td>adenomatous proliferation, ulceration</td>
<td>Campylobacter sputorum</td>
<td></td>
</tr>
<tr>
<td>Chickens</td>
<td>granulomatous</td>
<td>Escherichia coli</td>
<td></td>
</tr>
<tr>
<td>enteritis</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Reviewed in (Rhodes 1990) and (Pfeiffer 1985)

*a Organism suspected but not proven.
<table>
<thead>
<tr>
<th>Model</th>
<th>Animal</th>
<th>Time Course</th>
<th>Injury</th>
<th>Extent</th>
<th>Inflammatory Mediators</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3H/HeJ mice</td>
<td>mice</td>
<td>4-17 weeks/1 year</td>
<td>mucosal submucosal</td>
<td>right-sided colitis caecum perianal pancolitis</td>
<td>not known</td>
<td>Spontaneous inflammation</td>
</tr>
<tr>
<td>IL-2 knockout</td>
<td>mice</td>
<td>6-15 weeks/10-25 weeks</td>
<td>mucosal submucosal</td>
<td>not known</td>
<td>Spontaneous inflammation</td>
<td></td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>rat, guinea pig, rabbit</td>
<td>1-2 days/3 weeks</td>
<td>transmural</td>
<td>local colitis</td>
<td>ROS, PGE&lt;sub&gt;2&lt;/sub&gt;, HETE</td>
<td>Anticolon antibodies</td>
</tr>
<tr>
<td>TNBS/Ethanol</td>
<td>rat, dog</td>
<td>1-2 days/8 weeks</td>
<td>transmural</td>
<td>local colitis or enteritis</td>
<td>ROS, IL-1, IL-2, IL-6, LTB&lt;sub&gt;4&lt;/sub&gt;, NO, IFN-γ</td>
<td>Granulomata</td>
</tr>
<tr>
<td>Formalin-Immune Complex</td>
<td>rabbit, guinea-pig</td>
<td>4-8 days/6 weeks</td>
<td>mucosal submucosal</td>
<td>local colitis</td>
<td>IL-1, LTB&lt;sub&gt;4&lt;/sub&gt;, PGE&lt;sub&gt;2&lt;/sub&gt;, TXB&lt;sub&gt;2&lt;/sub&gt;, PGI&lt;sub&gt;2&lt;/sub&gt;.</td>
<td>Responds to SSP</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>rat</td>
<td>3-4 days/6 weeks</td>
<td>transmural</td>
<td>small intestine caecum local colitis</td>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;, PGF&lt;sub&gt;2α&lt;/sub&gt;, TXA&lt;sub&gt;2&lt;/sub&gt;, TXB&lt;sub&gt;2&lt;/sub&gt;, PGI&lt;sub&gt;2&lt;/sub&gt;.</td>
<td>LTB&lt;sub&gt;4&lt;/sub&gt; not increased</td>
</tr>
<tr>
<td>Peroxynitrite</td>
<td>rat</td>
<td>1-3 days/2 weeks</td>
<td>mucosal submucosal</td>
<td>mucosal submucosal</td>
<td>LTB&lt;sub&gt;4&lt;/sub&gt;, LTC&lt;sub&gt;4&lt;/sub&gt;, NO, PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Residual fibrosis and luminal narrowing</td>
</tr>
<tr>
<td>DNCB</td>
<td>guinea-pig, rabbits, mice</td>
<td>3-5 days/5 weeks</td>
<td>mucosal</td>
<td>local colitis</td>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;, PGI&lt;sub&gt;2&lt;/sub&gt;, TXA&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Dysplasia in rabbits</td>
</tr>
<tr>
<td>Carrageenan</td>
<td>rat, mouse, guinea pig, rabbit, hamster monkey,</td>
<td>3-4 weeks/2-4 weeks</td>
<td>mucosal submucosal</td>
<td>right-sided colitis</td>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;, LTB&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Responds to SSP but not to 5-ASA</td>
</tr>
<tr>
<td>Dextran sulphate</td>
<td>mice, guinea-pig, hamster</td>
<td>48-72 hours/2-4 hours</td>
<td>mucosal submucosal</td>
<td>left-sided colitis</td>
<td>IL-1, IL-6, TNFα, PAF, PGE&lt;sub&gt;2&lt;/sub&gt;, LTB&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Responds to 5-ASA in rats Carcinogenic in hamsters</td>
</tr>
<tr>
<td>PG-PS</td>
<td>rats (strain sensitive)</td>
<td>3 weeks/4-6 months</td>
<td>transmural</td>
<td>local colitis</td>
<td>IL-1, NO, LTB&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Requires intramural injection. Spontaneous reactivation</td>
</tr>
<tr>
<td>fMLP</td>
<td>rabbit, rat, mouse</td>
<td>4 days/2 weeks</td>
<td>mucosal submucosal</td>
<td>local colitis</td>
<td>LTB&lt;sub&gt;4&lt;/sub&gt;, PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>5-ASA improves permeability</td>
</tr>
</tbody>
</table>
Spontaneous colitis in C3H/HeJ Bir Mice

A genetic basis for IBD has recently been supported by the discovery that the C3H/HeJ mouse strain develops recurrent perianal ulceration and right-sided colitis. Furthermore, cross-breeding of a severely affected female, from this strain, with a normal male has resulted in a substrain (C3H/HeJBir) with a particularly high incidence of colitis (Sundberg et al 1994). These animals develop a mild colitis in the third to fourth week of life which resolves by 10–12 weeks.

Experimental Models of Intestinal Inflammation

Development of experimental models of intestinal inflammation has two broad aims. The first is the reproduction of similar patterns of disease to those found in IBD, in terms of macroscopic and microscopic appearances, and in production of inflammatory mediators; examples are the acetic acid (MacPherson 1977) and TNBS/ethanol-induced models of colitis (Morris et al 1989). The second aim is to reproduce possible aetiopathological abnormalities found in IBD, examples being colitis induced by bacteria-derived polymers (Chester et al 1985; Sartor et al 1989) and peroxynitrite (Rachmilewitz et al 1993). Table 3.2 shows the most widely used of the models of experimental colitis/ileitis. The essential features of these models are described below, followed by a review of the literature of the involvement of ROS in experimental models colitis.

Transfection Models

The histological similarities of CD to human intestinal tuberculosis and to Johne’s disease in cattle has prompted research into the role of mycobacteria, and other infectious agents, in CD (Gitnick et al 1989; Wall et al 1993). Transfection of lymph node homogenates from patients with CD causes granulomata in the mouse footpad (Mitchell et al 1970). Furthermore, granulomatous inflammation, similar to CD, occurs after intra-mucosal inoculations of Mycobacterium Bacillus Calmette-Guerin in the rat (Pooley et al 1994).

Rectal inoculation of Chlamydia trachomatis causes proctitis in Cynomologus monkeys with inflammation characterised by mononuclear infiltrates, lymphoid follicle hyperplasia and crypt abscesses (Quinn et al 1981). The proctitis lasts for 4–6 weeks, and can be extended to 12 weeks by a second inoculation at 4–8 weeks. The practical difficulties of handling primates limits the usefulness of this model.

Ischaemic Models

Mesenteric infarction produces a dissimilar inflammatory response to IBD but it has been suggested that microvascular abnormalities may be responsible for the pathological features of IBD (Wakefield et al 1989). Experimental work in animals...
supports this hypothesis. Injection of glass and ceramic microspheres into the mesenteric circulation in dogs causes mucosal oedema and bleeding (Boley et al 1965). Mucosal inflammation, necrosis and ulceration develops in ferrets after mesenteric injection of styrene microspheres (Hudson et al 1992). A combination of a vascular and an immunological approach has been used to cause enteritis in ferrets, in which human albumin microspheres were injected into the mesenteric artery of animals pre-sensitised to human albumin (Hudson et al 1994). Although ROS production has not been investigated in these models, free radical production is well recognised in other models of ischaemic colitis (Douglas et al 1989) and free radical scavengers modulate the associated granulocyte infiltration (Zimmerman et al 1990) and mortality (Dalsing et al 1983).

**Immunologic Models**

The premise of these models is that colonic inflammation can be produced by reproducing a component of the immunological response, conventionally classified as Type I-IV humoral and cell-mediated responses. There are few models based on type I or type II hypersensitivity, though in an early study, colitis was induced in mice by sensitising them to human colonic extracts (Callaghan et al 1963), and colitis was induced in dogs by intravenous injection of anti-colon antiserum prepared in ducks and rabbits (Leveen et al 1961).

Type III hypersensitivity (immune complex-mediated) is thought responsible for the short-lived, colonic inflammation produced by the “Auer procedure” in rabbits. An acute colitis develops in rabbits pre-immunised with egg albumin and subsequently administered a formalin enema, followed 2 hours later by a further injection of egg albumin (Kraft et al 1963). Subsequent models, using injection of pre-formed immune-complexes into rabbits following a dilute formalin enema, produced inflammation that peaked at 2-4 days, but lasting in a milder form for up to 6 weeks (Hodgson et al 1978b). In a limited study, replacement of the egg albumin with an antigen derived from *E. coli* produced inflammation lasting several months (Mee et al 1979).

Colitis induced by Type IV hypersensitivity is thought to underlie the pro-inflammatory effects of the hapten, 2,4-dinitrochlorobenzene (DNCB) in guinea pigs (Bicks et al 1969; Glick et al 1981) and rabbits (Rabin et al 1978) and dinitrofluorobenzene in mice (Brkic et al 1992). These models produce right-sided colonic inflammation with mucosal oedema, ulceration, a chronic inflammatory infiltrate, mucous depletion and crypt branching. An interesting parallel with IBD is the association of ileal inflammation and pericholangitis in DNCB-induced colitis (Banic et al 1993).
Further examples of Type IV-mediated inflammation, namely TNBS-induced colitis in rats, and liposaccharide and formalin enemas in lipopolysaccharide-sensitised rabbits, are discussed below.

**Trinitrobenzene Sulphonic Acid**

The TNBS model of colitis, which lasts up to 8 weeks, was first described by Morris *et al* (Morris *et al* 1989) and is widely used because of ease of induction and good reproducibility. The aetiology is not well understood, though TNBS may be acting as a hapten which gains entry to the mucosa when administered with the mucosal barrier breaker, ethanol. A typical regime is the intrarectal administration of 30mg TNBS in 250μl of 50% ethanol (Rachmilewitz *et al* 1989). The resulting inflammatory response develops over 1–4 days, lasts up to 8 weeks, and is characterised by mucosal and submucosal infiltration by neutrophils, macrophages, lymphocytes, mast cells and fibroblasts. In the original description of this model, granulomata were found in 57% of the experimental animals and Langerhan’s-type giant cells also noted (Morris *et al* 1989). Thus, the inflammation in TNBS-induced inflammation more closely resembles CD than UC. A granulomatous enteritis also be induced in rats by intra-jejunal application of TNBS (Selve 1992).

**Bacterial Cell Wall Products**

The pro-inflammatory nature of bacterial cell wall derivatives is well demonstrated in two experimental models in which peptidoglycan-polysaccharide (PG-PS) polymers and chemotactic peptides are injected into the gastrointestinal wall.

In the former model, PG-PS polymers, derived from streptococcal cell walls, are sonicated, sterilised and injected suberosally into the distal ileum or caecum of rats; causing a local acute and chronic granulomatous inflammation which extends to the mesenteric lymph nodes (Sartor *et al* 1985). The acute inflammation resolves in about 2 weeks but a mild, chronic, granulomatous inflammation lasts for at least 6 months. Of particular relevance to human disease is the spontaneous reactivation that occurs in genetically susceptible breeds, such as Lewis rats (Sartor *et al* 1989; Yamada *et al* 1993). Interestingly, PG-PS complexes have recently been identified in the bowel wall in CD (Klasen *et al* 1994).

Chester *et al* described colonic inflammation in mice and rats induced by chemotactic peptides, particularly fMLP, though others such as formylnorleucyl-leucyl-phenylalanine and formylmethionine were also pro-inflammatory, causing mucosal oedema, ulceration and necrosis and increased mucosal leukotrienes (Chester *et al* 1985). Luminal fMLP also exacerbates leukotriene release in formalin enema/immune complex colitis in rabbits (Zipser *et al* 1987).
Interestingly fMLP undergoes an enterobiliary circulation in both rats with fMLP-induced colitis, (Hobson et al 1988) and in man (Roberts et al 1990). The rectal administration of a related chemotactic peptide, N-formyl-L-methionine L-leucine L-tyrosine induces cholangitis in rats (Yamada et al 1994), suggesting that bacterial peptides may be responsible for the hepatobiliary complications in IBD.

Colitis can be induced in rabbits, previously skin-sensitised to lipopolysaccharide, that are administered rectal lipopolysaccharide following a 1% formalin enema to increase permeability (Hotta et al 1986).

There is thus good evidence that inflammation ensues if bacterial products gain access to the mucosa. However, the experimental models demonstrating this phenomenon are relatively cumbersome if large numbers of animals are required for drug screening experiments.

Carrageenan

Carrageenan is a sulphated polygalactoside (100-800 kDa) derived from the red sea-weed Euchema spinosum. Degradation by acid hydrolysis results in a smaller molecule (<30 kDa) (Pfeiffer 1985). A right-sided colitis is induced by the addition of both degraded and undegraded carrageenan to the drinking water (1-10%) in a variety of species, after a latent period of 3 to 4 weeks. The inflammation is characterised by an acute and chronic inflammatory infiltrate, epithelial atrophy, crypt abscesses and superficial ulceration (Sartor 1992). Administration of a further derivative of carrageenan, dextran sulphate sodium (DSS) (3-10%) in the drinking water, causes a left-sided colitis, thus more closely resembling UC (Okayasu et al 1990). Inflammation in both models appears related to the overgrowth of anaerobic bacteria; Bacteroides vulgatus in the case of carrageenan (Breeling et al 1988), and B. distasonis in DSS-induced colitis (Okayasu et al 1990). Furthermore, prophylactic metronidazole prevents colitis in both models (Sartor 1992).

Genetic Models

Colitis develops in a proportion of mice expressing HLA-B27 (Hammer et al 1990) and in 100% of interleukin-2 knockout mice (Sadlack et al 1993). These models require no experimental manipulation and their use is likely to increase once they are more available and less expensive.

Acetic Acid-Induced Colitis

Acetic acid-induced colitis was first described by MacPherson and Pfeiffer (MacPherson et al 1978). The acetic acid is applied to the mucosa at a concentration of 2-10% for varying lengths of time (10-30sec) and is given either as an intraluminal infusion per rectum (MacPherson et al 1978; Sharon et al 1985), or injected through the colonic wall at laparotomy (Fedorak et al 1992).
The mechanism of colonic injury in acetic acid-colitis is unknown, but appears unrelated purely to the pH of the acetic acid as an equivalent pH of hydrochloric acid (pH 2.3) does not induce colitis, nor purely due to the acetate moiety, as acetate alone is similarly ineffective (Zeitlin et al 1984). However, the pH of the acetic acid is an important determinant of the degree of inflammation (Fabia et al 1992b). Intraluminal casein worsens inflammation in acetic acid-induced colitis, suggesting that organic acids damage the epithelial barrier allowing entry of luminal toxins (Miller et al 1991). An alternative explanation might be that, as other short-chain fatty acids, such as butyrate, are readily taken up into the colon and can cause inflammation in acidic form (McCafferty et al 1989), hydrogen ions may gain entry to the epithelium by active mucosal uptake of acetate. However, this hypothesis remains to be tested.

Acetic acid produces a diffuse ulceration of the colon associated with formation of pseudopolyp-like structures, alterations in crypt depth and mucous secretion and a transmural, non-specific inflammatory response (MacPherson et al 1978). The degree of neutrophil infiltration correlates well with myeloperoxidase activity (Fretland et al 1989; Rachmilewitz et al 1989). Table 3.3 shows the similarities between acetic acid-induced colitis and human UC. Particularly relevant are similarities in the mucosal histological changes and the production of lipid mediators and ROS (Sharon et al 1985; Keshavarzian et al 1990).

<table>
<thead>
<tr>
<th>Feature</th>
<th>Ulcerative Colitis (Ref)</th>
<th>Acetic Acid-Induced Colitis (Ref)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Produces loose stool with blood</td>
<td>(MacPherson et al 1978)</td>
<td></td>
</tr>
<tr>
<td>Endoscopic appearances</td>
<td>(Mann et al 1980)</td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td>(MacPherson et al 1978)</td>
<td></td>
</tr>
<tr>
<td>Excess production of ROS</td>
<td>(Simmonds et al 1992a)</td>
<td>(Keshavarzian et al 1990)</td>
</tr>
<tr>
<td>Production of eicosanoids</td>
<td>(Stenson 1990)</td>
<td>(Sharon et al 1985; Mascolo et al 1995)</td>
</tr>
<tr>
<td>Increased colonic blood flow</td>
<td>(Hulten et al 1977)</td>
<td>(Sekizuka et al 1988)</td>
</tr>
<tr>
<td>Improvement with sulphasalazine</td>
<td></td>
<td>(MacPherson 1977; Keshavarzian et al 1990)</td>
</tr>
<tr>
<td>Changes in bacterial flora</td>
<td>(Fabia et al 1993a)</td>
<td>(Fabia et al 1993a)</td>
</tr>
<tr>
<td>Worsening with indomethacin therapy</td>
<td>(Rampton et al 1981)</td>
<td>(LeDuc et al 1993)</td>
</tr>
</tbody>
</table>
The major dissimilarities are the differences in aetiology and the spontaneous recurrence which occurs in UC but not acetic acid-induced colitis. Table 3.4 shows the wide range of agents with efficacy in acetic acid-induced colitis.

### Table 3.4 Drugs compounds with a beneficial effect in acetic acid-induced colitis.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Action</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>5-ASA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulphasalazine</td>
<td>Multiple (See Table 1.4)</td>
<td>(Keshavarzian et al 1990)</td>
</tr>
<tr>
<td><strong>Glucocorticoids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Budesonide</td>
<td>Non-absorbable steroid</td>
<td>(Fabia et al 1994)</td>
</tr>
<tr>
<td>Budesonide-β-D-glucuronide</td>
<td>Budesonide pro-drug</td>
<td>(Cui et al 1994)</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>Non-absorbable steroid</td>
<td>(McLeod et al 1994)</td>
</tr>
<tr>
<td><strong>Antioxidant Therapy</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catalase</td>
<td>Metabolises $\text{H}_2\text{O}_2$</td>
<td>(Keshavarzian et al 1992a)</td>
</tr>
<tr>
<td>WR-2721</td>
<td>Antioxidant</td>
<td>(Keshavarzian et al 1992a)</td>
</tr>
<tr>
<td>Cu(II)2(3,5-DIPS)4</td>
<td>SOD mimetic</td>
<td>(Keshavarzian et al 1992a)</td>
</tr>
<tr>
<td>Methoxypolyethylene:SOD</td>
<td>SOD bound to carrier</td>
<td>(Keshavarzian et al 1990)</td>
</tr>
<tr>
<td>Allopurinol</td>
<td>Xanthine oxidase inhibitor</td>
<td>(Keshavarzian et al 1990)</td>
</tr>
<tr>
<td><strong>Anti-Inflammatory</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WEB 2086</td>
<td>PAF-antagonant</td>
<td>(Will et al 1991)</td>
</tr>
<tr>
<td>MK 886</td>
<td>Inhibits leukotriene synthesis</td>
<td>(Empey et al 1992b)</td>
</tr>
<tr>
<td>SC-41930</td>
<td>LTB$_4$ receptor antagonist</td>
<td>(Fretland et al 1990)</td>
</tr>
<tr>
<td>NPC 15669</td>
<td>Inhibits leukocyte recruitment</td>
<td>(Noronha-Blob et al 1993)</td>
</tr>
<tr>
<td>IL-1 receptor antagonist</td>
<td>Inhibitor of IL-1</td>
<td>(Thomas et al 1991; Will et al 1991)</td>
</tr>
<tr>
<td>Ketotifen</td>
<td>Mast cell stabiliser</td>
<td>(Eliakim et al 1992)</td>
</tr>
<tr>
<td>Verapamil</td>
<td>Calcium antagonist</td>
<td>(Fabia et al 1993b)</td>
</tr>
<tr>
<td></td>
<td>PLA$_2$ inhibitor</td>
<td>(Fedorak et al 1992)</td>
</tr>
<tr>
<td>Quinacrine</td>
<td>PLA$_2$ inhibitor</td>
<td>(Fabia et al 1993b)</td>
</tr>
<tr>
<td><strong>Miscellaneous</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucralfate</td>
<td>Mucosal protectant</td>
<td>(Zahavi et al 1989)</td>
</tr>
<tr>
<td>Misoprostol</td>
<td>Prostaglandin analogue</td>
<td>(Yamada et al 1991)</td>
</tr>
<tr>
<td>Octreotide</td>
<td>Somatostatin analogue</td>
<td>(Eliakim et al 1993)</td>
</tr>
<tr>
<td>Phostatidylcholine inositol</td>
<td>Anti-inflammatory</td>
<td>(Fabia et al 1992a)</td>
</tr>
<tr>
<td>Exogenous lactobacillus</td>
<td>Replaces bowel flora</td>
<td>(Fabia et al 1993a)</td>
</tr>
<tr>
<td>Fatty acid dietary restriction</td>
<td>Inhibits eicosanoid synthesis</td>
<td>(Mascolo et al 1995)</td>
</tr>
</tbody>
</table>

Phospholipase A$_2$ (PLA$_2$), interleukin-1 (IL-1), superoxide dismutase (SOD), leukotriene B$_4$ (LTB$_4$), platelet activating factor (PAF).
Role of Free Radicals and use of Antioxidant Therapy in Experimental Models of Colitis

ROS and Antioxidant Therapy in Animal Models of Colitis

The role of oxidant-mediated inflammation has been best established in animal models of intestinal ischaemia (Granger et al 1981; McCord 1985; Grisham et al 1986). ROS production, as measured by mucosal levels of conjugated dienes and malondialdehyde, is increased rats with intestinal ischaemia (Douglas et al 1989) and intestinal permeability in cats is attenuated by allopurinol, DMSO (Parks et al 1983) and SOD (Granger et al 1981). Induction of intestinal ischaemia by intra-arterial injection of platelet activating factor (PAF) is attenuated by a combination of CuZnSOD and catalase and with allopurinol (Cueva et al 1988). SOD also improves intestinal permeability and eicosanoid release in a model of necrotizing enterocolitis (Miller et al 1988), though allopurinol has no effect (Clark et al 1988). ROS induced by ischaemia-reperfusion in cats is dependent on neutrophil activity, as anti-neutrophil monoclonal antibodies reduced the tissue damage (Nilsson et al 1994).

The evidence for a role for ROS in experimental intestinal ischaemia will be of relevance to human disease if theories of vascular impairment prove valid.

The importance of ROS has been studied in both acetic acid and TNBS-induced colitis. Keshavarzian et al showed increased production of ultra-weak and luminol-enhanced chemiluminescence in rats with colitis induced by both intraluminal acetic acid and intra-peritoneal mitomycin C (Keshavarzian et al 1992b). Colonic homogenates from rats with TNBS-induced colitis produce superoxide which is further enhanced by addition of NADH and NADPH, and is inhibited by superoxide dismutase and catalase (Grisham et al 1991).

ROS production and mucosal inflammation in acetic acid-induced colitis is attenuated by catalase, azide, the antioxidant WR-2721, Cu(II)2(3,5-DIPS)4 (a SOD mimic) (Keshavarzian et al 1992a), and in other studies, allopurinol, sulphasalazine and SOD bound to methoxypolyethylene glycol (Keshavarzian et al 1990). Oxypurinol, the active metabolite of allopurinol, reduces inflammation and mucosal TBARS in TNBS/ethanol-induced colitis (Siems et al 1992). Carrageenan-induced intestinal injury is also improved by the antioxidants, SOD-polyethylene glycol, allopurinol and DMSO (Moyana et al 1991).

In other, preliminary, studies the antioxidant lazaroid, U-74006F, reduced colonic myeloperoxidase activity and superoxide production in acetic acid-induced colitis (Yue et al 1993), the cyclic nitrooxide, Tempol, a chain-breaking antioxidant, reduced mucosal injury and eicosanoid production in both acetic acid and TNBS-induced colitis (Karmeli et al 1995) and both MnSOD (Yoshikawa et al 1992) and α-tocopherol attenuated TNBS-induced colitis (Blumenstein et al 1993).
Empey et al demonstrated the ability of vitamin E to convert the secretory state induced by radiation-induced enteritis into an absorptive one, though there was no significant effect on the histological appearances (Empey et al 1992a).

Allopurinol, SOD bound to polyethylene glycol and, to a lesser extent, DMSO all reduced mucosal damage in carrageenan-induced intestinal inflammation (Moyana et al 1991), suggesting that ROS may be of pathogenic importance in this model.

The importance of the free radical, NO in models of intestinal inflammation is suggested by the increased levels of NO observed in a variety of animal models of colitis (Yamada et al 1993) and the anti-inflammatory effects of the NO synthase inhibitor L-NAME in TNBS-induced ileitis in the rat; however L-NAME increased myeloperoxidase activity and intestinal secretion in control animals (Miller et al 1993) suggesting that NO may be both pro- and anti-inflammatory.

Free Radical-Mediated Colitis

A pro-inflammatory role for ROS in experimental colitis has been further implied by the development of two models in which free radicals, or free radical generating compounds, induce colonic inflammation.

2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) generates free radicals in vitro and induces mucosal oedema and neutrophil infiltration when administered intra-rectally to rats, though ulceration is not a feature (Tamai et al 1992). There were associated increases in mucosal levels of TBARS and decreases in sulphydryls. Furthermore, sulphasalazine, 5-ASA, an LTB4 antagonist and glutathione, improved the inflammation in this model (Tamai et al 1992). The other new model of interest has been created by the intracolonic instillation of peroxynitrite, the radical created by the reaction of NO and superoxide (Rachmilewitz et al 1993).

Summary

It is perhaps remarkable that such a wide variety of exogenous insults causes gastrointestinal inflammation in animals that so closely resembles human IBD, suggesting that the inflammatory response represents a final common pathway of response to injury. The excess production of ROS in many experimental models of colitis, and in UC, serves to emphasise the need to establish whether they are central to the pathogenesis of gastrointestinal inflammation or mere epiphenomena.

In the absence of the ‘perfect model’ based on the known aetiology of IBD, the choice of an experimental model depends on the nature of the experimental work for which it is intended.
Choice of an animal model of colitis

Strober described useful criteria for an ideal model of inflammatory bowel disease (Table 3.5). The acetic acid-induced model fulfilled the requirements for such studies in its similarity to human UC and the known quality of excess production of mucosal ROS. The ease, speed and cheapness of this model were added advantages. Although TNBS-induced colitis shares many of the advantages of acetic acid-induced colitis, its similarity to CD was felt to be a disadvantage in the present studies which were carried out to compare results with those previously obtained from patients with UC.

Table 3.5 The ideal model of inflammatory bowel disease.

<table>
<thead>
<tr>
<th>Identical to human IBD, having:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Same cause</td>
</tr>
<tr>
<td>Same pathology and pathophysiology</td>
</tr>
<tr>
<td>Same clinical spectrum</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>The model should:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Be accessible and inexpensive</td>
</tr>
<tr>
<td>Have a defined genetic background</td>
</tr>
<tr>
<td>Have a similar immune system to humans</td>
</tr>
<tr>
<td>Allow the researcher to alter its:</td>
</tr>
<tr>
<td>- Dietary intake</td>
</tr>
<tr>
<td>- Immunologic status</td>
</tr>
<tr>
<td>- Exposure to infectious agents</td>
</tr>
</tbody>
</table>

The model should be susceptible to various forms of treatment

Adapted from (Strober 1985).

HYPOTHESIS

It was hypothesised that the ROS production by colonic biopsies from acetic acid-induced colitis would demonstrate elevated ROS production and that this would be proportional to the degree of inflammation.

AIM OF THIS STUDY

The purpose of this study was to develop an acetic acid-induced model of colitis in rats for the in vitro and in vivo assessment of novel antioxidant therapies for use in IBD and to demonstrate that the macroscopic and microscopic grade of inflammation correlated with the luminol-amplified chemiluminescence of colonic biopsies in vitro.
The methods for assessing the macroscopic and microscopic scores of the colitis are described first, as the macroscopic scoring system, in particular, was used to assess the grade of inflammation during the step-wise development of the protocol for acetic acid-induced colitis used in subsequent experiments and Chapters. The development of this model is then detailed, concluding with the final protocol, which is also outlined in Chapter 2, p. 46. The results are then presented for the macroscopic and microscopic scores, and the luminol- and lucigenin-amplified chemiluminescence, using the methods described in Chapter 2, for colonic biopsies from acetic acid-induced colitis, saline-perfused controls and untreated controls.

METHODS

Animals

Male Wistar rats (150–200g, Charles River, UK), were housed in light/dark controlled conditions and fed a standard chow diet. Prior to induction of colitis animals and controls were fasted for 16 hours with access to water ad libitum. Each rat was sedated by brief respiration of 20–25% CO$_2$ followed by anaesthesia with 35mg/kg intraperitoneal pentobarbital. The rats were killed at 24 hours by CO$_2$ asphyxiation.

Materials

The materials used for these experiments are described in Chapter 2.

Macroscopic Assessment of Acetic Acid-Induced Colitis

At post mortem laparotomy, 6cm of colon extending proximally from 2cm above the anal margin was removed, split longitudinally, pinned out on card and the macroscopic appearances of the colonic mucosa scored on a scale adapted from Morris et al (Morris et al 1989) ranging from 0 – 5 as shown in Table 3.6.

Plate 3.1 shows the macroscopic appearances of 5 colons from acetic acid-induced colitis and 3 from saline-perfused controls. Plate 3.2 shows these same 5 colons, and 5 others, from acetic acid-induced colitis with their respective macroscopic scores and demonstrates individual features of the inflamed colon in this model.

For in vitro experiments in subsequent Chapters, colons with a macroscopic score of 5 were not used because the presence of extensive necrosis might have confounded the results.
Table 3.6 Scoring system for the macroscopic appearances of acetic acid-induced colitis.

<table>
<thead>
<tr>
<th>Scoring System</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>No macroscopic change</td>
<td>0</td>
</tr>
<tr>
<td>Mucosal erythema alone</td>
<td>1</td>
</tr>
<tr>
<td>Mild mucosal oedema, slight bleeding or small erosions</td>
<td>2</td>
</tr>
<tr>
<td>Moderate oedema, bleeding ulcers or erosions</td>
<td>3</td>
</tr>
<tr>
<td>Severe ulceration/erosions, oedema without tissue necrosis</td>
<td>4</td>
</tr>
<tr>
<td>Severe ulceration/erosions, oedema with extensive tissue necrosis</td>
<td>5</td>
</tr>
</tbody>
</table>

Intermediate values reflected intermediate appearances and were given half point increments. A score of >4 (i.e. either 4.5 or 5) was given if tissue necrosis was present, 4.5 if it involved <1cm of the length of the colon, 5 if >1cm.

Inter-observer Coefficient of Repeatability of the Macroscopic Score

Because of the necessity to process colonic tissue rapidly following killing of the rats, separate macroscopic scores by different investigators were not routinely recorded. The score was thus agreed by two investigators (the author and A Claxson) who were blinded as to the origin of the rats. Blinding was carried out by a third investigator (C Chander) who coded the rat cages according to treatment group.

To calculate the inter-observer repeatability the macroscopic scoring of 20 photographic records of colons from rats with acetic acid-induced colitis (Table 3.7) was independently recorded by 2 investigators (A Claxson) and the author). The coefficient of repeatability (CR) was 0.75, thus demonstrating that adequate repeatability according to standard criteria (British Standards Institution 1979) (see Chapter 2, p. 45).
Table 3.7  Coefficient of repeatability of macroscopic scoring of acetic acid-induced colitis between 2 observers A and B.

<table>
<thead>
<tr>
<th>Histology Score A</th>
<th>Histology Score B</th>
<th>Differences between A and B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2.5</td>
<td>2</td>
<td>-0.5</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>2.5</td>
<td>-0.5</td>
</tr>
<tr>
<td>2.5</td>
<td>2</td>
<td>-0.5</td>
</tr>
<tr>
<td>1</td>
<td>1.5</td>
<td>0.5</td>
</tr>
<tr>
<td>3.5</td>
<td>3.5</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>4.5</td>
<td>0.5</td>
</tr>
<tr>
<td>3.5</td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>2.5</td>
<td>-0.5</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1.5</td>
<td>1</td>
<td>-0.5</td>
</tr>
<tr>
<td>2</td>
<td>1.5</td>
<td>-0.5</td>
</tr>
<tr>
<td>2.5</td>
<td>2</td>
<td>-0.5</td>
</tr>
<tr>
<td>2.5</td>
<td>2</td>
<td>-0.5</td>
</tr>
<tr>
<td>2</td>
<td>1.5</td>
<td>-0.5</td>
</tr>
</tbody>
</table>

Mean: -0.15  
Standard Deviation: 0.37  
Coefficient of Repeatability: 0.75

Plate 3.1  Macroscopic appearances of colons 2-8cm from the rectum removed and opened longitudinally from acetic acid-induced colitis (n=5) shown on the left and 0.9% saline-perfused controls (n=3) on the right.
Plate 3.2 Macroscopic appearances of acetic acid-induced colitis. The macroscopic scores of the colons shown were as follows; 1 (3.5), 2 (4), 3 (1.5), 4 (1.5), 5 (1), 6 (2), 7 (1.5), 8 (3.5), 9 (4.5), 10 (4). N = area of necrosis.
Histological Assessment of Acetic Acid-Induced Colitis

Randomly distributed full-thickness biopsies were fixed in 10% formol saline prior to wax embedding, sectioning and staining with haematoxylin and eosin (see below). Biopsies were obtained from rats with acetic acid-induced colitis, prepared according to the protocol in Chapter 2, p. 46. Coded biopsies were scored by a single, experienced histopathologist (Dr A Coumbe) blinded to the macroscopic score and chemiluminescence responses of the sections, using the scoring system shown in Table 3.8. In the absence of an established scoring system for assessing the histological changes in acetic acid-induced colitis, a well-established system developed for UC (Saverymuttu et al 1986) was adapted for use in acetic acid-induced colitis. The adaptations were made to include more objective variables, such as the presence or absence of fibrin and mucosal ulceration.

Table 3.8  Histological assessment of full-thickness biopsies from acetic acid-induced colitis in rats.

<table>
<thead>
<tr>
<th>Histological Feature</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophil Infiltrate</td>
<td></td>
</tr>
<tr>
<td>None 0  Slight increase 1  Marked Increase 2</td>
<td>(0-2)</td>
</tr>
<tr>
<td>Epithelium</td>
<td>(0-2)</td>
</tr>
<tr>
<td>Lamina Propria</td>
<td>(0-2)</td>
</tr>
<tr>
<td>Muscularis Mucosa</td>
<td>(0-2)</td>
</tr>
<tr>
<td>Submucosa</td>
<td>(0-2)</td>
</tr>
<tr>
<td>Muscularis Propria</td>
<td>(0-2)</td>
</tr>
<tr>
<td>Serosa</td>
<td>(0-2)</td>
</tr>
<tr>
<td>Fibrin Deposition</td>
<td></td>
</tr>
<tr>
<td>Absent 0  Present 1</td>
<td>(0-1)</td>
</tr>
<tr>
<td>Mucosa</td>
<td>(0-1)</td>
</tr>
<tr>
<td>Submucosa</td>
<td>(0-1)</td>
</tr>
<tr>
<td>Submucosal Neutrophil Margination</td>
<td></td>
</tr>
<tr>
<td>Absent 0  Present 1</td>
<td>(0-1)</td>
</tr>
<tr>
<td>Submucosal Oedema</td>
<td></td>
</tr>
<tr>
<td>Nil 0  Patchy 1  Confluent 2</td>
<td>(0-2)</td>
</tr>
<tr>
<td>Epithelial Necrosis</td>
<td></td>
</tr>
<tr>
<td>Nil 0  Localised 1  Extensive 2</td>
<td>(0-2)</td>
</tr>
<tr>
<td>Epithelial Ulceration</td>
<td></td>
</tr>
<tr>
<td>Absent 0  Present 1</td>
<td>(0-1)</td>
</tr>
<tr>
<td>Maximum Score</td>
<td>20</td>
</tr>
</tbody>
</table>
Neutrophil Infiltrate

The neutrophil infiltrate was assessed separately in the epithelium, lamina propria, muscularis mucosa, submucosa, muscularis propria and serosa. A score of 0 was applied if there was no increase in neutrophil infiltrate, 1 if there was a mild-moderate increase and 2 if there was a marked increase. As there are normally only a very few neutrophils found in the submucosa and lamina propria of the gastrointestinal wall the subjective component of this scoring system is restricted to the assessment of a moderate or marked increase.

Fibrin Deposition

Fibrin deposition is a local accumulation of a proteinaceous exudate, which therefore stains pink with a haematoxylin and eosin stain, and is a prominent feature of the inflammatory response, commonly seen in the peri-vascular space and the ulcerated mucosa (Hurley 1985). It was assessed as being present or absent in the mucosa and submucosa.

Submucosal Neutrophil Margination

Submucosal neutrophil margination, otherwise known as ‘pavementing’ is the appearance of neutrophils in apposition to the venular wall, or penetrating endothelium (Hurley 1985), and is a marker of the degree of active neutrophil recruitment to the gastrointestinal wall from the mesenteric circulation; it was assessed as being absent (0) or present (1).

Submucosal Oedema

Submucosal oedema was scored as absent (0), patchy (1) or confluent (2) throughout the section. Confluent oedema was marked only if the submucosa throughout the section was oedematous. Any intermediate state between this and total absence of oedema was scored as patchy oedema.

Epithelial Necrosis

Epithelial necrosis is defined as loss of nuclear staining with retention of epithelial continuity and is a precursor to ulceration. Localised necrosis (1) was scored as that involving five glands or less, if it extended for more than 5 glands it was defined as being extensive (2).

Epithelial Ulceration

Epithelial ulceration was defined according to the standard criteria as a loss of epithelial continuity and was assessed as being absent (0) or present (1).
The photomicrographs shown in Plates 3.3–3.10 are all of the rat colon and stained with haematoxylin and eosin. The original magnification is shown in parentheses. If appropriate, the direction of the mucosal surface is indicated (MS).

Plate 3.3 Normal rat colon (x4).

Plate 3.4 Saline-perfused rat (x10). The histological appearances are normal except for mild, superficial oedema of the lamina propria (O).
Plate 3.5  Acetic acid-induced colitis (x4). The inflammation is mild and demonstrates the presence of confluent submucosal oedema (O).

Plate 3.6  Acetic acid-induced colitis (x10). Mild inflammation with confluent submucosal oedema (O) and a mild-moderate neutrophil infiltrate in the lamina propria, submucosa and serosa (N) (score 1).
Plate 3.7  Acetic acid-induced colitis (x10). Marked infiltrate of neutrophils in the lamina propria and particularly in the submucosa (N) (score 2), focal necrosis of the mucosa (NC) and submucosal fibrin deposition (F).

Plate 3.8  Acetic acid-induced colitis (x20). There is a marked neutrophil infiltrate in the submucosa (score 2) and submucosal oedema (O). Fibrin deposition (F) is present in the perivascular space in the lamina propria and in the submucosa. There is marked epithelial necrosis (NC).
Plate 3.9  Acetic acid-induced colitis (x40). Submucosal neutrophil margination in a vessel (M) and shows fibrin deposition which characteristically is peri-vascular (F).

Plate 3.10  Acetic acid-induced colitis (x40). Moderate neutrophil (N) infiltrate in the muscularis propria (MP).
Intra- and Inter-observer Coefficient of Repeatability of the Histological Score

To assess the repeatability of the histological scoring system developed for use in these studies the intra- and inter-observer variability was calculated using the coefficient of repeatability (CR) (Bland et al 1986) (see Chapter 2, p. 45).

A total of 16 coded sections were scored on two separate occasions by a single experienced histopathologist (Dr. Coumbe, observer A), who was blinded as to their source. The CR for the intra-observer variability is shown in Table 3.9 and was 6.5.

The inter-observer variability was assessed by a further observer (observer B, the author) who assessed 13 coded sections previously scored by observer A. Observer B was blinded to the origins of the sections by an independent investigator using coded card taped to the slide identifier. The CR was calculated between the scores of Observer B and the first and second scoring of Observer A, and was 6.9 and 7.6, respectively (Table 3.9). Since the differences in all cases do not exceed the figure for the CR, the method can be considered to have adequate repeatability (British Standards Institution 1979).

Table 3.9 Coefficient of repeatability of histological scoring of acetic acid-induced colitis between 2 observers, A and B. Observer A scored sections twice, A1 and A2.

<table>
<thead>
<tr>
<th>Histology</th>
<th>Histology</th>
<th>Histology</th>
<th>Differences</th>
<th>Differences</th>
<th>Differences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Score - A1</td>
<td>Score - A2</td>
<td>Score - B</td>
<td>between A1 and A2</td>
<td>between A1 and B</td>
<td>between A2 and B</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>-1</td>
<td>-1</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>5</td>
<td>1</td>
<td>-2</td>
<td>-3</td>
</tr>
<tr>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>-2</td>
</tr>
<tr>
<td>0</td>
<td>2</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>1</td>
<td>-1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>2</td>
<td>-1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>13</td>
<td>13</td>
<td>14</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>13</td>
<td>15</td>
<td>1</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>7</td>
<td>3</td>
<td>0</td>
<td>-3</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>5</td>
<td>-3</td>
<td>-1</td>
<td>2</td>
</tr>
<tr>
<td>14</td>
<td>13</td>
<td>14</td>
<td>-1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>18</td>
<td>17</td>
<td>14</td>
<td>-1</td>
<td>-4</td>
<td>-3</td>
</tr>
<tr>
<td>10</td>
<td>13</td>
<td>12</td>
<td>3</td>
<td>2</td>
<td>-1</td>
</tr>
</tbody>
</table>

Mean: 0.38, -0.15, -0.38
Standard Deviation: 3.26, 3.45, 3.79
Coefficient of Repeatability: 6.51, 6.90, 7.58
Establishment of Method for Acetic Acid-Induced Model of Colitis

Initial experiments were carried out to establish the optimal method of achieving a reproducible inflammatory and chemiluminescence mucosal response prior to proceeding to *in vitro* testing of conventional antioxidants and conventional therapies for IBD (Chapter 4), *in vivo* and *in vitro* testing of rh-MnSOD (Chapter 6) and *in vitro* assessment of desferrioxamine and ferric citrate (Chapter 7).

**Instillation of Acetic Acid into the Colon**

Previous investigators have used a variety of methods for introducing acetic acid into the colonic lumen. Because the present studies required the use of colorectal biopsies, a method was sought that would maximise the amount of inflamed tissue per animal. A series of experiments were therefore carried out to assess the optimal method of tube insertion, acetic acid volume and the time course for acetic acid instillation.

**Choice of Tube and Assessment of Tube Position**

An infant feeding tube with a cross-sectional diameter of 2mm (Pennine Healthcare Products, UK) was chosen for its flexibility and smooth tip, thus limiting trauma from tube insertion. This tube is closed off at the distal end and has two perforations at 8 and 15mm from the tip (Plate 2.1).

The maximal length of tube that could easily be inserted into the colon of the rat was assessed in 3 control rats in which a midline abdominal incision was made after they had been killed. The infant feeding tube was then inserted *per rectum* and the progression of the tube along the colon noted. In each animal the tube was seen to progress easily into the colon to 8cm in two animals and in one to 10cm. For subsequent experiments, therefore, the infant feeding tube was marked at 8cm from tip and inserted until the mark was at the level of the anus.

**Assessment of Acetic Acid Volume**

Using the same three animals as in the previous experiment the maximal volume that could be introduced into the colon without causing over-inflation was assessed. The colon was slowly instilled with 0.9% saline (150mM) over 10-30sec until the haustral markings began to flatten. This point was arbitrarily taken as the limit of acceptable colonic distension. Three instillations of normal saline in each of three animals were carried out. The results and mean (SD) are shown in Table 3.10.

As a result of these experiments the volume was maintained at 3ml of either acetic acid, or normal saline, for subsequent experiments. It was also noted during these studies that it was necessary to gently hold the folds of the anus around the tube to prevent the fluid being expelled prematurely.
Table 3.10  Colonic volumes after injection of normal saline to the point at which serosal markings are lost (taken as the limit of acceptable distension).

<table>
<thead>
<tr>
<th>Volume of Saline Infused to the Limit of Acceptable Distension</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAT 1</td>
</tr>
<tr>
<td>3.3</td>
</tr>
<tr>
<td>2.6</td>
</tr>
<tr>
<td>2.4</td>
</tr>
<tr>
<td><strong>MEAN</strong></td>
</tr>
<tr>
<td><strong>SD</strong></td>
</tr>
</tbody>
</table>

Assessment of Speed of Injection of Acetic Acid into the Colon

A further observation was made during the above experiment, relating to the speed of instillation of the acetic acid. Instillation of 3ml fluid into the colon at a rate faster than 20sec resulted in loss of hastral markings before the full volume was reached. An instillation time of 30sec allowed the saline to distribute evenly throughout the left colon but longer time periods were of no additional benefit in this respect. The time course of instillation of acetic acid was thus chosen as 30sec.

Assessment of Necessity to Withdraw Excess Acetic Acid After Exposure

An experiment was carried out to confirm the necessity of adequate removal of acetic acid after the initial exposure to the colonic lumen. In 16 rats, starved of food but allowed water for 16 hours, 3ml of 3% acetic acid was infused into the colon via an infant feeding tube inserted to 8cm, as described above. The acetic acid was instilled slowly over 30sec, but not actively withdrawn, and the animal returned to the cage which was lined with absorbent paper. The time taken for rectal expulsion of the fluid was recorded. At 24 hours the macroscopic score of the colonic inflammation was assessed, as described earlier, p. 73 and Table 3.6.

There was a positive correlation between the time taken for expulsion of acetic acid and the resulting macroscopic score, Rho=+0.5, P=0.0085 (Fig 3.1). This experiment demonstrated that short exposure times led to high variability of the inflammatory response and long exposure times to a high grade of inflammation. As a result, in subsequent experiments acetic acid/saline was retained in the colonic lumen for 30sec and then withdrawn and a flush of 2ml saline used to remove residual acetic acid.
Fig 3.1 Relationship of the macroscopic score of acetic acid-induced colitis in 16 rats at 24 hours to the time taken for spontaneous expulsion of acetic acid to occur after instillation of 3% acetic acid. R=+0.6, P=0.0085, Spearman's rank correlation coefficient. The high variability of the macroscopic score with short retention times and the high degree of inflammation with long times is demonstrated.

Assessment of Acetic Acid Concentration

In early descriptions of the acetic acid-induced model of colitis (MacPherson et al 1978) it was recognised that the degree of inflammation was related to the concentration of acetic acid used. More recently it has also been shown that the time that the acetic acid is in contact with the mucosa is also a critical factor (Fabiet al 1992b) (and see above). In these latter studies the animals underwent laparotomy, two colostomies fashioned, and the acetic acid injected after washing out of luminal contents with warmed normal saline, further normal saline was used to remove the acetic acid at specified times. Under these conditions, in which the acetic acid contact time could be kept very constant, the optimal time and concentration were determined to be 4% applied for 15sec.

The macroscopic score in response to 2.5, 3 and 4% acetic acid at 24 hours was assessed in 3 groups of rats (Table 3.11). In these experiments, colons exposed to 4% acetic acid for 30sec developed severe necrosis and dilatation (score 5) with unacceptable frequency and, therefore, 3% was used for subsequent experiments. This matches the optimal concentration suggested by other studies in which 1, 2 or 3% acetic acid were compared (Keshavarzian et al 1992b).

However, as seen in Table 3.11, the variability of the macroscopic score remained large. The coefficient of variation (CV) of the macroscopic scores of the
rats given 3% in Table 3.11 is 95%. This compares with a CV of 32% for the 119 rats shown in Fig 3.3, indicating that the variability of acetic acid-induced colitis improved in subsequent experiments.

Table 3.11 Macroscopic score in acetic acid-induced colitis at varying concentrations of acetic acid instilled for 30sec.

<table>
<thead>
<tr>
<th>Rat</th>
<th>Macroscopic Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5% Acetic Acid</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
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<td>3</td>
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<td>9</td>
<td>-</td>
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<td>10</td>
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</tbody>
</table>

Final Protocol for Acetic Acid-Induced Colitis

Prior to induction of colitis animals and controls were fasted for 16 hours with access to water *ad libitum*. Each rat was sedated by brief respiration of 20–25% CO$_2$ followed by anaesthesia with 35mg/kg intraperitoneal pentobarbital. An infant feeding tube (Pennine Healthcare FT-1608/40, OD 2mm (Plate 2.1) was inserted into the colon to 8cm and 2ml of acetic acid (3% v/v in 0.9% saline) or saline alone (control animals) infused into the colon. The acetic acid/saline was retained in the colon for 30sec, after which fluid was withdrawn. The rats were killed at 24 hours by CO$_2$ asphyxiation.

Establishment of Method of Sampling the Colonic Mucosa

Previous studies have demonstrated ROS production by mucosal scrapings from acetic acid-induced colitis (Keshavarzian *et al* 1990). In the present study it was hypothesised that mucosal biopsies would respond to antioxidants in a similar manner to colorectal biopsies from UC (Simmonds *et al* 1992a). As mucosal scrapings disrupt the mucosa, it was felt that they were inappropriate for such comparisons and therefore a method was sought for sampling the colorectal mucosa that would maintain mucosal integrity.
Initial attempts to take mucosal biopsies using a 9 inch endoscopic forceps demonstration model of standard endoscopy forceps with a 2mm cup (KeyMed UK) were unsuccessful as there was no consistency as to the size or composition of the biopsy and sampling was technically difficult, largely because the thickness of the colonic wall in the rat was insufficient to allow the forceps to take consistently sized mucosal samples. As a result of these investigations, full-thickness transverse sections, 5-10mm in width, were taken from dissected colon that had been opened longitudinally and pinned to cork matting with the serosal surface downwards, as shown in Fig 3.2.

---

**Chemiluminescence Response of Full-Thickness Biopsies from Acetic Acid-Induced Colitis**

Biopsies were initially placed in pre-oxygenated (95% O₂, 5% CO₂ for 10min) D-PBS, with added calcium (1.13mM) and glucose (5mM) at ambient temperature and then transferred to 300μM luminol or lucigenin immediately prior to assessment of the chemiluminescence response which was measured in a Packard Tri Carb 1600 CA liquid scintillation counter analyser operated in ‘the out-of-coincidence’ mode for 2min (see Chapter 2, p 52).
CALCULATIONS AND STATISTICS

To assess the repeatability of the macroscopic and histological scoring systems developed for use in these studies the inter-observer variability and, for the histological scoring, the intra-observer variability was calculated using the coefficient of repeatability (CR) (Bland et al 1986).

The chemiluminescence is expressed as the number of photons/min/mg of wet weight of tissue after subtraction of the background count. The background count was taken as the average photons per minute for two vials out of each twelve analysed, each containing 300μM luminol or lucigenin in oxygenated D-PBS alone. Comparisons between groups of control animals, saline-treated animals and acetic acid-induced colitis were made using a Kruskal-Wallis test, followed by the Mann-Whitney U test (two-tailed) with the Bonferroni correction for multiple comparisons (Godfrey 1985).

The coefficient of variation (CV) of the macroscopic scores are calculated for the macroscopic and histological scores for acetic acid-induced colitis and for the chemiluminescence response for luminol and lucigenin. The macroscopic and histological scores were calculated from a single value for each animal. Because a number of values (2-6) were available for the chemiluminescence response, the CV was initially calculated for each individual animal and then expressed as the median (IQR) of the CV between animals.

Correlations between the macroscopic appearances, histological score and chemiluminescence response were assessed using Spearman’s rank correlation test.

RESULTS

Macroscopic Changes

Full thickness biopsies were taken at random by cutting cross-sectional strips (median (IQR) wet weight; 44 (31–78) mg) of the dissected, inflamed colon. The macroscopic scores were agreed by two investigators blinded to the treatment category of the animal. The median (IQR) of the macroscopic scores of colons from 45 untreated controls, 16 saline-perfused controls and 103 colons is shown in Fig 3.3.

The changes were compared to the chemiluminescence and histological scores, see below.
Fig 3.3  Macroscopic scores of inflammatory damage in rats pre-treated with 3% intra-colonic acetic acid (Acetic Acid-induced Colitis) or 0.9% saline (Saline Controls), assessed at 24 hours and in untreated control animals (Controls). Scores for rats with acetic acid-induced colitis were compared with colons from rats given intra-colonic 0.9% saline and untreated controls. Differences are shown using the Mann-Whitney U test, two-tailed, with the Bonferroni correction for multiple comparisons.

Variability of the Macroscopic Score
The coefficient of variation of the macroscopic score of 103 rats with acetic acid colitis was 32%.

Histological Score
The histological scores of 18 biopsies from untreated controls, 6 from saline-perfused controls and 44 from acetic acid-induced colitis are shown in Fig 3.4. Acetic acid-induced colitis gave a greater histological score than both untreated control biopsies (P=0.003) and saline-perfused controls (P=0.015), though untreated controls were not significantly different from saline-perfused controls (P=0.2).
Fig 3.4  Histological scores of inflammatory damage in rats pre-treated with 3% intra-colonic acetic acid (Acetic acid-induced colitis) or 0.9% saline (Saline Controls), assessed at 24 hours and in untreated control animals (Controls). Scores for 64 biopsies from rats with acetic acid-induced colitis were compared with 6 biopsies from saline-perfused rats and 21 untreated controls. Acetic acid-induced colitis produced significantly more damage than saline controls, $P=0.012$ and controls, $P=0.003$. Mann-Whitney U test, two-tailed, with the Bonferroni correction for multiple comparisons. There was no significant difference between Controls and Saline Controls, $P=0.2$.

Variability of the Histological Score

The coefficient of variation of the histological score of 64 rats with acetic acid-induced colitis was 35%.

Chemiluminescence Response in Acetic Acid-Induced Colitis and Controls

The luminol-amplified chemiluminescence response by 116 biopsies from 38 animals with acetic acid-induced colitis was compared with 8 biopsies from 4 animals in which 0.9% saline was used in place of acetic acid and in 34 biopsies from 17 untreated control animals (Fig 3.5). A similar comparison was made for lucigenin-amplified chemiluminescence using 31 biopsies from 15 animals from acetic acid-induced colitis, 8 biopsies from 4 animals in which 0.9% saline was used in place of acetic acid and in 19 biopsies from 16 untreated control animals (Fig 3.6).
The median (IQR) of the luminol-amplified chemiluminescence for acetic acid-induced colitis, saline-treated and untreated controls were 6454 (1721-19125) photons/min/mg, 74 (1–154) and 1 (1–64) respectively (Fig 3.5). The median (IQR) for the lucigenin-amplified chemiluminescence for acetic acid-induced colitis, saline-treated and controls were 716 (428–1380) photons/min/mg, 347 (157–759) and 80 (19–163), respectively (Fig 3.6).

Biopsies from acetic acid-induced colitis produced significantly more luminol-amplified chemiluminescence than biopsies from saline-treated and untreated controls (P=0.003) (Fig 3.5). Biopsies from acetic acid-induced colitis produced significantly more lucigenin-amplified chemiluminescence than biopsies from untreated controls (P=0.003), and biopsies from saline-perfused controls produced significantly more lucigenin-amplified chemiluminescence than biopsies from untreated controls (P=0.014) (Fig 3.6).

![Fig 3.5](image-url) Log-transformed luminol-amplified chemiluminescence counts in full-thickness colonic biopsies from rats with acetic acid-induced colitis (116 biopsies from 38 animals), saline controls (8 biopsies from 4 animals) and untreated controls (34 biopsies from 17 animals). Groups were compared using the Mann-Whitney U test (two-tailed) with the Bonferroni correction for multiple comparisons.
Fig 3.6 Log-transformed lucigenin-amplified chemiluminescence counts in full-thickness colonic biopsies from rats with acetic acid-induced colitis (31 biopsies from 14 animals), saline-perfused controls (8 biopsies from 4 animals) and untreated controls (15 biopsies from 7 animals). Groups were compared using the Mann-Whitney U test (two-tailed) with the Bonferroni correction for multiple comparisons.

Variability of the initial chemiluminescence response

The median (IQR) of the CV of the initial chemiluminescence response of 116 biopsies from 38 animal rats with acetic acid-induced colitis to luminol was 56 (38–82)% and of 31 biopsies from 15 animals rats to lucigenin with acetic acid-induced colitis was 29 (17–49)% . These variations are small in comparison to the percentage increase in luminol and lucigenin-amplified chemiluminescence response of acetic acid-induced colitis biopsies compared to control (median +20,000% and +500%, respectively).

Chemiluminescence Compared to the Grade of Inflammation

To assess the adequacy of the luminol-amplified chemiluminescence response as a measure of inflammation, the luminol-amplified chemiluminescence was compared with the macroscopic and histology scores in colons from 58 rats pre-treated with 3% acetic acid-induced colitis at 24 hours and 13 untreated controls. Similarly, lucigenin-amplified chemiluminescence and the macroscopic and histology scores were compared between colons from 18 rats pre-treated with 3% acetic acid-
induced colitis at 24 hours and 14 untreated controls. Comparisons were carried out using Spearman’s rank correlation coefficient and were made between the macroscopic score, the histological score and the chemiluminescence response from each animal.

**Luminol-Amplified Chemiluminescence Compared With Macroscopic Score**

In all the rats studied, the luminol-amplified chemiluminescence correlated with the macroscopic score (Rho=+0.6, P=0.0001). The relationship between the macroscopic score and the luminol-amplified chemiluminescence in rats with acetic acid-induced colitis alone also demonstrated a positive correlation (Rho=+0.5, P=0.0002). However as shown in Fig 3.7 the relationship between the macroscopic score and luminol-amplified chemiluminescence was not definitely linear. All biopsies from colons with a macroscopic score of ≥1.5 produced more chemiluminescence than those with a score of 0 (P=0.005) apart from those with a score of 3.5 of which there were only 3. There was also a significant difference between those with a score of 2 and 2.5 (P=0.04).

![Fig 3.7](image-url)

**Fig 3.7** Relationship of luminol-amplified chemiluminescence to the macroscopic score in colons from 116 from 38 rats pre-treated with 3% acetic acid-induced colitis at 24 hours, 8 biopsies from 4 rats saline-perfused rat and 34 biopsies from 17 untreated controls. Data shown as macroscopic score against the log of the chemiluminescence. *P=0.005 compared to a score of 0, †P=0.04 compared to a score of 2, Mann-Whitney U test (two-tailed) with the Bonferroni correction for multiple comparisons.
Lucigenin-Amplified Chemiluminescence Compared With Macroscopic Score

In all rats studied the lucigenin-amplified chemiluminescence correlated with the macroscopic score (Rho=+0.6, P=0.0001). As with luminol-amplified chemiluminescence, the relationship between the macroscopic score and lucigenin-amplified chemiluminescence is not clearly linear (Fig 3.8). Biopsies from colons with a macroscopic score of 2.5 and 3 produced significantly more chemiluminescence than those with a score of 0 (P=0.03 and 0.004, respectively).

Fig 3.8 Relationship of lucigenin-amplified chemiluminescence to the macroscopic score in colons from 31 biopsies from 15 rats pre-treated with 3% acetic acid-induced colitis at 24 hours, 8 biopsies from 4 saline-perfused rat and 19 biopsies from 16 untreated controls. Data shown as macroscopic score against the log of the chemiluminescence. *P=0.03 compared to a score of 0, †P=0.004 compared to a score of 0, Mann-Whitney U test (two-tailed) with the Bonferroni correction for multiple comparisons.

Luminol-Amplified Chemiluminescence Compared with the Histological Score

In all the rats studied, luminol-amplified chemiluminescence correlated with the histology score (Rho=+0.7, P=0.0001) (Fig 3.9). Examination of biopsies from acetic acid induced colitis alone showed there was also a significant correlation between the luminol-amplified chemiluminescence response and the histological score in this group, Rho=+0.5, P=0.0001.
Fig 3.9 Relationship of luminol-amplified chemiluminescence to the histology score in biopsies from 64 rats pre-treated with 3% acetic acid-induced colitis at 24 hours, 6 saline-perfused controls and 21 untreated controls, Rho=+0.7, P=0.0001, Spearman's rank correlation coefficient.

**Multiple Linear Regression Analysis of Luminol-Amplified Chemiluminescence and the Histology Score**

To determine which factor of the histology score (Table 3.8) most closely correlates with luminol-amplified chemiluminescence, multiple linear regression was carried out using log_{10}-transformed values for luminol-amplified chemiluminescence as the co-variable and each of the histological features as the variables (Table 3.12). The only variable which demonstrated an independent, significant relationship with luminol-amplified chemiluminescence was the degree of submucosal neutrophil infiltration, though the presence or absence of submucosal neutrophil margination just failed to reach significance.

**Lucigenin-Amplified Chemiluminescence Compared with the Histological Score**

The lucigenin-amplified chemiluminescence correlated with the histology score (Rho=+0.6, P=0.003) (Fig 3.10); however the correlation between the lucigenin-amplified chemiluminescence response of biopsies from acetic acid induced colitis alone and the histological score just failed to reach significance, Rho=+0.5, P=0.053.
Table 3.12  Multiple linear regression analysis of luminol-amplified chemiluminescence (co-variable) against elements of the histological score.

<table>
<thead>
<tr>
<th>Histological Feature</th>
<th>Coefficient</th>
<th>Standard Error</th>
<th>Standard Coefficient</th>
<th>t-value</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophil Infiltrate</td>
<td>-0.3</td>
<td>0.2</td>
<td>-0.1</td>
<td>1.3</td>
<td>0.18</td>
</tr>
<tr>
<td>Epithelium</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.7</td>
<td>0.51</td>
</tr>
<tr>
<td>Lamina Propria</td>
<td>0.1</td>
<td>0.2</td>
<td>0.1</td>
<td>0.8</td>
<td>0.43</td>
</tr>
<tr>
<td>Muscularis Mucosa</td>
<td>0.5</td>
<td>0.2</td>
<td>0.4</td>
<td>2.6</td>
<td>0.01</td>
</tr>
<tr>
<td>Submucosa</td>
<td>0.1</td>
<td>0.2</td>
<td>0.1</td>
<td>0.5</td>
<td>0.62</td>
</tr>
<tr>
<td>Serosa</td>
<td>-0.3</td>
<td>0.2</td>
<td>-0.2</td>
<td>1.5</td>
<td>0.13</td>
</tr>
<tr>
<td>Lamina Propria</td>
<td>0.1</td>
<td>0.2</td>
<td>0.2</td>
<td>1.5</td>
<td>0.13</td>
</tr>
<tr>
<td>Muscularis Mucosa</td>
<td>0.5</td>
<td>0.2</td>
<td>0.3</td>
<td>1.8</td>
<td>0.07</td>
</tr>
<tr>
<td>Submucosal Neutrophil</td>
<td>0.5</td>
<td>0.2</td>
<td>0.2</td>
<td>2</td>
<td>0.05</td>
</tr>
<tr>
<td>Margination</td>
<td>-0.2</td>
<td>0.1</td>
<td>-0.2</td>
<td>1.7</td>
<td>0.1</td>
</tr>
<tr>
<td>Submucosal Oedema</td>
<td>0.1</td>
<td>0.2</td>
<td>0.1</td>
<td>0.4</td>
<td>0.66</td>
</tr>
<tr>
<td>Epithelial Necrosis</td>
<td>-0.1</td>
<td>2</td>
<td>-0.1</td>
<td>0.7</td>
<td>0.50</td>
</tr>
</tbody>
</table>

Fig 3.10  Relationship of lucigenin-amplified chemiluminescence to the histology score in biopsies from 16 rats pre-treated with 3% acetic acid-induced colitis at 24 hours and 9 untreated controls. Rho=+0.6, P=0.003, Spearman's rank correlation coefficient.
SUMMARY OF MAIN FINDINGS

- The method for inducing colitis in rats using rectal infusion of acetic acid and for sampling the inflamed colonic mucosa was established.
- The macroscopic scores of acetic acid-induced colitis were higher than for saline-perfused controls which was, in turn, higher than untreated controls.
- The histological score of acetic acid-induced colitis was greater than that for saline-perfused controls and untreated controls. There was no significant difference between saline-perfused and untreated controls.
- The luminol-amplified chemiluminescence response of colonic biopsies from acetic acid-induced colitis was higher than for saline-perfused controls and untreated controls.
- The lucigenin-amplified chemiluminescence response of colonic biopsies from acetic acid-induced colitis and saline-perfused controls was greater than for untreated controls.
- Both the luminol- and lucigenin-amplified chemiluminescence correlated with the macroscopic score and histological score when all groups of animals (untreated, saline-perfused and acetic acid-treated) were studied. When only acetic acid-induced colitis was included there was a significant correlation of the histological score with luminol-, but not lucigenin-amplified chemiluminescence.
- Multiple-linear regression of the variables of the histological score demonstrated a significant correlation between luminol-amplified chemiluminescence and the degree of neutrophil infiltration into the submucosa.

DISCUSSION

In summary, the acetic acid-induced model of experimental colitis in rats has been successfully established using a technique which maximises the amount of available tissue for in vitro experimental work. Use of full-thickness biopsies is a practical method of obtaining colonic tissue and minimises damage to the mucosal architecture. Chemiluminescence measurements are thus more likely to reflect in vivo function. Furthermore, this technique allows histological analysis of the tissue; in this latter respect the technique is similar to colorectal endoscopic biopsies from patients. ROS production by colonic biopsies, measured by luminol- and lucigenin-amplified chemiluminescence, from this model correlated with macroscopic and microscopic scores, in particular the degree of neutrophil infiltration into the submucosa.

Full-thickness colonic biopsies from rats with acetic acid-induced colitis have been shown here to produce increased levels of ROS, as detected by amplified...
chemiluminescence, compared with control tissue. This confirms previous work by Keshavarzian et al using mucosal scrapings (Keshavarzian et al 1990). In addition, it has been demonstrated that the level of ROS production correlates with the grade of mucosal inflammation, as assessed by the macroscopic and histological score. This compares with similar work in this laboratory showing that luminol-amplified chemiluminescence of colorectal biopsies from patients with UC correlated well with the sigmoidoscopic and histological grade of inflammation (Simmonds et al 1992a). Furthermore, the ROS production was most closely related to the degree of neutrophil infiltration into the submucosa, supporting previous evidence that neutrophils are chiefly responsible for luminol-amplified chemiluminescence (De Chatelet et al 1982).

There were small but statistically significant differences in the macroscopic score and luminol- and lucigenin-amplified chemiluminescence response between untreated and saline-perfused controls. It is not clear whether the infusion of 3ml saline into the colon causes these changes in macroscopic appearance and chemiluminescence response as a result of direct trauma by the tube, stretching of the colonic wall, or some other mechanism. In any event, the results reinforce the need to use saline-perfused controls in experiments with acetic acid-induced colitis.

Lucigenin-amplified chemiluminescence in saline-perfused and untreated controls was apparently greater than luminol-amplified chemiluminescence in the same groups. As lucigenin is more responsive to superoxide than luminol (Halliwell 1989a), this suggests that the uninflamed colon in the rat produces predominantly superoxide, whereas inflamed tissue produces predominantly luminol-amplified chemiluminescence, which is neutrophil-dependent (De Chatelet et al 1982).

There was considerable variability in the degree of inflammation from animal to animal because of the difficulties in keeping the acetic acid in contact with the mucosa for a defined length of time using the rectal route of administration. Other workers have achieved better reproducibility using intra-colonic injection at laparotomy (Keshavarzian et al 1990) and direct application of acetic acid to the mucosal surface at laparotomy and opening of the colon (Fedorak et al 1992). The importance of the time that the acetic acid is in contact with the mucosa was confirmed by the demonstration of a correlation between the time taken to expel the acetic acid and the resulting macroscopic score. However these techniques require the animals to undergo laparotomy which increases the level of complexity of the experiments and is unnecessary for studies requiring only a supply of inflamed colonic tissue for in vitro studies. Despite this variability there was a reasonable correlation between the degree of macroscopic damage, the histological grade and the chemiluminescence response.
THE ANTIOXIDANT POTENTIAL OF THERAPEUTIC AGENTS FOR INFLAMMATORY BOWEL DISEASE

INTRODUCTION

The potential pro-inflammatory role for ROS in IBD is well established (Babbs 1992; Simmonds et al 1992a; Sedghi et al 1993; Grisham 1994a), and is presented in detail in Chapter 1, as is the evidence that current therapy may act via antioxidant mechanisms. In addition, there is preliminary clinical evidence that specific antioxidant therapy may be effective in inflammatory bowel disease. In steroid-resistant Crohn’s disease (CD) a combination of superoxide dismutase (SOD) and desferrioxamine was reportedly effective in an uncontrolled study (Emerit et al 1991), while allopurinol has been used successfully in acute and chronic pouchitis (Levin et al 1992). Excess ROS may also be pathogenic in experimental models of intestinal inflammation (Keshavarzian et al 1990; Grisham et al 1991); antioxidants ameliorate inflammation in acetic acid-induced colitis and TNBS-induced colitis (Yoshikawa et al 1992; Keshavarzian et al 1992a).

Thus, it would be useful to have an easily applicable method for assessing the ability of proposed therapies for IBD, to reduce ROS production by the inflamed colonic mucosa. This chapter describes the application of the acetic acid-induced colitis model in rats for this purpose.

Use of Colorectal Biopsies and in Vitro Techniques for Detection of Antioxidant Activity of Test Compounds

ROS production is increased by colorectal biopsies from patients with UC and CD and in UC, ROS production is diminished by conventional antioxidants and by 5-ASA (Simmonds et al 1992a). However, application of human tissue for screening purposes is hampered by the limited availability of biopsy material from patients with active UC. Use of inflamed colorectal biopsies from an animal model of IBD would therefore be a useful alternative.

As already discussed, the advantages of the acetic acid-induced colitis are the similarity of the inflammatory response to UC with respect to histology, eicosanoid production (Sharon et al 1985), and its response to sulphasalazine (Keshavarzian et al 1992a). Furthermore, as has been described in Chapter 3, the inflamed colonic mucosa
in acetic acid-induced colitis produces excess ROS (Keshavarzian et al 1990). The ease, rapidity and cheapness of inducing an inflammatory response in acetic acid-induced colitis are further advantages.

**Choice of Compounds to Validate this Model of Assessing the Antioxidant Potential of Novel Therapies for Inflammatory Bowel Disease**

Studies using a similar approach to that described here, but limited by the availability of tissue, have been carried out using colorectal biopsies from patients with active UC (Simmonds et al 1992a; Simmonds 1992b; Simmonds et al 1992c). These human data were obtained in this laboratory, and thus were available for direct comparison with those derived from this study in rats. A range of conventional antioxidants (sodium azide, catalase, taurine, CuZnSOD, dimethyl sulphoxide, N-acetylcysteine and ascorbate), and two standard therapies for IBD (5-aminosalicylate (5-ASA) and hydrocortisone), have been studied using human tissues (Simmonds et al 1992a; Simmonds 1992b; Simmonds et al 1992c). These experiments were therefore repeated using colonic biopsies from acetic acid-induced colitis.

**Potential New Therapeutic Agents for Inflammatory Bowel Disease**

These studies were extended to examine the effects of potential new antioxidant approaches with two novel antioxidants, LY231617 and amflutizole. Results using rh-MnSOD and desferrioxamine are presented in chapters 6 and 7, respectively.

**LY231617**

The novel antioxidant compound, LY231617 (Eli-Lilly & Co, Indiana, USA) has been shown to protect against cerebral ischaemia in rats *in vivo*, and hydrogen peroxide-induced neuronal damage *in vitro* (Clemens et al 1993). The chemical structure of LY231617 is shown in Fig 4.1. The suggested role of the microvasculature in IBD (Knutson et al 1968; Geller et al 1983; Wakefield et al 1991) made this an interesting compound for further study.

**Amflutizole**

Ischaemia-reperfusion increases superoxide generation via the conversion of xanthine dehydrogenase to xanthine oxidase (McCord 1985), and may have a pathogenic role in IBD, particularly if mucosal ischaemia results from the known vascular abnormalities in IBD (Wakefield et al 1989). A related disorder, pouchitis occurring after ileo-anal anastomosis in UC, is ameliorated by the competitive xanthine oxidase inhibitor, allopurinol (Levin et al 1992). The active metabolite of allopurinol, oxypurinol, improves morphological changes and reduces tissue levels of TBARS in TNBS-induced colitis (Siems et al 1992).
Amflutizole is a novel non-competitive inhibitor of xanthine oxidase (Eli-Lilly & Co, Indiana, USA), which has efficacy in neuronal ischaemia in rats (Phillis et al 1994). The structure of this molecule is shown in Fig 4.1.

![Chemical structures of Amflutizole and LY231617](image)

Fig 4.1 The chemical structures of Amflutizole and LY231617

**HYPOTHESIS**

It was hypothesised that the amplified-chemiluminescence response to a range of standard antioxidants and two conventional therapies for IBD, 5-ASA and hydrocortisone, would be similar in inflamed colonic biopsies from acetic acid-induced colitis, to that previously shown for UC.

**AIMS OF STUDY**

The aim of this study was to develop a method for screening compounds for antioxidant effects on inflamed colon using colonic biopsies from the acetic acid model of colitis in rats. We aimed to validate the technique by comparing the results obtained with previous data using biopsies from patients with active UC (Simmonds et al 1992a; Simmonds 1992b; Simmonds et al 1992c). A range of conventional antioxidants and two standard therapies for IBD were evaluated. In addition, we studied the effects of two novel antioxidants, LY231617 and amflutizole.

**MATERIALS AND METHODS**

**Reagents**

All chemical reagents were of analytical grade and were obtained from Sigma Chemical Co, Poole, Dorset, unless otherwise stated. The CuZnSOD was from human erythrocytes (specific activity: 3610 U/mg protein). Catalase was from bovine liver attached to 4% agarose beads (specific activity: 150,000-200,000 U/g agarose).
Table 4.1 Study compounds - mechanism of antioxidant activity.

<table>
<thead>
<tr>
<th>Drug/Compound</th>
<th>Antioxidant Action</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Conventional Antioxidants</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium Azide</td>
<td>Inhibits myeloperoxidase</td>
<td>(Klebanoff 1992)</td>
</tr>
<tr>
<td></td>
<td>Scavenges OH*</td>
<td>(Nurcombe et al 1989)</td>
</tr>
<tr>
<td>Catalase</td>
<td>Metabolises H₂O₂</td>
<td></td>
</tr>
<tr>
<td>Taurine</td>
<td>Scavenges HOCl</td>
<td>(Dallegri et al 1990)</td>
</tr>
<tr>
<td></td>
<td>Scavenges OH*</td>
<td>(Green et al 1991)</td>
</tr>
<tr>
<td>CuZnSOD</td>
<td>Metabolises O₂·</td>
<td>(Fridovich 1974)</td>
</tr>
<tr>
<td>Dimethylsulphoxide</td>
<td>Scavenges OH*</td>
<td>(Beilke et al 1987)</td>
</tr>
<tr>
<td>N-Acetylcysteine</td>
<td>Provides substrate for glutathione</td>
<td>(Halliwell et al 1987)</td>
</tr>
<tr>
<td></td>
<td>peroxidase; scavenges HOCl</td>
<td></td>
</tr>
<tr>
<td>Ascorbate</td>
<td>Scavenges HOCl</td>
<td>(Bendich et al 1987)</td>
</tr>
<tr>
<td></td>
<td>Scavenges thiyl/peroxyl/O₂·</td>
<td>(Nishikimi 1975)</td>
</tr>
<tr>
<td><strong>Standard Therapies for IBD</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-ASA</td>
<td>Scavenges OH*</td>
<td>(Miyachi et al 1987; Allgayer et al 1992)</td>
</tr>
<tr>
<td></td>
<td>Scavenges O₂·</td>
<td>(Miyachi et al 1987; Gionchetti et al 1991)</td>
</tr>
<tr>
<td></td>
<td>Scavenges HOCl</td>
<td>(Aruoma et al 1987a; Williams et al 1989)</td>
</tr>
<tr>
<td>Iron chelator</td>
<td></td>
<td>(Winrow et al 1990)</td>
</tr>
<tr>
<td>Inhibits lipid peroxidation</td>
<td></td>
<td>(Greenfield et al 1991)</td>
</tr>
<tr>
<td>Inhibits neutrophil respiratory burst</td>
<td></td>
<td>(Allgayer et al 1994)</td>
</tr>
<tr>
<td>Iron chelator</td>
<td></td>
<td>(Yamada et al 1990)</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>Inhibits neutrophil function</td>
<td>(Baltch et al 1986)</td>
</tr>
<tr>
<td><strong>Proposed Therapies in IBD</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amflutizole</td>
<td>Inhibits xanthine oxidase</td>
<td>(Phillis et al 1994)</td>
</tr>
<tr>
<td>LY231617</td>
<td>Not known</td>
<td></td>
</tr>
</tbody>
</table>

Amflutizole (3-(3-trifluoromethyl)-4-aminoisothiazole-5-carboxylic acid) and LY231617 (2,6-bis(1,1-dimethylethyl)-4-[[1-ethyl]amino]methyl]phenol hydrochloride) were a gift from Dr. Jill Panetta (Eli-Lilly & Co, Indiana, USA). The compounds used and their main mechanisms of antioxidant activity are shown in Table 4.1.
All compounds and appropriate controls were made up on the day of the experiment and adjusted to pH 7.4 by addition of 1M NaOH or HCl prior to use. Controls for these experiments were vehicle for all compounds except CuZnSOD for which the heat-inactivated enzyme was prepared by heating in D-PBS to 100°C for two hours (Heikkila 1985). Luminol was used as the chemiluminescence amplifier for all compounds apart from CuZnSOD for which lucigenin was used. The experimental conditions for the test compounds is shown in Table 4.2.

<table>
<thead>
<tr>
<th>Drug/Compound</th>
<th>Concentration tested</th>
<th>Soluble in Aqueous Solution</th>
<th>Chemiluminescence Amplifier</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Conventional Antioxidants</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium Azide</td>
<td>1mM</td>
<td>+</td>
<td>Luminol</td>
</tr>
<tr>
<td>Catalase</td>
<td>3000U/ml</td>
<td>+</td>
<td>Luminol</td>
</tr>
<tr>
<td>Taurine</td>
<td>20mM</td>
<td>+</td>
<td>Luminol</td>
</tr>
<tr>
<td>CuZnSOD*</td>
<td>30/300U/ml</td>
<td>+</td>
<td>Lucigenin</td>
</tr>
<tr>
<td>Dimethylsulphoxide</td>
<td>1/5/10%</td>
<td>+</td>
<td>Luminol</td>
</tr>
<tr>
<td>N-Acetylcysteine</td>
<td>20mM</td>
<td>+</td>
<td>Luminol</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>20mM</td>
<td>+</td>
<td>Luminol</td>
</tr>
<tr>
<td><strong>Standard Therapies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>for IBD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-ASA</td>
<td>1/10/20mM</td>
<td>+</td>
<td>Luminol</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>1mM</td>
<td>+</td>
<td>Luminol</td>
</tr>
<tr>
<td><strong>Proposed Therapies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>for IBD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amflutizole</td>
<td>1/10/20mM</td>
<td>-</td>
<td>Luminol</td>
</tr>
<tr>
<td>LY231617</td>
<td>1/10mM</td>
<td>-</td>
<td>Luminol</td>
</tr>
</tbody>
</table>

All compounds and controls were dissolved in Dulbecco’s phosphate buffered saline (D-PBS). *Control for all compounds was D-PBS apart from CuZnSOD for which the control was the heat-inactivated enzyme.

**Induction of Experimental Colitis in Rats**

The method of induction of acetic acid-induced colitis in rats is described in Chapter 2, p. 45.
Assessment of Antioxidant Activity of Test Compounds Using Colonic Biopsies from Acetic Acid-Induced Colitis

Water-soluble Compounds

Fig 4.2 illustrates, schematically, the experimental technique for discerning the effect of water-soluble compounds on the amplified chemiluminescence of colonic biopsies from acetic acid-induced colitis and UC in humans.

Biopsies were placed in pre-oxygenated (95% O₂, 5% CO₂ for 10min) D-PBS, with added calcium (1.13mM) and glucose (5mM) at ambient temperature. They were then transferred to scintillation vials containing 1.8ml of 300µM luminol or lucigenin and chemiluminescence measured in a Packard Tri Carb 1600 CA liquid scintillation counter analyser operated in ‘the out-of-coincidence’ mode for 2min (see Chapter 2). 200µl of the test compound or appropriate control, at 10 times the final concentration, was then added to the vial and after vortexing for two seconds, the chemiluminescence re-counted. For each compound at each concentration a pair of biopsies from each of ≥5 rats, one treated with the test compound and one with vehicle, was used to assess the percentage change in chemiluminescence. After counting, samples were blotted, weighed and, where appropriate, placed in 10% formol saline for subsequent histology.

Sodium azide, catalase, taurine, reduced glutathione, CuZnSOD, DMSO, N-acetylcysteine, ascorbate and hydrocortisone were dissolved in D-PBS and adjusted to pH 7.4 as described above. 5-ASA was initially dissolved in 1M NaOH then made up in D-PBS and adjusted to pH 7.4.

Water-insoluble Compounds

LY231617 and amflutizole were insoluble in aqueous solution at the desired concentrations. A suspension of each compound was therefore prepared in 10% acacia, dissolved in D-PBS, oxygenated for 10min in 95% O₂, 5% CO₂ and adjusted to pH 7.4 with 1M NaOH. Colonic biopsies were prepared as above. Following the initial chemiluminescence count the biopsies were placed in 10% acacia, with or without the addition of the test compound, in suspension (see Fig 4.3). Incubation was carried out for 30min in a shaking water bath at 37°C. At the end of this time the samples were removed, washed twice in oxygenated D-PBS to remove excess acacia, placed in 1.8ml of 300µM luminol and recounted in the chemiluminometer. Controls were incubated in 10% acacia, in D-PBS, alone. To compare these results with those from water-soluble compounds, and to provide a positive control, 5-ASA (20mM) was analysed in a similar way.
Calculations And Statistics

Effect of Water-Soluble Compounds

For each compound at each concentration a pair of biopsies from each of ≥5 rats was used to assess the percentage change in chemiluminescence. The chemiluminescence counts before and after addition of test compound are given as \( t_0 \) and \( t_1 \), respectively, and for the biopsy treated with control, \( c_0 \) and \( c_1 \), respectively. To take account of small changes in control values during the assay, the change in chemiluminescence produced by the test compound on one biopsy was adjusted by the change in chemiluminescence produced by the control solution with its pair. Accordingly, the percentage change in chemiluminescence response induced by the test compound (\( \Delta\%t \)) is calculated as follows: 
\[ \Delta\%t = \left( \frac{(t_1 \times c_0/c_1) - t_0)}{t_0} \right) \times 100. \]

The results are expressed as median (IQR)% change in the chemiluminescence response. The difference in chemiluminescence response between biopsies treated with test compounds and controls is calculated by comparing the percentage change in chemiluminescence induced by the test agent and control using the Wilcoxon signed-rank test for paired variables. A P-value of less than 0.05 (two-tailed) was taken as significant.

To investigate the stability of the change in chemiluminescence response produced by vehicle alone, the CV of the response to D-PBS (control for most of the compounds tested) was calculated for 2-6 biopsies from each of 10 animals and the median (IQR) expressed for the CV of the luminol-amplified chemiluminescence response to D-PBS in all 10 (Snedecor 1956).

Effect of Water-Insoluble Compounds

Results are expressed as the mean percentage change in chemiluminescence for each group of biopsies exposed to the test compound after correction of the initial count for the percentage change induced by acacia alone (control). No range is given because grouping the biopsies together resulted in loss of animal identity. The chemiluminescence counts before and after exposure to the test compound were compared using the Mann-Whitney U test. A P-value of less than 0.05 (two-tailed) was taken as significant.

To investigate the variability of the chemiluminescence response induced by incubation of biopsies in 10% acacia, the median CV was calculated for 8 groups of 5 biopsies from 5 rats incubated in 10% acacia alone that were used as controls in experiments assessing the effect of water-insoluble compounds (Fig 4.3).
Excised rat colon

Biopsies are cut from the dissected rat colon and placed in Dulbecco's PBS with 1.13mM CaCl$_2$ and 5mM glucose, oxygenated with 95% oxygen, 5% CO$_2$ for 10 min.

**Control Biopsy**

The biopsy is transferred to a scintillation vial containing luminol/lucigenin (300μM) and counted in the scintillation counter for 2 min.

After the initial count the vial is removed and 200μl of either vehicle or the test compound, at 10x the final concentration added.

The vial is vortexed for 2 seconds to ensure mixing of the vehicle or test compound.

Each biopsy is counted in the scintillation counter for 2 min.

**Test Biopsy**

The biopsy is transferred to a scintillation vial containing luminol/lucigenin (300μM) and counted in the scintillation counter for 2 min.

Vial containing test compound

Fig 4.2 Method for assessing the effect of water-soluble test compounds on the amplified chemiluminescence produced by inflamed colonic biopsies from acetic acid-induced colitis. The chemiluminescence counts before and after addition of test compound are t0 and t1, respectively, and for control biopsies, c0 and c1, respectively. The change in chemiluminescence produced by the test compound on one biopsy was adjusted by the change in chemiluminescence produced by the control solution with its pair, as follows: $\Delta%t = (((t1 \times c0/c1) - t0)) / t0) \times 100.$
Biopsies are cut from the dissected rat colon and placed in Dulbecco's PBS with 1.13mM CaCl$_2$ and 5mM glucose, oxygenated with 95% oxygen, 5% CO$_2$ for 10min (D-PBS).

Control Biopsies from ≥5 rats

The biopsies are transferred to a scintillation vial containing luminol/lucigenin (300μM) and counted in the scintillation counter for 2min.

Test Biopsies from ≥5 rats

After the initial count the biopsies are placed in 10% acacia dissolved in D-PBS, with or without a suspension of the test compound for 30min.

10% Acacia in D-PBS

The biopsies are removed and washed in 2 cycles of D-PBS.

Each biopsy is counted in the scintillation counter for 2min.

Fig 4.3 Method for assessing the effect of water-insoluble test compounds on the amplified chemiluminescence produced by inflamed colorectal biopsies from acetic acid-induced colitis. Compounds were prepared in 10% acacia, dissolved in D-PBS, oxygenated for 10min in 95% O$_2$, 5% CO$_2$ and adjusted to pH 7.4 with 1M NaOH. Following the initial chemiluminescence count the biopsies were placed in 10% acacia, ± test compound and incubated for 30min in a shaking water bath at 37°C. The samples were then removed, washed twice in oxygenated D-PBS to remove excess acacia, placed in 1.8ml of 300μM luminol and recounted in the chemiluminometer.
RESULTS

Variability of the Chemiluminescence Assay

Variability of the change in chemiluminescence after addition of vehicle

The variability of the response to D-PBS was calculated to give an indication of the reproducibility of the assay. The CV (mean (IQR)) of the change in luminol-amplified chemiluminescence resulting from incubation in D-PBS (control for most of the experiments) calculated from the 10 animals in which there were two or more control experiments was 18 (11–41)%. This indicates that a change in the chemiluminescence response of at least 18% might be needed to detect a statistically significant difference between the vehicle and test compound.

Variability of the change in chemiluminescence after incubation of acetic acid biopsies in 10% acacia

Because the assay was adapted for use with insoluble agents a similar calculation was made for the variability of chemiluminescence response to vehicle alone (10% acacia). The CV for the mean change in chemiluminescence of biopsies incubated in 10% acacia alone was 48%. This reflects the increased variability of this system compared to incubation in D-PBS.

Comparison of Response of Biopsies from Acetic Acid-Induced colitis and UC to Conventional Antioxidants

Significant reductions in luminol-amplified chemiluminescence in the acetic acid-induced colitis model were observed with sodium azide (median (IQR)) (-83 (-85 - -73)%; P=0.03); catalase (-43 (-47 - -41)%; P=0.03); 10% DMSO (-67 (-78 - -50%); P=0.03) and ascorbate (-54 (-69 - -35)%; P=0.01) and in lucigenin-amplified chemiluminescence with CuZnSOD (-26 (-32 - -15)%; P=0.03) (Fig 4.4). A limited dose response for DMSO gave an IC_{50} of 6%. N-acetylcysteine produced no significant change in luminol-amplified chemiluminescence. The profile of responses in acetic acid-induced colitis demonstrate a striking resemblance to that previously observed in UC (Simmonds et al 1992a; Simmonds 1992b; Simmonds et al 1992c). With one compound, taurine, a small, but significant, reduction was produced in UC biopsies (Simmonds et al 1992a) but not in acetic acid-induced colitis.
Fig 4.4  The percentage change in chemiluminescence response of inflamed biopsies from acetic acid-induced colitis to conventional antioxidants compared with results using mucosal biopsies from active UC (Data redrawn from Simmonds et al 1992a; Simmonds 1992b; Simmonds et al 1992c). Chemiluminescence was measured before and after exposure to test compound and compared to a second biopsy exposed to vehicle (n ≥ 5; *denotes P < 0.05 drug compared with vehicle, Wilcoxon signed rank test). Luminol was used as the amplifier for chemiluminescence for all compounds except CuZnSOD, for which lucigenin was used. NAC = N-acetylcysteine.
Comparison of Response of Biopsies from Acetic Acid-induced Colitis and UC to Standard Therapies

As for UC biopsies, 5-ASA produced a significant reduction in luminol-amplified chemiluminescence response in acetic acid-induced colitis biopsies (median (IQR)) -88 (-89 – -70)% at 20mM 5-ASA, P=0.03) with a limited dose-response producing an estimated IC50 of 4mM (Fig 4.5). Hydrocortisone did not alter luminol-amplified chemiluminescence in either acetic acid-induced colitis or UC biopsies (Simmonds 1992b) (Fig 4.5).

![Graph showing percentage change in luminol-amplified chemiluminescence response](image)

**Fig 4.5** The percentage change in the luminol-amplified chemiluminescence response of inflamed biopsies from acetic acid-induced colitis to standard treatments in IBD compared with results using mucosal biopsies from active UC (†Data redrawn from (Simmonds et al 1992a; Simmonds 1992b; Simmonds et al 1992c). Chemiluminescence was measured before and after exposure to test compound and compared to a second biopsy exposed to vehicle (n≥5 ; *P<0.05 drug compared with vehicle, Wilcoxon signed rank test, two-tailed).
Response of Biopsies from Acetic Acid-Induced Colitis to Potential New Therapies for Inflammatory Bowel Disease

Two novel, water-insoluble, antioxidant compounds, LY231617 and amflutizole, produced marked reductions in luminol-amplified chemiluminescence after incubation of the biopsies in suspensions of the compounds in 10% acacia (means, -98%, \( P=0.009 \) and -88%, \( P=0.028 \), respectively) (Fig 4.6). 5-ASA (20mM), which had previously been shown to reduce luminol-amplified chemiluminescence in aqueous solution, reduced the chemiluminescence by a similar amount (mean, 83% \( P=0.002 \)) when tested in acacia.

Fig 4.6 Mean percentage change in luminol-amplified chemiluminescence response of inflamed biopsies from acetic acid-induced colitis to proposed new therapies, LY231617, amflutizole, and to 5-ASA. Chemiluminescence was measured before and after incubation of colonic biopsies in a suspension of the test compound in 10% acacia for 30min and compared with biopsies exposed to acacia alone \( (n=5) \); * denotes \( P<0.05 \) drug compared with vehicle, Mann-Whitney U test).

SUMMARY OF MAIN FINDINGS

- The profile of effects on the chemiluminescence response of acetic acid-induced colitis biopsies given by conventional antioxidants (sodium azide, catalase, copper-zinc superoxide dismutase, dimethyl sulfoxide, N-acetylcysteine and ascorbate) and standard therapies (5-aminosalicylate and hydrocortisone) resembled that previously reported using biopsies from ulcerative colitis.
- Two novel compounds, LY231617 and amflutizole substantially reduced chemiluminescence (98% and 88%, respectively).
DISCUSSION

The results demonstrate that colonic biopsies from the acetic acid-induced colitis model respond to conventional antioxidants and standard therapies for IBD, 5-ASA and hydrocortisone, in a similar fashion to mucosal biopsies from patients with active UC (Simmonds et al 1992a; Simmonds 1992b; Simmonds et al 1992c). The estimated IC$_{50}$ of 5-ASA in this model compares favourably with the intraluminal concentrations recorded after oral administration of aminosalicylates (5-10mM) in UC (Staerk-Laursen et al 1990). The concentrations are also similar to those required to reduce LTB$_4$ production by colorectal UC biopsies, in vitro (Peskar et al 1987).

Hydrocortisone has no direct antioxidant activity in vitro in this model or with UC biopsies (Simmonds 1992b). However, other studies, using prolonged incubation with dexamethasone in vitro, have demonstrated inhibition of superoxide generation by stimulated phagocytes (Maridonneau-Parini et al 1989). This suggests that the therapeutic effect of hydrocortisone in vivo may thus be partly due to an indirect antioxidant action by inhibition of neutrophil function (Baltch et al 1986).

Of the conventional antioxidants examined, the greatest inhibition occurred with azide, a potent inhibitor of myeloperoxidase (Allen 1982). Azide also inhibits other haem proteins such as catalase, though this would lead to an increase in ROS production, and also quenches singlet oxygen and the hydroxyl ion (Nurcombe et al 1989). Although the antioxidant action of azide could result from a cytotoxic effect, azide has been shown to reduce the luminol-amplified chemiluminescence of stimulated neutrophils to a greater effect when added before the stimulus (100%) than after (20%) (Hallett et al 1983), an effect which would not occur if the reduction in chemiluminescence was purely due to cytotoxicity. Furthermore, azide does not alter the luminol-amplified chemiluminescence response of activated rat macrophages, which lack myeloperoxidase (Wang et al 1993), nor does it inhibit superoxide production by activated neutrophils (Tauber et al 1979).

The present studies using acetic acid-induced colitis biopsies support previous findings showing that CuZnSOD reduces lucigenin-amplified chemiluminescence by mucosal biopsies in vitro from UC (Simmonds 1992b). These findings also provide further support for an in vivo effect, based on limited clinical studies in CD (Emerit et al 1991), in UC (Niwa et al 1985), and in acetic acid-induced colitis in rats (Keshavarzian et al 1992a).

An antioxidant effect for catalase can be demonstrated in both acetic acid-induced colitis and UC biopsies (Simmonds et al 1992a) further supporting the suggestion that neutrophil-mediated ROS are important in IBD, and in acetic acid-induced colitis in rats, in which catalase is of proven benefit (Keshavarzian et al 1992a).
N-acetylcysteine replenishes intracellular stores of glutathione, is a weak scavenger of superoxide and hydroperoxide, but reacts avidly with hypochlorous acid and the hydroxyl radical (Aruoma et al 1989). However, N-acetylcysteine was ineffective in reducing ROS production by acetic acid-induced colitis or UC biopsies under the present experimental conditions (Simmonds 1992b).

In this investigation ascorbate produced similar reductions in chemiluminescence in acetic acid-induced colitis to that previously shown with UC biopsies (Simmonds 1992b). However, ascorbate is readily oxidised to the potentially damaging ascorbate radical in the presence of iron (Rowley et al 1983) which is present in the bowel lumen in millimolar quantities (Babbs 1992). This reaction could limit the potential usefulness of ascorbate as a therapy in UC.

The predominant antioxidant action of DMSO in vitro is scavenging of the hydroxyl ion (Repine et al 1979). As the hydroxyl ion is extremely reactive the local concentration of any scavenger would have to be prohibitively high to compete with biological molecules in the vicinity of its production. This may explain evidence from in vivo studies in which oral 5% DMSO failed to improve acetic acid-induced colitis (Keshavarzian et al 1990).

The hypochlorite scavenger taurine had little effect on ROS production by UC biopsies (Simmonds et al 1992a). This may be because the reaction product of taurine and hypochlorous acid, taurine monochloramine, retains oxidant activity, sufficient, for example, to inactivate α1-antiproteinase (Aruoma et al 1988).

The potent antioxidant activity of the novel agent, LY231617, demonstrated here, suggests that it should be further evaluated as therapy in IBD. The other novel compound studied, amflutizole, also showed potent antioxidant activity, which was at least as effective as 5-ASA in reducing the chemiluminescence response of biopsies from acetic acid-induced colitis. Given the apparent clinical benefit of the competitive xanthine oxidase inhibitor, allopurinol, in pouchitis (Levin et al 1992), amflutizole is a further candidate for research into the role of xanthine oxidase inhibition as a therapeutic option in IBD.

In conclusion, this in vitro system using biopsies taken from rats with acetic acid-induced colitis provides a convenient method for the screening of the antioxidant potential of new therapies for IBD. Of the agents assessed in the present study, dimethyl sulfoxide, ascorbate, amflutizole and LY231617 seem most suitable for further evaluation in IBD.
5
CHEMILUMINESCENCE OF HUMAN COLORECIAL MUCOSAL BIOPSIES

INTRODUCTION

Previous work in this laboratory has demonstrated that luminol-amplified chemiluminescence correlates with the macroscopic and microscopic scores of colorectal mucosal inflammation in UC (Simmonds et al 1992a). However, a similar correlation with a recognised disease activity score, based on the patients symptoms and haematological and biochemical markers of activity, has not been shown. In this context, it is of interest that the production of other inflammatory mediators, such as TNFα (Gardiner et al 1995), and LTB₄ (Lauritsen et al 1986), which may be of pathogenic importance in UC, correlate with clinical disease activity. The aim of this Chapter was to repeat and extend the earlier studies to further validate the technique of amplified chemiluminescence using colorectal biopsies; in particular, to examine the relationship between mucosal ROS production and a score for clinical disease activity. The scoring system used was a modification of one previously described (McCarthy et al 1991; Cambridge et al 1992; Stevens et al 1992; Stevens et al 1993), and is based on the Harvey-Bradshaw Index for CD together with haematological and biochemical variables of relevance to UC, namely haemoglobin (Hb), erythrocyte sedimentation rate (ESR), and serum albumin.

HYPOTHESIS

Luminol-amplified chemiluminescence of colorectal biopsies from patients with UC correlates with the clinical disease activity and with the sigmoidoscopic and histological scores.

AIM

The aim of this study was to extend the studies previously performed in this laboratory (Simmonds et al 1992a) to examine the relationship between mucosal ROS production and disease activity measured using a clinical score, as well as sigmoidoscopically and histologically.
MATERIALS AND METHODS

Clinical Scoring of Disease Activity in Ulcerative Colitis

Scoring of disease activity in patients with UC was carried out using a modification of a previously described system (McCarthy et al 1991; Cambridge et al 1992; Stevens et al 1992; Stevens et al 1993), which combines the clinical criteria of the Harvey-Bradshaw Index, originally designed for use in CD (Harvey et al 1980), and the haematological and biochemical variables which are of relevance to UC; ESR, Hb and serum albumin (Table 5.1). The previous system scored the total number of liquid stools and arbitrarily scored the number of bloody stools as 0,1,2 or 3. This system was modified so that the total number of bloody stools was recorded as for liquid stools. A score of >5 was used to define remission as previously described (McCarthy et al 1991; Cambridge et al 1992; Stevens et al 1992; Stevens et al 1993).

Table 5.1 Clinical scoring system for disease activity in ulcerative colitis.

<table>
<thead>
<tr>
<th>Clinical/Laboratory Feature</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of liquid or mushy stools</td>
<td>number/day</td>
</tr>
<tr>
<td>Number of bloody stools</td>
<td>number/day</td>
</tr>
<tr>
<td>Number of formed stools</td>
<td>number&gt;3/day</td>
</tr>
<tr>
<td>Severity of abdominal pain</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Mild</td>
<td>1</td>
</tr>
<tr>
<td>Moderate</td>
<td>2</td>
</tr>
<tr>
<td>Severe</td>
<td>3</td>
</tr>
<tr>
<td>General well-being</td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>0</td>
</tr>
<tr>
<td>Slightly under par</td>
<td>1</td>
</tr>
<tr>
<td>Poor</td>
<td>2</td>
</tr>
<tr>
<td>Very poor</td>
<td>3</td>
</tr>
<tr>
<td>Terrible</td>
<td>4</td>
</tr>
<tr>
<td>Complications (arthralgia, uveitis, erythema nodosum, pyoderma gangrenosum)</td>
<td>1 each</td>
</tr>
<tr>
<td>Haemoglobin &lt;12g/dl (men) &lt;11.5g/dl (women)</td>
<td>1</td>
</tr>
<tr>
<td>ESR&gt;20mm/hour</td>
<td>1</td>
</tr>
<tr>
<td>Albumin&lt;35</td>
<td>1</td>
</tr>
</tbody>
</table>

Modified from (Stevens et al 1992).

Remission using this scoring system is defined as those with a score of <5.

Sigmoidoscopic Scoring

The assessment of the sigmoidoscopic appearances was made using the method of Baron (Baron et al 1964) as shown in Table 5.2.
Table 5.2 Scoring system for sigmoidoscopic appearances in UC.

<table>
<thead>
<tr>
<th>Sigmoidoscopic Appearances</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Mucosa oedema (loss of vascular pattern)</td>
<td>1</td>
</tr>
<tr>
<td>Contact bleeding</td>
<td>2</td>
</tr>
<tr>
<td>Spontaneous bleeding and/or mucosal ulceration</td>
<td>3</td>
</tr>
</tbody>
</table>

**Histological Scoring**

The assessment of the histological appearances was made using a modification of that described by Saverymuttu (Saverymuttu *et al* 1986), and identical to a previously published method (Simmonds *et al* 1992a) (Table 5.3).

Table 5.3 Histological assessment of colorectal biopsies from patients with UC.

<table>
<thead>
<tr>
<th>Histological Feature</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goblet Cell Depletion</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Slight</td>
<td>1</td>
</tr>
<tr>
<td>Moderate</td>
<td>2</td>
</tr>
<tr>
<td>Marked</td>
<td>3</td>
</tr>
<tr>
<td>Crypts Inflammation</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Single inflammatory cells</td>
<td>1</td>
</tr>
<tr>
<td>Cryptitis</td>
<td>2</td>
</tr>
<tr>
<td>Crypt abscesses</td>
<td>3</td>
</tr>
<tr>
<td>Neutrophil infiltration</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Slight increase</td>
<td>1</td>
</tr>
<tr>
<td>Moderate increase</td>
<td>2</td>
</tr>
<tr>
<td>Marked increase</td>
<td>3</td>
</tr>
<tr>
<td>Mononuclear cell infiltration</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Slight increase</td>
<td>1</td>
</tr>
<tr>
<td>Moderate increase</td>
<td>2</td>
</tr>
<tr>
<td>Marked increase</td>
<td>3</td>
</tr>
<tr>
<td><strong>Maximum Score</strong></td>
<td><strong>12</strong></td>
</tr>
</tbody>
</table>
Variability of the Histological Score of Colorectal Biopsies from Patients with Ulcerative Colitis

To assess the repeatability of the histological scoring system developed for use in these studies, the intra-observer variability was calculated using the coefficient of repeatability (Bland et al 1986) (see Chapter 2, p. 45).

10 sections were scored by a single experienced histopathologist (Dr Coumbe), blinded to their source. The coefficient of repeatability (2 SD of the differences between the measures (Bland et al 1986)) for the intra-observer variability (Table 5.4) was 2.2. The differences between the two sets of scorings were all lower than this figure, thus validating the scoring method (British Standards Institution 1979).

Table 5.4 Coefficient of repeatability in histological scoring of colorectal biopsies from patients with ulcerative colitis.

<table>
<thead>
<tr>
<th>Histology</th>
<th>Histology</th>
<th>1st and 2nd compared</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st Score</td>
<td>2nd Score</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>-1</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>-1</td>
</tr>
<tr>
<td>13</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>13</td>
<td>1</td>
</tr>
</tbody>
</table>

Means 0.50
Standard Deviation 1.08
Coefficient of Repeatability 2.16

Chemiluminescence Assay

The luminol-amplified chemiluminescence response was measured in a minimum of two biopsies, from adjacent sites, from each of 77 patients with UC and 13 controls. The procedure for collection of biopsies and for luminol-amplified chemiluminescence is described in Chapter 2 (p. 45).

Patients

Mucosal colorectal biopsies were obtained from patients undergoing routine sigmoidoscopy or colonoscopy. Each patient gave informed consent and the study was approved by the Tower Hamlets Health Authority Ethics Committee.
Controls were patients, with normal sigmoidoscopic appearances, undergoing investigation for lower gastrointestinal tract symptoms. In patients with adenomatous polyps, biopsies were taken at least 10cm from the lesion. The diagnosis of UC was based on conventional clinical, radiological, endoscopic and histological criteria.

Table 5.5 shows the demographic data of the patients studied. The patients with UC were subdivided according to the extent of the disease and the disease activity.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>Ulcerative Colitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>13</td>
<td>77</td>
</tr>
<tr>
<td>Age (range)</td>
<td>58 (47-67)</td>
<td>49 (45-52)</td>
</tr>
<tr>
<td>Male:Female</td>
<td>8:5</td>
<td>31:46</td>
</tr>
<tr>
<td>Disease Extent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distal</td>
<td>-</td>
<td>17</td>
</tr>
<tr>
<td>Left-sided</td>
<td>-</td>
<td>41</td>
</tr>
<tr>
<td>Sub-total/total</td>
<td>-</td>
<td>19</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td>5-ASA</td>
<td>0</td>
<td>41</td>
</tr>
<tr>
<td>Oral/rectal steroids alone</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>5-ASA + azathioprine</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>5-ASA + steroids + azathioprine</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>5-ASA + steroids</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>Sigmoidoscopic Score</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 0</td>
<td>13</td>
<td>8</td>
</tr>
<tr>
<td>Grade 1</td>
<td>0</td>
<td>27</td>
</tr>
<tr>
<td>Grade 2</td>
<td>0</td>
<td>35</td>
</tr>
<tr>
<td>Grade 3</td>
<td>0</td>
<td>7</td>
</tr>
</tbody>
</table>

**Calculation and Statistics**

The chemiluminescence is expressed as the number of photons/min/mg wet weight of tissue after subtraction of the background count. The background count of each vial containing 300μM luminol in Dulbecco’s PBS alone was measured prior to addition of the biopsy. Comparisons between groups for clinical score, luminol-amplified chemiluminescence response and sigmoidoscopic score in controls and patients were made using a Kruskal-Wallis test, and individual pairs were compared...
using a Mann-Whitney U test (two-tailed) with the Bonferroni correction for multiple comparisons (Godfrey 1985).

The coefficient of variation (CV) of the response to luminol by 2-4 biopsies was compared in 13 control and 77 UC patients (Snedecor 1956) (see Chapter 2, p. 45).

Correlation between the clinical disease activity score, sigmoidoscopic appearances, histological score and chemiluminescence response was assessed using Spearman’s rank correlation test.

RESULTS

**Variability of the Luminol-Amplified Chemiluminescence Response in Colorectal Biopsies**

To assess the variability of the luminol-amplified chemiluminescence response in colorectal biopsies, 26 colorectal biopsies from 13 controls and 233 biopsies from 77 patients with UC were assessed. The median (IQR) of the CV in controls was 28 (18–54)% and in UC was 49 (32–78)%. Given the higher variability in the UC biopsies than the control biopsies, a correlation was undertaken between the mean value of the luminol-amplified chemiluminescence and the variability for each patient, however there was no proven relationship between the two measures (Rho=+0.1, P=0.54).

**Clinical Score Compared with the Sigmoidoscopic Score**

A prime aim of this study was to investigate the relationship between the clinical disease activity and the ROS production by colorectal biopsies from patients with UC and to confirm previous findings of a correlation between sigmoidoscopic score and ROS production (Simmonds et al 1992a). The clinical disease score was therefore also compared to the sigmoidoscopic score in 77 patients and 13 controls (Fig 5.1). There was no significant difference between UC with a sigmoidoscopic score of 0, and controls, P=0.49. For all other sigmoidoscopic grades in UC, the clinical grade was significantly greater than controls; grade 1, P=0.035; grade 2, P=0.001; grade 3, P=0.008. Grade 1 colitis was also associated with significantly lower clinical scores than grade 2, P=0.003 and grade 3, P=0.016. However, there was no difference between the clinical score in grades 2 and 3 appearances, P=0.65.
Fig 5.1 Clinical Score in 77 patients with UC and 13 controls according to the sigmoidoscopic score. *P≤0.001 compared with normals and for UC, score 2 compared with score 0. †P≤0.01 for UC, score 3 compared with score 0 and for score 2 compared with score 1. ‡P≤0.05 for UC, score 1 compared with score 0 and for UC score 3 compared with score 1. Comparisons between groups were made using the Kruskal-Wallis test, followed by the Mann-Whitney U test, 2-tailed, with the Bonferroni adjustment for multiple comparisons (Godfrey 1985).

Luminol-amplified Chemiluminescence According to the Sigmoidoscopic Score

Fig 5.2 shows the log_{10}-transformed luminol-amplified chemiluminescence for 77 patients and 13 controls. The absolute values in median (IQR) for the chemiluminescence response in photons/min/mg wet weight of tissue after subtraction of background were as follows: normals, 4250 (3558–10896); UC with a sigmoidoscopic score of 0, 17392 (12934–50334); UC with a sigmoidoscopic score of 1, 36198 (15173–93814); UC with a sigmoidoscopic score of 2, 92563 (45620–282413); UC with a sigmoidoscopic score of 3, 96245 (34917–300897).

The luminol-amplified chemiluminescence response of colorectal biopsies from patients with UC with a sigmoidoscopic score of 1 was not significantly greater than controls, just failing to reach significance, P=0.051). Those from patients with a
sigmoidoscopic score of 2 and 3 produced significantly greater chemiluminescence than those from normal controls (P=0.003 and 0.0051 respectively). The chemiluminescence response of biopsies from patients with UC with a sigmoidoscopic grade of 2 were significantly greater than from patients with grade 0 and 1 colitis (P=0.03).

Fig 5.2 Log₁₀-transformed luminol-amplified chemiluminescence in 77 patients with UC and 13 controls according to the sigmoidoscopic score. *P≤0.001 compared to Normals, †P≤0.01 compared to Normals, ‡P=0.03 compared to UC, sigmoidoscopic score=0 and 1. All comparisons made using the Kruskal-Wallis test, followed by the Mann-Whitney U test, 2-tailed, with the Bonferroni adjustment for multiple comparisons (Godfrey 1985).

Luminol-amplified Chemiluminescence According to Clinical Score

Fig 5.3 illustrates the comparison between the clinical score with the luminol-amplified chemiluminescence of colorectal biopsies in 77 patients with UC. There was a weak, though significant, correlation between the two scores Rho=+0.2, P=0.013.
Multiple Linear Regression Analysis of Luminol-Amplified Chemiluminescence and Clinical and Sigmoidoscopic Scores

To determine whether the sigmoidoscopic or clinical scores correlates best with luminol-amplified chemiluminescence, multiple linear regression was carried out using $\log_{10}$ luminol-amplified chemiluminescence as the co-variable and the clinical and sigmoidoscopic scores as the variables (Table 5.6). Only the sigmoidoscopic score demonstrated an independent, significant relationship with luminol-amplified chemiluminescence ($P=0.0001$).

Table 5.6 Multiple linear regression analysis of luminol-amplified chemiluminescence (co-variable) against the sigmoidoscopic and clinical scores in patients with ulcerative colitis.

<table>
<thead>
<tr>
<th>Disease Activity Variable</th>
<th>Coefficient</th>
<th>Standard Error</th>
<th>Standard Coefficient</th>
<th>t-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sigmoidoscopic Score</td>
<td>4.0</td>
<td>0.8</td>
<td>0.6</td>
<td>5.3</td>
<td>0.0001</td>
</tr>
<tr>
<td>Clinical Score</td>
<td>-0.1</td>
<td>1.0</td>
<td>-0.1</td>
<td>0.1</td>
<td>0.91</td>
</tr>
</tbody>
</table>
Luminol-Amplified Chemiluminescence According to the Histological Score of Adjacent Biopsies

There was no correlation between the chemiluminescence and the histological score of 26 biopsies from 9 UC patients with grade 1 colitis, 15 with grade 2, and 2 with grade 3, Spearman’s rank correlation coefficient, $\text{Rho}=+0.1, P=0.55$ (Fig 5.4).

![Graph](image)

Fig 5.4 Log$_{10}$-transformed luminol-amplified chemiluminescence of biopsies from 26 patients with UC according to the histological score of an adjacent biopsy. No significant correlation, Spearman’s rank correlation coefficient, $\text{Rho}=+0.1, P=0.55$. Regression $y = 0.04x + 4.5$.

Multiple Linear Regression Analysis of Luminol-Amplified Chemiluminescence and Elements of the Histology Score

In Chapter 3 multiple linear regression analysis of the luminol-amplified chemiluminescence against the elements of the histological score showed that submucosal neutrophil infiltration independently correlated with chemiluminescence of the colonic biopsies from acetic acid-induced colitis. To determine which factor of the histology score for UC most closely correlates with luminol-amplified chemiluminescence, multiple linear regression was carried out using log$_{10}$-transformed values for luminol-amplified chemiluminescence as the co-variable and each of the histological features as the variables (Table 5.7). The only variable which demonstrated an independent, significant relationship with luminol-amplified chemiluminescence was the degree of neutrophil infiltration, which is in agreement with the results for acetic acid-induced colitis.
Table 5.7  Multiple linear regression analysis of luminol-amplified chemiluminescence (co-variable) against elements of the histological score for ulcerative colitis.

<table>
<thead>
<tr>
<th>Histological Feature</th>
<th>Coefficient</th>
<th>Standard Error</th>
<th>Standard Coefficient</th>
<th>t-value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goblet Cell Depletion</td>
<td>-0.04</td>
<td>0.3</td>
<td>-0.1</td>
<td>0.2</td>
<td>0.86</td>
</tr>
<tr>
<td>Crypts Inflammation</td>
<td>-0.20</td>
<td>0.2</td>
<td>-0.2</td>
<td>0.9</td>
<td>0.38</td>
</tr>
<tr>
<td>Neutrophil infiltration</td>
<td>0.50</td>
<td>0.2</td>
<td>0.6</td>
<td>2.4</td>
<td>0.03</td>
</tr>
<tr>
<td>Mononuclear cell infiltration</td>
<td>-0.10</td>
<td>0.2</td>
<td>-0.1</td>
<td>0.4</td>
<td>0.70</td>
</tr>
</tbody>
</table>

**Luminol-Amplified Chemiluminescence According to Therapy**

Fig 5.5 shows the chemiluminescence in 77 patients with UC divided according to treatment. There was no significant differences between the groups using a Kruskal-Wallis test. In order to test the possibility that this lack of difference between the groups is due to the inclusion of all disease grades in the comparisons, the largest subdivision of disease activity, namely those with a sigmoidoscopie score of 2, was re-analysed after division into treatment groups. The largest groups within this category were 17 patients treated with 5-ASA-containing preparations alone, 6 treated with 5-ASA-containing preparations and rectal and/or oral steroids and 6 on no treatment. Analysis by a Kruskal-Wallis test showed no differences between these groups, P=0.35.

**Luminol-Amplified Chemiluminescence According to Disease Extent**

Fig 5.6 shows the chemiluminescence in 77 patients with UC divided according to disease extent. There were no significant differences between the groups on analysis using a Kruskal-Wallis test, P= 0.93.

**Luminol-Amplified Chemiluminescence Compared with Age**

It has been suggested that ROS production may increase, and antioxidant defence decrease, in the elderly (Cutler 1991). Fig 5.7 shows the luminol-amplified chemiluminescence in 77 patients with UC according to age. There was no significant correlation between age and luminol-amplified chemiluminescence.
Fig 5.5 Log$_{10}$-transformed luminol-amplified chemiluminescence in 77 patients with UC sub-divided by treatment. AZA = azathioprine There were no significant differences between the groups using a Kruskal-Wallis test.

Fig 5.6 Log$_{10}$-transformed luminol-amplified chemiluminescence in 77 patients with UC sub-divided by the extent of the disease. There were no significant differences between the groups using a Kruskal-Wallis test.
Fig 5.7 Log<sub>10</sub>-transformed luminol-amplified chemiluminescence compared with age in 77 patients with UC. Correlation not significant Rho=-0.006, P=0.51, Spearman’s Rank Correlation. Regression $y = -0.0029x + 4.9$.

**SUMMARY OF MAIN FINDINGS**

- The clinical disease activity score was significantly higher in all grades of sigmoidoscopic inflammation than normals and a score of 0 for UC patients. Each sigmoidoscopic score had a significantly higher clinical score than lesser grades of inflammation apart from a sigmoidoscopic grade of 3 compared to 2.
- The luminol-amplified chemiluminescence response in UC was significantly higher than normals for all sigmoidoscopic grades of inflammation apart from 0.
- There was a significant positive correlation between the clinical score and the luminol-amplified chemiluminescence in UC using Spearman’s rank correlation coefficient.
- There was no correlation between the overall histological score and the luminol-amplified chemiluminescence; however, multiple linear regression analyses revealed a significant association between the degree of mucosal neutrophil infiltration and the chemiluminescence response.
- Luminol-amplified chemiluminescence did not vary significantly with the age of the patients, their diseases’ extent nor with therapy.
DISCUSSION

The finding that chemiluminescence correlates with sigmoidoscopic appearances assessed semi-quantitatively in UC is in agreement with previous studies (Simmonds et al 1992a; Keshavarzian et al 1992b).

It was not possible to confirm previous findings of a correlation between the luminol-amplified chemiluminescence response of endoscopic biopsies in UC with the same histological score as employed in the present studies (Simmonds et al 1992a). However in the study by Simmonds et al, 1992, 60 biopsies were examined, of which 8 had no detectable ROS production and there is little further increase in the chemiluminescence response above a histological score of 3 (Simmonds et al 1992a). In the current study only two biopsies had a score of less than 3. It is possible therefore that a positive correlation is found between the histological score and ROS production only if a substantial proportion of inactive biopsies are included in the sample. However, it was shown here using multiple linear regression analysis that the degree of neutrophil infiltration significantly and independently correlated with the luminol-amplified chemiluminescence response. The inclusion of other variables, such as the degree of goblet cell depletion and mononuclear cell infiltration, in an overall histological score may obscure the correlation with chemiluminescence response. The finding that the neutrophil count correlates with chemiluminescence is consistent with the hypothesis that this cell type is the major source of mucosal ROS in IBD.

It has now been demonstrated that luminol-amplified chemiluminescence correlates with a score based on clinical and laboratory indices, compatible with the proposal that ROS production is pathogenic in UC. It may also be a marker of disease activity, although it is unlikely to have sufficient reproducibility, or ease of usage, to be clinically useful. Furthermore, the correlation of clinical score with luminol-amplified chemiluminescence was lost under the more rigorous conditions of multiple regression analysis.

Antioxidant protection diminishes with age and thus the relationship between age and luminol-amplified chemiluminescence of colorectal biopsies in UC was examined; however no significant correlation was found in the present study. This may be because ROS production also decreases with age (Nagel et al 1982).

If it is hypothesised that some therapies of proven efficacy in IBD are primarily antioxidant, then a relationship might have been expected between the treatment type and luminol-amplified chemiluminescence in UC; this was not seen in this study, probably because patients taking similar drug regimens had varying clinical and sigmoidoscopic scores (data not shown).
The data presented here provide further support for the hypothesis that ROS are pathogenic in UC by showing that the luminol-amplified chemiluminescence response of colorectal biopsies is related to clinical disease activity and the intensity of the mucosal neutrophil infiltrate.
INTRODUCTION

If superoxide is of pathogenic importance in IBD, then therapeutic use of the antioxidant enzyme, superoxide dismutase, may ameliorate gastrointestinal inflammation. Manganese superoxide dismutase (MnSOD) is found in both prokaryotes, and in the mitochondria of eukaryotic cells. Deletion of the MnSOD gene in mice causes neonatal death in mice from cardiomyopathy (Li et al 1996).

A protective role for MnSOD in intestinal inflammation is suggested by its increased expression in intestinal epithelial cell lines exposed to LPS and TNFα (Valentine et al 1992) and by IL-1-stimulated endothelial cells (Susuki et al 1993; Lontz et al 1995). In contrast, CuZnSOD is not up-regulated by pro-inflammatory mediators in intestinal epithelial cell lines (Valentine et al 1992).

Results from in vivo studies also support the hypothesis that MnSOD might be more important than CuZnSOD in endogenous protection against oxidant stress. In a study by Seo et al, mRNA levels of MnSOD were increased, though protein levels were diminished, whereas both the mRNA and protein levels of CuZnSOD were decreased, suggesting that MnSOD expression is the more important in the inflammatory response (Seo et al 1995). In addition, increased levels of CuZnSOD, but elevated levels of MnSOD, are found in the gastric mucosal inflammation induced by Helicobacter pylori in humans (Gotz et al 1996), and in rat glomerular cells exposed to hydrogen peroxide (Yoshioka et al 1994).

MnSOD expression is increased in the mucosa of acetic acid (Tannahill et al 1995) and TNBS-induced colitis (Seo et al 1995), whereas CuZnSOD levels are decreased in both circulating neutrophils (Verspaget et al 1988) and in the mucosa in active IBD (Mulder et al 1991).

CuZnSOD has been assessed in models of intestinal ischaemia and been shown to have a beneficial effect. Injection of CuZnSOD into the superior mesenteric artery, during a 1 hour period of ischaemia in cats, limited the resulting intestinal injury and increase in permeability (Granger et al 1981). CuZnSOD also protects against ischaemia-induced gastric mucosal damage in cats (Perry et al 1986), reduces intestinal infarction and perforation due to intraperitoneal aminophylline in rats (Dalsing et al 1986).
1983), inhibits lipid peroxidation in the post-ischaemic intestine in cats (Younes *et al* 1987), and attenuates inflammation in a model of necrotizing enterocolitis in rabbits (Miller *et al* 1988).

MnSOD, which has been less well studied in such models, reduces leukocyte adhesion in experimental intestinal ischaemia in cats (Suzuki *et al* 1991). In a preliminary study, TNBS-induced colitis was improved by the subcutaneous administration of 50,000 U/kg MnSOD one hour prior to the induction of colitis (Yoshikawa *et al* 1992).

Rh-MnSOD is a recombinant form of human manganese superoxide dismutase which has a half-life in the rat of 6-8 hours (Baret *et al* 1984), considerably longer than CuZnSOD, which has a half-life of 6-10 minutes (Gorecki *et al* 1991). In comparative studies, *in vivo*, rh-MnSOD has shown greater efficacy than CuZnSOD. Rh-MnSOD is more effective in adjuvant-induced arthritis (Parizada *et al* 1991; Shingu *et al* 1994) and in bleomycin-induced lung damage (Parizada *et al* 1991). Other potential advantages of MnSOD over CuZnSOD as a therapeutic agent include its ability to equilibrate well with interstitial fluid, whereas CuZnSOD has a negative charge and thus equilibrates less well and, unlike CuZnSOD (Yim *et al* 1990), it is resistant to inactivation by hydrogen peroxide (Clare *et al* 1984).

Thus MnSOD may have a more important protective role in gastrointestinal inflammation than the limited role suggested for CuZnSOD in therapeutic trials in patients with IBD, to date (Niwa *et al* 1985; Emerit *et al* 1991). These latter studies of SOD in humans employed parenteral administration. Previous studies in this laboratory demonstrated that ROS production by inflamed colorectal biopsies from patients with IBD was reduced by the direct application of CuZnSOD. It was therefore hypothesised that topical administration of MnSOD to the inflamed mucosa might have a potent antioxidant effect.

**HYPOTHESIS**

Rh-MnSOD is an effective scavenger of superoxide in cell-free systems and is effective in reducing production of ROS when applied directly to inflamed colonic tissue from acetic acid-induced colitis *in vitro* and is anti-inflammatory, *in vivo*, in the same model.

**AIM**

To compare the antioxidant effects of rh-MnSOD in cell-free systems and using inflamed colorectal biopsies from the acetic acid-induced model of colitis. To extend these experiments to examine the anti-inflammatory effect of topical rh-MnSOD in acid-induced colitis *in vivo*. 

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METHODS AND MATERIALS

The experimental procedures for the xanthine/xanthine oxidase cell-free system is described in Chapter 2, p52. The procedure for assessment of the antioxidant activity of compounds using colorectal biopsies from acetic acid-induced colitis and patients are described in Chapter 4, p. 102–108. Details of materials and methods in addition to, or at variance with, these are described below.

Materials

Rh-MnSOD (specific activity: 3,500 U/mg protein) was provided by Bender & Co. Ges mbH, Vienna, Austria. Hydrogel is an inert colloid in which the particles are in the external or dispersion phase and water in the internal or dispersed phase. Rh-MnSOD was supplied in a polyethylene glycol-based hydrogel suspension by Bender & Co. Ges mbH, Vienna, Austria and the hydrogel base was also supplied as a control. It is commonly used as a transport medium for therapeutic agents in experimental models.

Methods

Cell-Free Systems

The antioxidant effects of rh-MnSOD and CuZnSOD and their heat inactivated equivalents (HI-rh-MnSOD, HI-CuZnSOD) were assessed according to the protocol described in Chapter 2. Both enzymes were inactivated by the method of Heikkila et al (1985), as described in Chapter 4, p. 104 (Heikkila 1985). Active enzymes were assessed at 0.001, 0.01, 0.1, 1.0, 10, and 100U/ml. Each concentration was assessed in triplicate. The chemiluminescence response was assessed using lucigenin (300μM) which responds primarily to superoxide radicals.

Acetic Acid-Induced Colitis In Vitro

The protocol for acetic acid-induced colitis is given in Chapter 2, p. 45. Rh-MnSOD was soluble in aqueous solution and was tested according to the protocol described for water soluble agents described in Chapter 4. The chemiluminescence response was assessed using lucigenin (300μM). These results demonstrate the efficacy of rh-MnSOD in a cell free system.

Acetic Acid-Induced Colitis In Vivo

The method for acetic acid-induced colitis is described in Chapter 2 and varied only in that groups of animals were administered test agents 1 hour prior to induction of colitis. Test compounds, as described below, were administered in 0.2ml of hydrogel by gavage needle inserted perrectum to 6cm. A small volume was chosen to avoid early expulsion of the fluid, to maximise tissue penetration.
Groups were coded so that the two investigators performing the macroscopic scoring and chemiluminescence counts were unaware of the treatment group. Microscopic scoring was carried out by a single histopathologist (Dr A Coumbe) blinded to the treatment group of the animals.

One hour before induction of colitis 0.2ml hydrogel with or without rh-MnSOD was administered. The selected doses of rh-MnSOD were approximately equivalent to those used in rat models of arthritis in experiments carried out in this laboratory (Dowling et al 1993). In the latter experiments the dose was expressed in μg rh-MnSOD /weight of rat (kg) and administered as a single intra-articular injection. The use of a single local injection into the area of inflammation was thus comparable to the present studies. Using the quoted figures for the activity of the rh-MnSOD (3625U/mg protein) and the weight of the rats (150-200mg), the calculated doses administered ranged from to 15U to 1200U, which is broadly similar to the doses used in the present study.

The 7 groups of animals studied were as follows:

1. Absolute controls - these animals underwent no intervention
2. Controls treated with 600U rh-MnSOD - these animals had no intervention to induce colitis but were given 600U rh-MnSOD in 0.2ml hydrogel 25 hours prior to being killed.
3. Acetic acid-induced colitis controls - these animals had acetic acid administered as per protocol.
4. Acetic acid-induced colitis, as per protocol, pre-treated with hydrogel alone - these animals were given acetic acid as per protocol but 1 hour beforehand 0.2ml hydrogel was administered by gavage needle to 6cm intra-rectally. The gavage needle passed easily to 6cm but further progress into the rat colon could not be achieved without difficulty.
5. Acetic acid-induced colitis pre-treated with 6U rh-MnSOD - these animals were given acetic acid as per protocol but 1 hour beforehand 6U MnSOD in 0.2ml hydrogel was administered by gavage needle to 6cm intra-rectally.
6. As (5) but animals were pre-treated with 60U rh-MnSOD.
7. As (5) but animals were pre-treated with 600U rh-MnSOD.

All groups were killed at 24 hours by CO₂ respiration and the colon from 2-8cm proximal to the anus was removed, opened longitudinally, laid out on card and the macroscopic score assessed, and biopsies taken from the portion 2-6cm from the anus. At least 3 random cross-sectional full-thickness sections were then cut; two were assessed for luminol-amplified chemiluminescence and a further one transferred to 10% formalin for subsequent wax embedding, sectioning, staining with
haematoxylin and eosin and scored using the method described in Chapter 2, p. 45. Luminol-amplified chemiluminescence was used to assess the maximum level of ROS production by these biopsies as the chemiluminescence response of biopsies from acetic-acid-induced colitis is greater with this amplifier, as shown in Chapter 2 (p. 53), thus enhancing the sensitivity of the assay.

STATISTICS

For *in vitro* studies, comparisons between compound and control were made using the Wilcoxon signed rank test, two-tailed, as described in Chapter 4.

The results are expressed as median (IQR)% change in the chemiluminescence response. The difference in chemiluminescence response between biopsies treated with test compounds and controls is calculated by comparing the percentage change in chemiluminescence induced by the test agent and control using the Wilcoxon signed-rank test for paired variables.

For *in vivo* studies, groups were compared using the Kruskal-Wallis test to detect a difference between the groups assessed as a whole and individual differences assessed using the Mann-Whitney test (two tailed) with the Bonferroni correction for multiple comparisons.

RESULTS

**Rh-MnSOD In a Cell Free System**

As shown in Fig 6.1, rh-MnSOD was an effective scavenger of superoxide with an IC₅₀ of 0.5 U/ml using xanthine and xanthine oxidase. A comparison with CuZnSOD was made at the highest concentration tested, showing equivalent efficacy. Heat-inactivated enzymes were also tested at 100U/ml demonstrating that, although not all activity was lost, the residual was approximately equivalent to the lowest dose of rh-MnSOD tested (Fig 6.1).
Fig 6.1 Effect of human recombinant manganese superoxide dismutase (rh-MnSOD) and copper zinc superoxide dismutase (CuZnSOD) on lucigenin-amplified chemiluminescence produced by the xanthine-xanthine oxidase. The results are corrected for the background count of the vial and expressed as the percentage difference from vehicle (Dulbecco's PBS).

Effect Of Rh-MnSOD On ROS Production Of In Vitro Colonic Biopsies From The Acetic Acid-Induced Colitis Model In Rats

Fig 6.2 shows the effect of rh-MnSOD and CuZnSOD on the lucigenin-amplified chemiluminescence produced by rat biopsies from the acetic acid-induced colitis model. Only CuZnSOD at a concentration of 300U/ml altered the chemiluminescence response significantly differently from control (heat-inactivated enzyme), P=0.03.
Fig 6.2 The percentage change in the lucigenin-amplified chemiluminescence response of inflamed biopsies from acetic acid-induced colitis to CuZnSOD and rh-MnSOD. Chemiluminescence was measured before and after exposure to test compound and compared to a second biopsy exposed to vehicle. CuZnSOD (300U/ml) reduced chemiluminescence significantly compared to control (heat-inactivated enzyme) *P=0.03 by the Wilcoxon rank signed test (two-tailed).

Effects of Pre-treatment with Intraluminal Rh-MnSOD In Vivo In Acetic Acid Colitis

Macroscopic Score of Colonic Biopsies from Acetic Acid-Induced Colitis Treated with Rh-MnSOD and Controls

Fig 6.3 shows the macroscopic score of the colon of 7 groups of rats comprising 2 control groups and 5 treatment groups. The median (IQR) of the macroscopic score for both control groups not given acetic acid was 0 (0–0). The median (IQR) for groups with acetic acid-induced colitis given nil, hydrogel, hydrogel with 6, 60 and 600U of rh-MnSOD in 0.2ml of hydrogel, was 2.5 (2–4), 2 (1–3), 2 (1–3), 3 (2–4), 3 (2–4), respectively. There was no significant difference between the 2 control groups or between those with acetic acid-induced colitis. There was a significant difference between untreated controls and all groups treated with acetic acid (P≤0.001), and between controls groups pre-treated with 600U rh-MnSOD and all groups treated with acetic acid (P≤0.03).
Microscopic Score of Colonic Biopsies from Acetic Acid-Induced Colitis Treated with Rh-MnSOD and Controls

Fig 6.4 shows the changes in the histological score of the colon of 7 groups of rats comprising 2 control groups and 5 treatment groups. Some histological specimens were lost during storage. The median (IQR) of the histological score for the absolute control group and that given 600U of rh-MnSOD were 0 (0–1.2) and 0 (0–0), respectively. The median (IQR) for groups with acetic acid-induced colitis given nil, hydrogel, hydrogel with 6, 60 and 600U of rh-MnSOD in 0.2ml of hydrogel, was 15 (11.5–18), 10 (7.5–14), 12 (8–14), 15 (10–16), 13 (5.5–17.2), respectively. There was no significant difference between the 2 control groups or between those with acetic acid-induced colitis. There was a significant difference between untreated controls and all groups treated with acetic acid (P≤0.001), and between controls groups pre-treated with 600U rh-MnSOD and all groups treated with acetic acid (P≤0.02).

Chemiluminescence of Colonic Biopsies from Acetic Acid-Induced Colitis Treated with Rh-MnSOD and Controls

Fig 6.5 shows the mean luminol-amplified chemiluminescence response of two colonic biopsies per animal in groups treated with rh-MnSOD and controls. The median (IQR) luminol-amplified chemiluminescence responses for the untreated control group and that given 600U of rh-MnSOD were 30 (0–67) and 274 (22–303), respectively. The median (IQR) for groups with acetic acid-induced colitis given nil, hydrogel, hydrogel with 6, 60 and 600U of rh-MnSOD in 0.2ml of hydrogel, was 10495 (4489–22862), 9318 (648–18357), 1575 (654–11101), 6767 (3113–10830), 4807 (2184–19243), respectively. There was no significant difference between the 2 control groups or between those with acetic acid-induced colitis. There was a significant difference between untreated controls and all groups treated with acetic acid (P≤0.001), and between controls groups pre-treated with 600U rh-MnSOD and all groups treated with acetic acid (P≤0.02).
CTL Control

CTL600 Control pre-treated with 600U rh-MnSOD

AAC Acetic acid-induced colitis control

AACH Acetic acid-induced colitis pre-treated with hydrogel alone

AAC6 Acetic acid-induced colitis pre-treated with 6U rh-MnSOD

AAC60 Acetic acid-induced colitis pre-treated with 60U rh-MnSOD

AAC600 Acetic acid-induced colitis pre-treated with 600U rh-MnSOD

The macroscopic score from acetic acid-induced colitis treated with rh-MnSOD in 0.2ml hydrogel administered by gavage intra-rectally 1 hour prior to induction of colitis and appropriate control groups (untreated controls, controls pre-treated with 600U rh-MnSOD, acetic acid-induced colitis alone and those with acetic acid-induced colitis administered 0.2ml hydrogel alone 1 hour prior to induction of the colitis). There was a significant difference between controls and acetic acid-treated groups but no difference between the 2 control groups or between those with acetic acid-induced colitis (Kruskal-Wallis test followed by the Mann-Whitney U test with the Bonferroni correction for multiple comparisons).
Fig 6.4 The histological score from acetic acid-induced colitis treated with rh-MnSOD in 0.2ml hydrogel administered by gavage intra-rectally 1 hour prior to induction of colitis and appropriate control groups (untreated controls, controls pre-treated with 600U rh-MnSOD, acetic acid-induced colitis alone and those with acetic acid-induced colitis administered 0.2ml hydrogel alone 1 hour prior to induction of the colitis). There was a significant difference between controls and acetic acid-treated groups but no difference between the 2 control groups or between those with acetic acid-induced colitis (Kruskal-Wallis test followed by the Mann-Whitney U test with the Bonferroni correction for multiple comparisons).
Fig 6.5  The chemiluminescence response by two full-thickness colonic sections from each animal from acetic acid-induced colitis treated with rh-MnSOD in 0.2ml hydrogel administered by gavage intra-rectally 1 hour prior to induction of colitis and appropriate control groups (untreated controls, controls pre-treated with 600U rh-MnSOD, acetic acid-induced colitis alone and those with acetic acid-induced colitis administered 0.2ml hydrogel alone 1 hour prior to induction of the colitis). There was a significant difference between controls and acetic acid-treated groups but no difference between the 2 control groups or between those with acetic acid-induced colitis (Kruskal-Wallis test followed by the Mann-Whitney U test with the Bonferroni correction for multiple comparisons).
SUMMARY OF MAIN FINDINGS

- CuZnSOD and rh-MnSOD were both effective scavengers of superoxide in a cell-free system.
- Rh-MnSOD did not reduce lucigenin-amplified chemiluminescence produced by inflamed colonic biopsies from acetic acid-induced colitis in vitro.
- Pre-treatment with intracolonic rh-MnSOD failed to improve the macroscopic or microscopic scores or ROS production, as assessed by luminol-amplified chemiluminescence, induced in rats by acetic acid.

DISCUSSION

Although rh-MnSOD had a potent anti-oxidant effect in cell-free systems it was not possible to demonstrate a reduction in ROS production by acetic acid colitis biopsies in vitro and there was no anti-inflammatory effect when it was administered rectally to rats prior to their exposure to acetic acid.

The most likely explanation of the lack of efficacy of the two SOD variants as antioxidants, or anti-inflammatory agents, in the inflamed colon is poor tissue penetration. Rh-MnSOD is a tetradimer, with a molecular weight of 80,000kD, and CuZnSOD has a molecular weight of 33,000kD. There is no data on the mucosal uptake of CuZnSOD and rh-MnSOD; however, differences in molecular weight might explain why the smaller CuZnSOD molecule moderately reduced ROS production by inflamed colonic biopsies from acetic acid-induced colitis, whereas rh-MnSOD failed to do so.

An alternative explanation for the negative results of this study may relate to the differential expression of MnSOD and CuZnSOD in gastrointestinal inflammation. MnSOD expression is increased in acetic acid-induced colitis (Tannahill et al 1995) and it is possible that addition of further enzyme has no additional antioxidant effect. Alternatively, MnSOD may not have a substantial role in antioxidant protection as, in epithelial lines exposed to interferon-γ and TNFα, ROS production by these cells did not alter, despite a concomitant marked increase in MnSOD protein and activity (Kinnula et al 1995).

If increased expression of MnSOD in intestinal mucosa explains the inability of exogenous enzyme to influence ROS production then the question arises as to the time course of this expression. Up-regulation in epithelial cell lines occurs within hours, suggesting that the 24 hour period between the onset and assessment of acetic acid-induced colitis, in the present study, would certainly have been sufficient for up-regulation of MnSOD to have occurred (Valentine et al 1992).

Another explanation for the lack efficacy of rh-MnSOD in vivo, may be the
relatively short time-course of this model. Repeating the study using a longer time-course or an alternative, more chronic model, might improve the results, but was beyond the scope of the Home Office Licence for these studies.

Although rh-MnSOD in vivo had no statistically significant anti-inflammatory effect with any of the study doses, others have described a bell-shaped response curve with MnSOD in other models of inflammation. Low doses (5μg (18U), intra-articularly) were anti-inflammatory in carrageenan-induced arthritis, but higher doses (12.5μg–50μg) had either no effect or worsened the inflammation (Dowling et al 1993). Intra-peritoneal administration in the adjuvant-induced foot pad oedema model in rats showed a similar pattern in which 50–200μg/kg reduced oedema but doses of 400μg–2mg/kg failed to do so (Dowling et al 1993). No conclusions can be drawn as to the occurrence of this effect in the present studies, although the difference between treated and untreated rats appeared to be greater in those receiving the lowest dose studied (6U/ml) with all three methods (macroscopic, microscopic and chemiluminescence) of assessment of inflammation employed.

There have been a number of proposed explanations for this bell-shaped response. It may be that excess SOD results in the over-production of hydrogen peroxide or that over-scavenging of superoxide prolongs the vasorelaxant effects of nitric oxide (Gryglewski et al 1986) leading to extravasation and oedema. Studies of SOD in models of myocardial ischaemia-reperfusion have demonstrated a bell-shaped response curve with high doses lacking efficacy and possibly worsening the injury (Bernier et al 1989; Omar et al 1990), and this may be due to the unopposed production of hydrogen peroxide as conjugating SOD with catalase ameliorates this effect (Mao et al 1993).

Another explanation for the apparent bell-shaped response to MnSOD may be that the protonated form of superoxide, the hydroperoxyl radical (HO$_2^-$), terminates lipid peroxidation reactions, as follows (Omar et al 1990):

\[
\text{LOO}^* + \text{HO}_2^- \rightarrow \text{LOOH} + \text{O}_2
\]

Excess scavenging of superoxide may, by reducing production of the hydroxylperoxyl radical, thus exacerbate lipid peroxidation. Supportive evidence for this is the protective effect of lipid peroxidation inhibitors against high dose SOD-induced injury to the reoxygenated, isolated rabbit myocardium (Nelson et al 1994).

In summary, the limited effectiveness of SOD as an anti-inflammatory agent, its dose-response characteristics, and the difficulties of delivery of this large protein make it unlikely that there will be a therapeutic application for rh-MnSOD, and possibly for any preparation of SOD in IBD.
IRON CHELATING AGENTS IN ULCERATIVE COLITIS

INTRODUCTION

Increased mucosal iron during inflammation may increase oxidant stress by promoting hydroxyl ion formation via Fenton chemistry. Increased mucosal permeability to luminal iron, breakdown of haem products (Babbs 1992) and superoxide-induced release of iron from ferritin (Biemond et al 1984) may overwhelm mucosal iron chelation releasing the ‘free iron’ necessary for this process (Grisham et al 1988; Grisham et al 1990c). If this occurs in vivo then exogenous use of iron chelators may reduce mucosal ROS production.

Human body iron stores are approximately 4.5g in the male (Bannerman et al 1962) and the loss of 1mg per day is kept in balance by absorption of a similar amount from the diet, about 1% of the total daily intake. Iron absorption involves active transport across the brush-border membrane into the mucosal cells (Topham et al 1991), from where it is transported into the plasma bound to iron chelators such as transferrin, ferritin, (Manis et al 1962), lactoferrin (Britigan et al 1991), and a recently described protein, mobilferrin (Conrad et al 1990). Non-protein bound tissue or free plasma iron is only found when the binding-capacity of transferrin is exceeded or if ferritin-bound iron is released by reductants, such as superoxide or ascorbic acid (Biemond et al 1984; Boyer et al 1987; Biemond et al 1988).

Disorders that cause iron overload, such as idiopathic haemochromatosis and iron overload from repeated transfusions in thalassaemia major, may be fatal. A comparison of cord blood plasma from babies with rhesus haemolytic disease and matched controls showed higher ferritin levels, lower iron binding capacity, increased lipid peroxidation (TBARS), low vitamin C levels and a reduced ability to inhibit peroxidation stress, though vitamin E levels were not altered (Berger et al 1990). In rheumatoid arthritis, oral (Blake et al 1981) and intravenous iron (Lloyd et al 1970) may worsen disease activity, possibly via an oxidant-dependent mechanism. Other disorders which may be mediated by raised levels of tissue iron are shown in Table 7.1.
Table 7.1 Disorders in which iron-mediated free radical release may be pathogenic.

<table>
<thead>
<tr>
<th>Disease/Disorder</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Haemochromatosis</td>
<td>(Halliwell et al 1989c)</td>
</tr>
<tr>
<td>Thalassaemia</td>
<td>(Modell et al 1982)</td>
</tr>
<tr>
<td>Malaria</td>
<td>(Gordeuk et al 1992)</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>(Yoshino et al 1984)</td>
</tr>
<tr>
<td>Post-ischaemic reperfusion injury</td>
<td>(Katoh et al 1992),</td>
</tr>
<tr>
<td>Paraquat toxicity</td>
<td>(Kohen et al 1985)</td>
</tr>
<tr>
<td>Chemotherapy toxicity</td>
<td>(Beare et al 1996)</td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>(Salonen et al 1992)</td>
</tr>
<tr>
<td>Cold-induced injury</td>
<td>(Maity et al 1992)</td>
</tr>
<tr>
<td>Rejection following organ transplantation</td>
<td>(Bradley et al 1986)</td>
</tr>
<tr>
<td>Muscular dystrophy</td>
<td>(Clark 1984)</td>
</tr>
<tr>
<td>Kwashiorkor</td>
<td>(Golden et al 1987)</td>
</tr>
<tr>
<td>Rhesus haemolytic disease</td>
<td>(Berger et al 1990)</td>
</tr>
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</table>

Iron as a Pro-Oxidant in Inflammatory Bowel Disease

The role of Fenton chemistry in the production of hydroxyl ions is described in Chapter 1. Briefly, hydrogen peroxide, produced by the dismutation of superoxide, may be converted to hydroxyl ions in the presence of ferrous ions via the Fenton reaction (Freeman et al. 1982). The body has no natural antioxidant defence against the hydroxyl radical thus causing tissue damage and the further accumulation of neutrophils and other pro-inflammatory cells (Esterbauer et al. 1989a). The most important question in biological systems is whether ‘free iron’ is present.

Surprisingly, there is no adequate data on levels of iron in the mucosa of IBD though a small study, published in abstract form, suggested there might be an increase (Liu-Brody et al. 1994). Evidence that iron-mediated oxidant stress is important in the pathogenesis of IBD thus rests on four questions. The first concerns the availability of free iron in the mucosa; is there evidence to suggest that iron, and in particular, free iron, might be increased in the inflamed gastrointestinal mucosa? Secondly, does current therapy have any iron chelation properties? Thirdly, does oral iron therapy worsen disease activity? Finally, is there evidence that iron chelation is anti-inflammatory or protects against tissue injury, particularly in the gastrointestinal tract?
Potential Sources for Increased Mucosal Free Iron in Inflammatory Bowel Disease

If free iron is present in the inflamed mucosa then the question arises as to its source. The increased permeability associated with IBD may result in luminal iron gaining access to the mucosa. Foods containing mixtures of iron and reducing agents, such as ascorbate, may be particularly potent sources of oxidant activity (Rowley et al 1983), as such reductants convert Fe^{3+} to Fe^{2+}, thus promoting both iron absorption and catalysing the Fenton reaction (Halliwell et al 1990).

Tissue iron is largely bound to ferritin which serves to sequester iron in a relatively inert form until it is required for metabolic processes such as haem synthesis (Reif 1992). As many as 4,500 iron atoms may be bound to the hydrous ferric oxide core of the ferritin molecule, though it is normally only 20% saturated (Theil 1983). This core is surrounded by a 24-subunit protein, apoferritin. Release of iron from ferritin requires a reductant and a suitable chelator (Theil 1983). The reaction sequence is as follows:

\[
ferritin:Fe^{3+}(n) + \text{reductant} \rightarrow ferritin:Fe^{3+}(n-i)Fe^{2+}
\]

\[
ferritin:Fe^{3+}(n-i)Fe^{2+} + \text{chelator} \rightarrow ferritin:Fe^{3+}(n-i) + \text{chelator:Fe}^{2+}
\]

Candidates for the role of such reductant activity are reduced flavins (Dognin et al 1973), superoxide (Williams et al 1974) and NO (Reif et al 1990). Activated neutrophils release iron from ferritin via superoxide (Biemond et al 1984). As the inflamed mucosa in IBD produces increased levels of superoxide (Simmonds et al 1992a), this mechanism may be of pathogenic importance in IBD. It has been suggested that release of the iron chelator, lactoferrin, by activated neutrophils may be part of a natural defence mechanism against this process (Aruoma et al 1987b); this mechanism may be impaired in IBD, but remains to be investigated.

NO is increased in the mucosa in UC (Boughton-Smith et al 1993; Middleton et al 1993), and is capable of releasing iron from ferritin (Reif et al 1990). However, NO may also have an antioxidant effect in iron-mediated oxidation by forming a ligand with iron (Ragsdale 1973) and thus be an antioxidant in biological systems (Kanner et al 1991). Its role in possible iron-mediated oxidant stress thus remains unclear.

Bleeding into inflamed areas can promote inflammation (Yoshino et al 1985). Haemoglobin, released by lysed erythrocytes, in inflamed tissues may be degraded to iron by hydrogen peroxide and lipid peroxides, thereby exacerbating oxidant stress (Gutteridge 1986). Haemoglobin may also directly accelerate lipid peroxidation via peroxidase activity (Cross et al 1987).
Iron Chelating Properties of Current Therapy

As discussed in Chapter 1, 5-ASA does have iron chelating properties (Grisham 1990a). This will have an antioxidant effect unless iron concentrations are high enough to chelate 5-ASA at a 1:1 ratio, in which case the combination becomes pro-oxidant (Grisham et al 1992). Neither steroids or immunosuppressive agents have any known chelating properties.

Iron Therapy in Inflammatory Bowel Disease

There are anecdotal reports of oral iron worsening disease activity in IBD (Halpin et al 1982; Kawai et al 1992) and this may be due to increased mucosal iron, as parenteral iron replacement appears to have no effect on disease activity in CD (Gasché et al 1994).

Evidence for an Anti-inflammatory Role for Iron Chelation

Desferrioxamine protects cultured intestinal epithelial cells against superoxide-mediated injury (Ma et al 1991) and both desferrioxamine and phenanthroline protect cultured gastric cells against hydrogen peroxide-induced damage (Hiraishi et al 1993). Other workers found that 1,10-phenanthroline protects against hydrogen peroxide-induced injury to cultured intestinal epithelial cells, but desferrioxamine was only protective if pre-incubated with the cells for 18 hours prior to injury (Watson et al 1994).

Table 7.2 summarises the in vivo models of inflammation in which iron chelation has been used. Desferrioxamine protects against indomethacin-induced gastric injury (Vaananen et al 1991), and that induced by intra-arterial infusion of nitric oxide donors, in rats (Lamarque et al 1995). It also has a protective effect in models of intestinal (Hernandez et al 1987; Andrews et al 1992) and myocardial (Reddy et al 1989) ischaemic injury.

Intra-peritoneal desferrioxamine is anti-inflammatory in foot-pad swelling induced by mono-sodium urate and carageenan in rats (Blake et al 1983). Desferrioxamine is also beneficial in post-ischaemic reperfusion myocardial injury (Katoh et al 1992), paraquat toxicity (Kohen et al 1985) and cold-induced injury (Maity et al 1992), in experimental models.

There is little data on the use of 1,10-phenanthroline in experimental inflammation. 1,10-phenanthroline inhibited inflammatory cell accumulation in carrageenan-induced pleural inflammation in rats (Ackerman et al 1980), and a related compound, o-Phenanthroline, has anti-inflammatory properties at a high dose in carrageenan-induced arthritis in rats (Nakagawa et al 1983), 1,10-Phenanthroline also inhibits cell proliferation in vitro, an effect abolished by addition of further Fe^{2+} or
Cu$^{2+}$ at concentrations sufficient to overcome its chelating actions (Mohindru et al 1983)

In iron overload, total body iron is massively increased and morbidity and mortality often high. Compliance with the necessarily frequent parenteral administration of desferrioxamine greatly improves the outcome. In humans, desferrioxamine has benefit in preventing rejection following organ transplantation (Bradley et al 1986), and hastened the recovery of children with cerebral malaria, in a controlled trial (Gordeuk et al 1992). Desferrioxamine, given intravenously to patients undergoing cardiac bypass surgery, reduced both neutrophil-mediated ROS production (Menasche et al 1988) and serum TBARS (Menasche et al 1990).

There is little data on the use of iron chelation in gastrointestinal inflammation in humans though, in an uncontrolled study, the combination of desferrioxamine and superoxide dismutase was of possible benefit in CD (Emerit et al 1991).

Table 7.2 Animal models of inflammation in which desferrioxamine has been used.

<table>
<thead>
<tr>
<th>Inflammatory Model</th>
<th>Animal</th>
<th>Benefit</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO-induced gastric injury</td>
<td>Rat</td>
<td>Reduced injury</td>
<td>(Lamarque et al 1995)</td>
</tr>
<tr>
<td>Indomethacin-induced gastric injury</td>
<td>Rat</td>
<td>Reduced injury</td>
<td>(Vaananen et al 1991)</td>
</tr>
<tr>
<td>Airway inflammation induced by xanthine/xanthine oxidase</td>
<td>Guinea Pig</td>
<td>Anti-inflammatory</td>
<td>(Misawa et al 1993)</td>
</tr>
<tr>
<td>Allergic air pouch</td>
<td>Rat</td>
<td>Worsens acute phase, improved chronic phase</td>
<td>(Yoshino et al 1984)</td>
</tr>
<tr>
<td>Urate-induced foot pad swelling</td>
<td>Rat</td>
<td>Low doses pro-inflammatory</td>
<td>(Blake et al 1983)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High doses anti-inflammatory</td>
<td></td>
</tr>
<tr>
<td>Carageenan-induced paw oedema</td>
<td>Rat</td>
<td>Anti-inflammatory</td>
<td>(Hirschelman et al 1986)</td>
</tr>
<tr>
<td>Bleomycin-induced lung fibrosis</td>
<td>Rat</td>
<td>No effect</td>
<td>(Cross et al 1985)</td>
</tr>
<tr>
<td>Pulmonary ozone toxicity</td>
<td>Rat</td>
<td>Low doses protective</td>
<td>(Louie et al 1993)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High doses no effect or pro-inflammatory</td>
<td></td>
</tr>
<tr>
<td>Adjuvant arthritis</td>
<td>Rat</td>
<td>Not anti-inflammatory</td>
<td>(Hirschelman et al 1986)</td>
</tr>
</tbody>
</table>
Iron Chelators Used in the Present Study

Desferrioxamine is derived from *Streptomyces pilosus*, chelates Fe$^{3+}$ (ferric) ions and is a powerful inhibitor of iron-induced lipid peroxidation (Wills 1969; Gutteridge *et al* 1979). Desferrioxamine forms a chelate with Fe$^{3+}$ with a relative ratio of 1:1 and is widely used in the treatment of iron overload. The use of desferrioxamine in human studies has been limited by the lack, until recently, of orally available agents. However, as discussed below, the lack of systemic effect of oral desferrioxamine may prove to be a positive benefit in the treatment of IBD.

1,10-phenanthroline chelates Fe$^{3+}$ ions and may thus inhibit Fenton chemistry induced production of hydroxyl ions.

HYPOTHESIS

Iron chelators will reduce, and iron salts increase, ROS production by the inflamed colon in acetic acid-induced colitis and UC.

AIM OF STUDY

To examine alterations in mucosal ROS production by colonic biopsies from acetic acid-induced colitis and UC *in vitro* produced by the iron chelators, desferrioxamine and 1,10-phenanthroline, and by exogenous iron in the form of ferric citrate.

METHODS AND MATERIALS

Materials

All reagents are of reagent grade and purchased from Sigma Chemical Co Ltd, Poole, Dorset unless otherwise stated. All compounds were dissolved in Dulbecco’s phosphate buffered saline (D-PBS) with added calcium (1.13mM) and glucose (5mM), pH adjusted to 7.4 with 1M NaOH. Controls for these experiments was the vehicle for the test compounds.

*Desferrioxamine and 1,10-Phenanthroline*

Desferrioxamine scavenges Fe$^{2+}$ and 1,10-phenanthroline scavenges Fe$^{3+}$ and were thus chosen to detect differential effects of chelating the two transition states of iron. Previous work suggested that low doses of desferrioxamine could exacerbate animal models of inflammation whereas higher doses were anti-inflammatory (Blake *et al* 1983). To avoid the risk of type II error from lack of penetration of the chelator into the biopsy a relatively high concentration of desferrioxamine was chosen (1mM). 1, 10-Phenanthroline was also assessed at a concentration of 1mM.
**Ferric citrate**

Preliminary investigations demonstrated that ferric chloride and ferrous sulphate precipitated in D-PBS, forming insoluble iron phosphates. Ferric citrate was therefore chosen as the source of exogenous iron in this assay. Ferric (Fe$^{3+}$) citrate is known to catalyse the superoxide-driven Fenton reaction at low concentrations (10μM) (Smith et al 1990) and complexes have been demonstrated by nuclear magnetic resonance spectroscopy in the synovial fluid in rheumatoid arthritis (Grootveld et al 1990). As with the iron chelators a relatively high concentration (100μM) of iron salts was chosen to avoid difficulties of tissue penetration.

**In Vitro Studies Using Rectal Biopsies From Patients With Ulcerative Colitis and Acetic Acid-Induced Colitis**

**Acetic Acid-Induced Colitis**

The procedure for inducing acetic acid-induced colitis and assessment of antioxidant activity with colonic biopsies is described in Chapter 4, p. 102–108.

**Ulcerative Colitis**

Rectal biopsies were taken using standard endoscopy biopsy forceps from patients with UC and controls undergoing sigmoidoscopy or colonoscopy at the Royal London and Newham General Hospitals. A telephone survey of manufacturers of endoscopic equipment failed to identify non-iron containing endoscopic biopsy forceps for use in this study.

Three groups of human subjects were studied. Active UC (sigmoidoscopic score 2 or 3, according to Baron criteria (Baron et al 1964), (see Table 5.2), inactive UC (sigmoidoscopic score 0 or 1), and a control group of patients, with no evident colonic disease, undergoing colonoscopic screening for polyps and colonic carcinoma (normals).

The procedure for assessment of the antioxidant activity of the test compounds with colorectal biopsies from UC is the same as that used for colonic biopsies from acetic acid-induced colitis, as described in Chapter 4, Fig 4.2. Briefly, colorectal biopsies from patients with active and inactive UC and controls were placed in 300μM luminol in pre-oxygenated phosphate buffered saline. Photon emission (chemiluminescence (CL)) was measured in a scintillation counter. Test agent or vehicle was then added and the biopsy recounted. The median percentage change was then calculated.
Calculations And Statistics

Calculations and statistical comparisons for each drug at each concentration were carried out according to the methods described in chapter 4, p. 106. The changes in the chemiluminescence response in control biopsies was compared to paired biopsies treated with the test compound using Wilcoxon signed-rank test (two tailed).

RESULTS

Chemiluminescence Response To Iron Chelators and Iron in Acetic Acid-Induced Colitis

Desferrioxamine at 1mM did not alter luminol-amplified chemiluminescence from inflamed biopsies from rats with acetic acid-colitis (Fig 7.1). Ferric citrate at 100μM also failed to affect the chemiluminescence response of inflamed biopsies from animals with acetic acid-induced colitis (Fig 7.1).

![Graph showing the percentage change in chemiluminescence response](image)

Fig 7.1 The percentage change in the luminol-amplified chemiluminescence response of inflamed biopsies from rats with acetic acid-induced colitis to desferrioxamine and ferric citrate. Chemiluminescence was measured before and after exposure to test compound and compared to a second biopsy exposed to vehicle, Wilcoxon signed rank test, two-tailed.

Chemiluminescence Response to Iron Chelators in Ulcerative Colitis

The effect of the iron chelators desferrioxamine and 1,10-phenanthroline at 1mM on luminol-amplified chemiluminescence produced by colorectal biopsies from
normals and patients with inactive and active UC are shown in Fig 7.2. Desferrioxamine (1mM) did not significantly alter luminol-amplified chemiluminescence in normals (P=0.40), but there were significant reductions in inactive UC (P=0.018) and active UC (P=0.008). 1,10-phenanthroline did not affect the luminol-amplified chemiluminescence in normals (P=0.50), but reduced it significantly in inactive UC (P=0.003), and in active UC (P=0.003).

![Graph showing percentage change in chemiluminescence response](image)

Fig 7.2  The percentage change in the luminol-amplified chemiluminescence response of inflamed biopsies from patients with UC and normal controls to the iron chelators, desferrioxamine and 1,10-phenanthroline and to ferric citrate. Chemiluminescence was measured before and after exposure to test compound and compared to a second biopsy exposed to vehicle.

**Chemiluminescence Response To Ferric Citrate in Ulcerative Colitis**

The effect of the iron citrate (100μM) on luminol-amplified chemiluminescence produced by colorectal biopsies from normals and patients with
inactive and active UC is shown in Fig 7.2. Ferric citrate (100μM) had no effect on luminol-amplified chemiluminescence in any patient group.

SUMMARY OF MAIN FINDINGS

- Desferrioxamine did not reduce, and exogenous iron (ferric citrate) failed to increase the luminol-amplified chemiluminescence response of inflamed colonic biopsies from acetic acid-induced colitis.
- The luminol-amplified chemiluminescence response of colorectal biopsies from patients with UC was significantly inhibited by both desferrioxamine and 1,10-phenanthroline; exogenous iron had no effect.
- There was no alteration in chemiluminescence in colorectal biopsies from human controls using desferrioxamine, 1,10-phenanthroline or ferric citrate.

DISCUSSION

Iron chelation significantly reduced ROS production by rectal biopsies from patients with active UC but not by colonic biopsies from rats with acetic acid-induced colitis. This suggests that ROS production in UC is, at least partly, dependent on the presence of chelatable iron, whereas in acetic acid-induced colitis it is not.

The present result are in agreement with the lack of a beneficial effect of desferrioxamine in vivo in acetic acid-induced colitis (Keshavarzian et al 1990). In the latter study the colitis was evaluated at 96 hours which may be insufficient time for the inflammatory process to raise levels of free mucosal iron. Repeating the present studies using more chronic models of colitis, such as TNBS- or dextran sulphate-induced colitis, or in iron-deficient and over-loaded rats, may be worthwhile.

Could the effects of desferrioxamine and 1,10-phenanthroline on chemiluminescence in the present studies be due to a direct antioxidant effect rather than iron chelation? Theoretically, desferrioxamine could have some direct antioxidant activity via its reaction with superoxide, but this proceeds at a slow rate of $10^3$ M⁻¹ s⁻¹, which is slower than the physiological dismutation rate (Halliwell 1985; Buetner 1987), and thus is unlikely to affect the results. Desferrioxamine also reacts in vitro with the hydroxyl ion at a rate controlled by diffusion with a second-order rate constant of $10^{10}$ M⁻¹ s⁻¹. However, the reactivity of the hydroxyl ion is such that it is unlikely to diffuse more than a few molecules distant before reacting with a biological molecule, and it is therefore unlikely that desferrioxamine, a relatively large molecule, is present in sufficient concentration in the tissue to compete with such target molecules. Desferrioxamine also reacts with hypohalous acids, such as hypochlorous acid (Wasil et al 1987a), and can inhibit hypochlorous acid-induced red blood cell lysis (Vissers et al 1990), but this reaction is slow and it is thought unlikely
that desferrioxamine is a significant scavenger of hypochlorous acid in biological systems (Halliwell 1989b). The lack of an antioxidant effect of desferrioxamine in acetic acid-induced colitis also suggests that the present results in humans were not due to a direct antioxidant mechanism. It is also unlikely that the antioxidant effect of desferrioxamine was due to cytotoxicity as no damage is seen in cell culture at a concentration of 800μM (Tan et al 1995). 1,10-phenanthroline is not known to have any direct scavenging properties and the effect observed in our studies is unlikely to be due to cytotoxicity as the agent is routinely used in cell culture to inhibit metalloproteinases (Vissers et al 1988).

It is therefore reasonable to conclude that the observed effect with biopsies from UC was due to iron chelation, and furthermore that free iron present in the mucosa of patients with UC may contribute to oxidant stress. In vivo, desferrioxamine has other effects on inflammation. Millimolar concentrations inhibit eicosanoid release by rat aortic rings (Jeremy et al 1988) and human cervical homogenates (Flatman et al 1988) though desferrioxamine stimulated eicosanoid levels in rat leukocytes and caecal fragments (Laughton et al 1989). In the latter experiments, desferrioxamine only increased PGF2α at concentrations above 500μM and had no effect on LTB4 production, even at 5mM, suggesting that in the colon, there would be little secondary effect on ROS production.

A caveat for the systemic use of desferrioxamine is its dose-dependent toxicity in non-iron overloaded patients, including ocular changes in thalassaemia (Davies et al 1983) and nausea, vomiting and visual impairment in rheumatoid arthritis (Polson et al 1985). However, the present results suggest that topical desferrioxamine therapy in IBD might be beneficial, in which case the lack of systemic absorption could be a positive advantage.

Since iron chelators reduce ROS production by inflamed biopsies from patients with UC, addition of iron salts might have been expected to increase it in normals, and possibly increase it still further in inactive and active UC biopsies. The maximum concentration of iron that could be dissolved, was used for these experiments. It is possible that this concentration was insufficient to overcome mucosal iron chelation in normals and that enough iron was already present in the inflamed biopsies to maximally drive the Fenton reaction.

In conclusion, the antioxidant effects of desferrioxamine and 1,10-phenanthroline in biopsies from patients with UC suggest that a clinical trial of topical iron chelation therapy in active disease is indicated.
ANTIOXIDANT NUTRIENT THERAPY IN ACTIVE ULCERATIVE COLITIS

INTRODUCTION

If ROS are pathogenic in IBD, then specific antioxidant therapy should be anti-inflammatory, and thus offer a new therapeutic approach. However, there have been few clinical studies in this area. In preliminary, uncontrolled trials, CuZnSOD in combination with desferrioxamine, had apparent efficacy in CD (Emerit et al 1991), and allopurinol alone was efficacious, again in uncontrolled studies, in acute and chronic pouchitis. (Levin et al 1992). Another preliminary study, of tazofelone, a novel antioxidant chemically related to the conventional antioxidant, butyrate hydroxytoluene (BHT), administered as an enema in active UC, produced remission in 59% of patients after four weeks of treatment (Beker et al 1995). Liposomal SOD injection (2.5mg twice weekly), apparently induced remission in 4/4 and 3/4 patients with CD and UC, respectively, in a wide-ranging study of patients with autoimmune diseases (Niwa et al 1985).

Antioxidant nutrients are required in small amounts for normal metabolism but have few other potential anti-inflammatory effects, apart from as antioxidants, and thus, a proven beneficial role in IBD would strongly support the hypothesis that ROS are pathogenic in IBD. In addition, the low toxicity of these exogenous substances makes them an attractive therapeutic possibility.

Antioxidant Nutrient Therapy

A number of vitamins and nutrients have antioxidant activity. These include β-carotene (a precursor of vitamin A), α-tocopherol (the most potent of the eight tocopherols that comprise vitamin E), ascorbic acid (vitamin C), glutathione and selenium. Patients suffering acute attacks of IBD have low serum levels of, and increased requirements for, the antioxidant vitamins, β-carotene, α-tocopherol and ascorbic acid (Abad-Lucruz et al 1988; Fernandez-Banares et al 1989).

The present study is the first to report a trial of a combination of antioxidant nutrients in IBD. The potential benefits and risks of such therapy are discussed.
**β-Carotene**

β-carotene, a precursor of vitamin A, is lipid-soluble and scavenges singlet oxygen (O') (Reilly et al 1991) and peroxyl radicals (Packer et al 1981; Canfield et al 1992). Although there is no data on its efficacy in gastrointestinal inflammation, β-carotene has been shown to limit photosensitization skin damage in porphyria (Mathews-Roth 1987) and inhibit urinary thioether excretion in smokers (Bos et al 1992). It has been suggested to have a role in cancer prevention (Diplock 1991). However, a possible role when used alone, or in combination with vitamins C and E, in limiting carcinogenesis has also been questioned after negative results in a four year, placebo-controlled, study to prevent colorectal adenomas (Greenberg et al 1994). Indeed, in a double-blind, placebo-controlled, study in prevention of lung cancer, the group receiving β-carotene alone had a higher incidence of cancer than the placebo group (Anonymous 1994).

The potential antioxidant benefit of oral β-carotene may be limited by its pharmacokinetics. Following absorption from the gut lumen, β-carotene is metabolised to retinol by intestinal carotene oxygenase; plasma levels of retinol are tightly controlled by retinol binding protein, with excess retinol being stored in the liver (Diplock 1991).

It is however possible that, in UC, raising luminal levels of β-carotene may nevertheless have a local antioxidant effect in colonic inflammation, as moderate doses (30mg orally per day) of β-carotene, taken for 3 months, significantly increase colonic mucosal levels (Mobarhans et al 1994). Furthermore, it may have a synergistic role with other antioxidant therapy in gastrointestinal inflammation.

**α-Tocopherol**

As with β-carotene, α-tocopherol is lipid soluble and a potent antioxidant (Harris et al 1992). It reduces neutrophil superoxide production (Engle et al 1988) and is the major inhibitor of lipid peroxidation chain reactions in human tissue (Ingold et al 1987), probably by scavenging peroxyl (LOO*) and alkoxy (LO*) radicals, generated from lipid peroxidation, and resulting in the production of the tocopherol-O*, as follows:

\[
\text{LOO}^* + \text{tocopherol-OH} \rightarrow \text{LOOH} + \text{tocopherol-O}^*
\]

and

\[
\text{LO}^* + \text{tocopherol-OH} \rightarrow \text{LOH} + \text{tocopherol-O}^*
\]

The tocopherol radical (tocopherol-O*) is poorly reactive, and these reactions
therefore break the chain reaction of lipid peroxidation. Tocopherol-\( \text{O}^\bullet \) is
re-converted to \( \alpha \)-tocopherol by reaction with water-soluble ascorbate at the lipid-
cytosol border (Esterbauer et al 1989b), suggesting that co-administration of these
vitamins may have a synergistic effect. Furthermore, co-administration of \( \alpha \)-
tocopherol and \( \beta \)-carotene increases the intestinal absorption of ascorbate (Wang et al
1995).

Clinical benefit with \( \alpha \)-tocopherol has been suggested in a number of
conditions associated with oxidant stress, including retrolental dysplasia, haemolytic
syndrome and intraventricular haemorrhage, reviewed in Halliwell, 1989 (Halliwell
1989a). It enhances cell-mediated immunity in the elderly (Meydani et al 1990) and
may slow progression of coronary disease (Rimm et al 1993; Stampfer et al 1993),
possibly by limiting oxidation of low-density lipoprotein (Esterbauer et al 1989a).
Plasma levels of TBARS in smokers are reduced by 10 weeks of vitamin E (200
IU/day) (Brown et al 1994).

The effect of vitamin E has been studied in experimental models of
inflammation, and there are limited studies in humans. Gastric artery injury in rats
leads to mucosal injury and increases in luminol-amplified chemiluminescence of
blood drawn from the gastric vein; both are attenuated by administration of
\( \alpha \)-tocopherol (Kurose et al 1993). A related study showed that ischaemia-reperfusion
injury to the gastric mucosa, in rats, was associated with increases in mucosal
TBARS, and was increased in vitamin E-deficient animals (Yoshikawa et al 1991).
Vitamin E and its analogue, the 21-aminosteroid (U-74500A) are of benefit in animal

One patient with active UC treated with another vitamin E analogue,
\( \alpha \)-tocopherylquinone, had a dramatic clinical response (Bennet 1986). Oral vitamin E
substantially raises faecal levels (Dion et al 1982) suggesting that, in addition to
raising the serum antioxidant level, there may be a local effect on the inflamed
colonic mucosa in IBD.

Vitamin E has few other potential anti-inflammatory actions apart from its
antioxidant properties though it does partially inhibit 5-lipoxygenase activity
(Reddanna et al 1985) and is a weak inhibitor of platelet activity (Jandak et al 1988).
Supplementation with 800 mg (592 IU)/day (in healthy, elderly adults increases the
delayed-type hypersensitivity skin test, the mitogenic response to concanavalin A and
IL-2 production (Meydani et al 1990).

Oral vitamin E at a dose of 1920 IU/day increased rectal dialysate
concentrations of the vitamin in patients with active ulcerative colitis but had no
effect on release of PGE\(_2\) or LTB\(_4\) from the rectal mucosa (Lauritsen et al 1987), and
thus changes in mucosal eicosanoid production are unlikely to contribute to the
potential therapeutic effect of α-tocopherol therapy IBD.

**Ascorbate**

Ascorbate effectively scavenges superoxide anions, singlet oxygen, hypochlorous acid and peroxyl radicals (Bendich *et al* 1987). Ascorbate is the main antioxidant in human plasma and peroxidation of plasma lipids cannot take place until available ascorbate has been consumed (Frei *et al* 1989). A probable major role *in vivo*, is restoration of the tocopherol radical to α-tocopherol, as described above. It may be protective in photo-oxidative retinal damage, ozone-induced lung damage, cataract formation and cigarette-induced lung damage (Bendich *et al* 1987).

Levels of reduced ascorbate are reduced in the mucosa of patients with active UC, and the ratio of ascorbate to dehydroascorbic acid is increased, suggesting that oxidative stress limits the levels of this antioxidant in the inflamed mucosa (Buffington *et al* 1995). In the carrageenan model of colitis in ascorbate-deficient guinea pigs, not only was mild colitis seen in the control animals but addition of large doses of ascorbic acid to the diet reduced the severity of colitis (Langman *et al* 1985). However a trial of ascorbate alone as maintenance therapy in UC showed no benefit (Hermanowicz *et al* 1985), suggesting that either it is ineffective, or is only effective in active disease, or in combination with other antioxidants. A daily dose of 1g ascorbate in volunteers reduces aspirin-induced gastric mucosal injury and ROS production (McAIlindon *et al* 1996).

**Selenium**

Selenium is an essential co-factor for glutathione peroxidase. Reduced plasma selenium levels and glutathione peroxidase activity in patients with severe, active rheumatoid arthritis were corrected by selenium supplementation, though glutathione peroxidase activity remained lower than controls (Tarp 1994).

Selenium deficiency causes Keshan Disease, a selenium-responsive cardiomyopathy, named after a village in the Keshan area of China where a fifth of the inhabitants died in an epidemic in 1935. Selenium also prevents the arthropathy characteristic of Kashin-Beck disease. Selenium deficiency causes cardiomyopathy in patients treated with long-term selenium-deficient total parenteral nutrition, including those with CD (Levy *et al* 1994). This cardiomyopathy is prevented and immune responsiveness improved by addition of selenium to total parenteral nutrition regimes (Peretz *et al* 1991).

Plasma selenium levels are reduced in active CD (Hinks *et al* 1988), though others only found low plasma levels in those with ileal resections greater than 200cm (Rannem *et al* 1992). Serum selenium levels are not significantly reduced in UC, compared to controls, though two studies have shown that levels are inversely
correlated with disease extent (Mortensen et al 1989; Ringstad et al 1993).

**Methionine**

Methionine is a precursor for glutathione which is the substrate for glutathione peroxidase, which metabolises hydrogen peroxide. Glutathione also plays a role in reducing tocopheroxyl radicals thus supporting the antioxidant function of vitamin E (Sies et al 1992). Rats fed with methionine have reduced liver oxidant activity (Selvam et al 1992) and arterial levels of oxidised glutathione are raised after intestinal ischaemia-reperfusion injury in rats (Abdulla et al 1990). Recent evidence shows that glutathione transferase activity is decreased in the rectal mucosa in UC (Bhaskar et al 1995), and mucosal glutathione levels are depressed in both healthy and diseased ileum in CD (lantomasi et al 1994). Blood glutathione transferase levels are depressed to a greater degree in UC in those with an onset before the age of 30 years compared to those with a later onset (Hertervig et al 1994). Furthermore, supplementing glutathione to cultured rat gastric cells increases the intra-cellular glutathione content and protects the cells from hydrogen peroxide-induced damage (Hiraishi et al 1994).

**Advantages of Combination Therapy**

There are several reasons why combination therapy is more likely to be effective than a single antioxidant nutrient compound. Firstly, the synergistic action of ascorbate and tocopherol, in reducing each other's radical moiety and secondly, the presence of both aqueous and lipid-soluble ROS, suggests that aqueous and lipid-soluble antioxidants should be given. Thirdly, production of ROS originates from a number of sources (Chapter 1), and it therefore seemed logical to offer antioxidant therapy which acts at several different sites.

A combination of β-carotene, α-tocopherol, ascorbate, methionine and selenium, similar to that used in the present study, was of benefit, and also non-toxic, in a placebo-controlled trial in recurrent pancreatitis (Sandilands et al 1990; Uden et al 1992). Treatment with ascorbate, α-tocopherol and β-carotene reduces plasma TBARS and inhibits copper-induced oxidation of low-density lipoprotein in healthy subjects (Jialal et al 1993).

**Potential Risks with Selenium-βCE/Methionine Therapy**

Selenium-βCE tablets are on sale to the general public in the UK and Germany and do not require a product licence. No reported adverse effects have been reported to the UK marketing company (Wassen International Ltd, Leatherhead) and the dose of Selenium-βCE and methionine proposed for this study has previously been administered to patients without any drug-related side-effects (Sandilands et al 1990;
Uden et al 1992). The total daily dose of compounds to be administered and the relevant drug safety information are shown in Table 8.1.

Table 8.1 Daily doses in antioxidant nutrient trial in active ulcerative colitis and safety limits *.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Study Daily Dose</th>
<th>Safety Information Limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-carotene</td>
<td>1500 units</td>
<td>≤ 25,000 units/day</td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>270 units</td>
<td>≤ 800 units/day</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>540mg</td>
<td>&lt;1500 mg/day</td>
</tr>
<tr>
<td>Selenium</td>
<td>600μg †</td>
<td>200 μg/day (800 μg †)</td>
</tr>
<tr>
<td>Methionine</td>
<td>500mg</td>
<td>Used in doses of 10g/day in paracetamol overdose may cause nausea, vomiting and irritability but these have not been reported at the study dose.</td>
</tr>
</tbody>
</table>

* based on information in Martindale's Pharmacopoeia.
† Recent study showing that fingernail changes associated with selenium toxicity do not occur with levels below 800μg/day (Yang et al 1995).
‡ The dose initially chosen was higher than the published limits, but was equivalent to those previously used without evidence of toxicity (Uden et al 1992).

Although the agents used in this study are all endogenous compounds, normally found in small quantities in mammalian systems, their use in high doses and as anti-inflammatory agents in IBD raises several concerns. High doses of vitamin E increase the bleeding time in rats (Takahashi 1995), which might worsen mucosal blood loss in active UC, though others have found that in humans, there is no alteration in platelet function, or bleeding time, with a dose of 800 units/day (Stampfer et al 1988).

Dehydroascorbate has marked pro-oxidant properties, and is readily formed from ascorbate in the presence of oxygen and iron (Stadtman 1991). This does not occur readily in plasma because of iron chelation (Minetti et al 1992); however, in iron overload, administration of ascorbate causes major toxicity, unless desferrioxamine is given at the same time (Nienhuis 1981). Thus the combined presence of iron and ascorbate in the lumen of the inflamed bowel may actually increase ROS production. Another potential problem with use of ascorbate in IBD is stimulation of neutrophil motility (Anderson 1982).

The risks of inducing the ascorbate radical may be reduced if moderate doses are given, as the faecal content of ascorbate is only raised with oral doses above 1-2g
(Hornig et al 1980). The risk of ascorbate radical formation by the catalytic action of luminal free iron should also be reduced by the concomitant administration of α-tocopherol which restores ascorbate to its reduced form (Harris et al 1992).

Thus the safety of antioxidant nutrient therapy in active UC has not previously been demonstrated, and it was one of the prime purposes of this trial to ensure safety of this combination, prior to proceeding to controlled studies.

HYPOTHESIS

The potent antioxidant activity of dietary supplements of a combination of the nutrients β-carotene, α-tocopherol, ascorbate, methionine and selenium will be safe for the treatment of active UC and improve clinical, haematological, biochemical and histological markers of inflammation.

AIM

To treat 10 patients, with moderate reactivations of UC, with a 2-8 week course of antioxidant nutrient therapy in an open-label study. Assessments will be made of the safety of the therapy, and of changes in clinical status, laboratory markers of inflammation and ROS production. The study will also serve as a preliminary investigation into the presence or absence of raised levels of TBARS in active UC (see Chapter 2).

METHODS

General Study Design

Ten patients, with moderate reactivations of UC, were treated in an open-label study with a 2-8 week course of antioxidant nutrient therapy. Patients were assessed at the start of the trial and at 1 and 2 weeks after. If there was clinical improvement at the end of the initial 2 week period, and the patient wished to continue, the study medication was continued, with 2 weekly review, for up to 8 weeks. If at any time during the trial there was clinical deterioration, or the patient requested withdrawal, then the antioxidant nutrient medication was stopped and the patient treated with appropriate conventional therapy for relapse in UC. Patients were also withdrawn from the trial if remission was achieved, and they then continued with their usual medication alone (Fig 8.1).
Mild/moderate active ulcerative colitis
(clinical core ≥ 5, ≥ 1 bloody stool/day
and sigmoidoscopic score > 1)

ENTRY INTO STUDY (WEEK 0)
• History and physical examination
• Sigmoidoscopy and rectal biopsy
• FBC, ESR, Albumin
• TBARS
• Commence a diary card (symptoms for week prior to entry into the study completed with patient at this visit)

Treatment commenced (with current therapy):
• Selenium βCE tablets, three tablets twice daily
• Methionine 250mg, one tablet, twice daily

WEEK 1
• As for Week 0 but no sigmoidoscopy/rectal biopsy
• Check for compliance and adverse effects

WEEK 2
• As for Week 0
• Check for compliance and adverse effects

WEEK 4
• As for Week 0 but sigmoidoscopy/rectal biopsy only carried out if leaving study
• Check for compliance and adverse effects

WEEK 6
• As for Week 0 but sigmoidoscopy/rectal biopsy only carried out if leaving study
• Check for compliance and adverse effects

WEEK 8
• As for Week 0
• Check for compliance and adverse effects

Stop SeβCE/Met and continue usual therapy or start prophylaxis
Stop SeβCE/Met and treat relapse with standard therapy

Fig 8.1 Study design for open-label study of antioxidant nutrient therapy in active ulcerative colitis. Selenium βCE and Methionine (SeβCE/Met), Thiobarbituric acid reactive substances (TBARS), Full blood count (FBC), Erythrocyte sedimentation rate (ESR).
Patients

Patients were recruited from the IBD out-patient clinic at the Royal London Hospital. Their current prophylactic therapy, if any, was not altered and they did not receive new additional therapy apart from antioxidant nutrient therapy. Ethical approval for the study was given by the Tower Hamlets Health Authority Ethics Committee.

Inclusion Criteria

- Moderate relapse (<8 stools per day and systemically well).
- Aged 18-75.
- Relapse confirmed using clinical and sigmoidoscopic criteria (clinical score ≥5, ≥1 bloody stool/day and sigmoidoscopic score ≥1).
- Patients willing to take part in the study after receiving verbal and written information about the trial.
- Informed, written and witnessed consent obtained.

Exclusion Criteria

- Previous diagnosis of pancolitis, macroscopically.
- Pregnant or breast feeding females.
- Those already taking vitamin/mineral supplementation.
- Patients who have received steroid therapy, either orally or rectally, in the month prior to the start of the study.
- Patients with serious concurrent disease such as renal, hepatic and heart failure or malignancy.
- Inability to cooperate with the study for psychosocial reasons, such as difficulties with language or mental dysfunction.

Patient Withdrawals

Patients were withdrawn if there was a significant deterioration in disease activity, sigmoidoscopic score or laboratory results or if remission was achieved. Remission was defined as less than one bloody stool in the previous week (based on dairy card reviewed at weekly or 2 weekly intervals), a sigmoidoscopic score ≤1 and a clinical disease activity score <5.

Study Methods

At entry into the study the following procedures were undertaken:

1. Screening medical history and physical examination.
2. Commence a diary card (Fig 8.2) documenting their general health and lower gastrointestinal symptoms, which will be continued on a daily
basis for the period of the trial. This is designed to generate a clinical score as described in Chapter 5 (Table 5.1). Patients were asked to report on the following symptoms:

- Number of liquid or soft stools passed per day. A soft stool was defined as a mushy or loose stool
- Number of bloody stools passed per day
- Number of formed stools passed per day
- Presence of abdominal pain that day and its severity, the options being, none, mild, moderate or severe.
- Description of how well they felt that day, options being well, slightly under par, poor, very poor or terrible.

Further space was given to describe any further symptoms whether attributable to the disease, study medication or intervening ill-health. Patients were instructed in its correct usage during the interview concerning their symptoms over the week prior to the first clinic visit.

(3) Sigmoidoscopy and rectal biopsy. The rectal mucosa was assessed according to recognised criteria, (see Chapter 5, Table 5.2) (Baron et al 1964). Biopsies were taken using KeyMed Hysteroscopy forceps A16. The histological score was assessed according to a previously published method (Simmonds et al 1992a), and is shown in Chapter 5 (Table 5.3).

(4) Blood was taken for full blood count, urea and electrolytes, liver function tests and erythrocyte sedimentation rate (ESR). Additional blood was taken for products of oxidative lipid metabolism (TBARS). The method for analysis of serum TBARS is described in Chapter 2.

(5) Treatment commenced with, in addition to current therapy (Table 8.2):

- Selenium βCE tablets, three tablets twice daily. Each tablet contains Selenium 100µg, β-Carotene 250 units, ascorbic acid 90mg and α-tocopherol 45 units.
- Methionine 250mg, one tablet, twice daily.

Referred to subsequently as SepCE/Met.

Patients were seen at one and two weeks and, in some patients, at two weekly intervals thereafter, at which stage (3) to (5), above, were repeated. Sigmoidoscopy was performed and rectal biopsies were taken at entry, 2 weeks and at withdrawal from the trial. A daily diary card (Fig 8.2) of symptoms was kept for the duration of the study for all subjects. At each subsequent visit after the start of the study patients were asked about any adverse symptoms and compliance with the study medication. This was checked by counting the remaining tablets in each supply of medication.
### Fig 8.2  Diary card for patients to record symptoms. The top page is a photocopy of the back of the bottom page. The card is folded vertically into three for the patient's convenience.
Plate 8.1  Study medication for trial of Selenium βCE and Methionine in active UC. Patients received 3 SeβCE and 1 Methionine tablet (250mg) twice daily.

Main Outcome Measures

• Achievement of remission (<1 bloody stool in the previous week, a sigmoidoscopic score of ≤1 and a clinical disease activity score <5).
• Improvement in disease activity based on clinical and sigmoidoscopic grading and laboratory measures of disease activity, namely biochemical markers of inflammation and mucosal histology.
• Improvement of markers of oxidant activity in the serum (TBARS) and the rectal mucosa (chemiluminescence).
• Adverse effects to the study medication.

Rectal biopsy ROS assay

Luminol amplified chemiluminescence of mucosal biopsy material as previously described (Simmonds et al 1992a) and in Chapter 2.

Serum oxidant activity

Measurement of products of lipid peroxidation (TBARS) as described in Chapter 2 (Rowley et al 1984).
STATISTICS

The data is analysed between weeks 0, 1 and 2 as subsequent withdrawals render direct comparisons at further time points invalid. A further statistical analysis was performed between the entry and exit points of the study. Comparisons between time points were carried out using the Wilcoxon signed-rank test, with Bonferroni’s correction. The data for further time points is shown graphically and the raw data is shown in tabular form.

Comparisons between time points of serially collected data in this manner has been criticised (Matthews et al. 1990), with the suggestion that analysis of summary data relating to each individual is more appropriate. This involves analysis techniques such as rates of change or integrals of individual variables. However these methods are only appropriate when two, or more, groups are compared and are inappropriate for the present study.

RESULTS

Patients Recruited

Table 8.2 shows the demographic details of the patients recruited into the study, including therapy on entry into the study.

<table>
<thead>
<tr>
<th>Subject Code</th>
<th>Age</th>
<th>Sex</th>
<th>Disease Extent</th>
<th>Disease Duration</th>
<th>Therapy For IBD at Study Entry</th>
<th>Other Therapy at Study Entry</th>
</tr>
</thead>
<tbody>
<tr>
<td>HH01</td>
<td>61</td>
<td>M</td>
<td>recto-sigmoid</td>
<td>7 years</td>
<td>Azathioprine 50mg bd</td>
<td>Nifedipine SR</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mesalazine 800 bd</td>
<td>Actraphane 30/70</td>
</tr>
<tr>
<td>BH02</td>
<td>49</td>
<td>F</td>
<td>splenic flexure</td>
<td>20 years</td>
<td>Mesalazine 800mg tds</td>
<td>Nil</td>
</tr>
<tr>
<td>AC03</td>
<td>54</td>
<td>M</td>
<td>recto-sigmoid</td>
<td>18 months</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>MG04</td>
<td>59</td>
<td>M</td>
<td>transverse</td>
<td>3 months</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>KP05</td>
<td>28</td>
<td>F</td>
<td>proctitis</td>
<td>1 year</td>
<td>Mesalazine 400mg bd</td>
<td>Nil</td>
</tr>
<tr>
<td>BE06</td>
<td>21</td>
<td>F</td>
<td>transverse</td>
<td>4 years</td>
<td>Mesalazine 800mg tds</td>
<td>Oral contraceptive Marvalon</td>
</tr>
<tr>
<td>RR07</td>
<td>49</td>
<td>F</td>
<td>transverse</td>
<td>24 years</td>
<td>Sulphasalazine 1g bd</td>
<td>Nil</td>
</tr>
<tr>
<td>YH08</td>
<td>38</td>
<td>F</td>
<td>distal</td>
<td>4 years</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>JL09</td>
<td>49</td>
<td>M</td>
<td>distal</td>
<td>18 years</td>
<td>Nil</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>HB10</td>
<td>36</td>
<td>F</td>
<td>left</td>
<td>15 years</td>
<td>Nil</td>
<td>Panadol, Migraleve, Imigran</td>
</tr>
</tbody>
</table>
Course of Disease, Achievement of Remission and Subsequent Therapy

Table 8.3 shows the number of patients in remission at the end of the study, the period of treatment with the study medication and the additional treatment required by each patient at the end of the study period.

Table 8.3  Study period, remission and post-study therapy in 10 patients with active UC treated with Se-βCE/Met for 2-8 weeks.

<table>
<thead>
<tr>
<th>Subject Code</th>
<th>Clinical Score on Entry - Exit</th>
<th>Duration of Antioxidant Therapy</th>
<th>Remission Achieved</th>
<th>Additional Therapy at End of Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>HHO1</td>
<td>22.0 - 4.3</td>
<td>8 weeks</td>
<td>NO</td>
<td>Predfoam enemas 1 nocte</td>
</tr>
<tr>
<td>BH02</td>
<td>9.8 - 1.5</td>
<td>2 weeks</td>
<td>YES</td>
<td>Nil</td>
</tr>
<tr>
<td>AC03</td>
<td>14.7 - 4.9</td>
<td>4 weeks</td>
<td>YES</td>
<td>Mesalazine 400mg tds (prophylaxis)</td>
</tr>
<tr>
<td>MG04</td>
<td>13.5 - 11.4</td>
<td>2 weeks</td>
<td>NO</td>
<td>Predfoam enemas 1 nocte, Mesalazine 800mg tds</td>
</tr>
<tr>
<td>KP05</td>
<td>7.0 - 1.3</td>
<td>4 weeks</td>
<td>YES</td>
<td>Nil</td>
</tr>
<tr>
<td>BE06</td>
<td>5.0 - 1.3</td>
<td>4 weeks</td>
<td>YES</td>
<td>Mesalazine suppository 500mg nocte, alt dies (prophylaxis)</td>
</tr>
<tr>
<td>RR07</td>
<td>27.5 - 1.4</td>
<td>6 weeks</td>
<td>NO</td>
<td>Prednisolone 20mg od</td>
</tr>
<tr>
<td>YH08</td>
<td>11.3 - 4.2</td>
<td>8 weeks</td>
<td>NO</td>
<td>Mesalazine 800mg tds + Predfoam 1 nocte</td>
</tr>
<tr>
<td>JL09</td>
<td>16.0 - 11.2</td>
<td>4 weeks</td>
<td>NO</td>
<td>Predfoam 1 nocte</td>
</tr>
<tr>
<td>HB10</td>
<td>12.6 - 9.7</td>
<td>3 weeks</td>
<td>NO</td>
<td>Mesalazine 800mg tds + Predfoam enemas 1 nocte</td>
</tr>
</tbody>
</table>

* The clinical score is recorded daily by the patient and then averaged on a weekly basis and then points added for abnormalities of the haematological and biochemical markers of inflammation at the end of that week (Table 5.1)

HH01 was treated for 8 weeks, with a gradual improvement in clinical symptoms, but was not in remission at the end of the 8 week period and was therefore prescribed Predfoam enemas 1 nocte, at the end of the study. BH02 was in remission after two weeks therapy of SeβCE/Met two weeks and required no additional therapy and was in remission when reviewed 3 months later. AC03 was treated for 4 weeks and, although there were improvements in clinical score, his sigmoidoscopic score remained 2, and he was therefore commenced on mesalazine 400mg tds. MG04, similarly showed improvements in clinical score but remained active at the end of 2

- 167 -
weeks and was commenced on mesalazine 800mg tds and Predfoam enemas 1 nocte. KP05 completed 4 weeks therapy with SeβCE/Met, and was in remission at the end of this time and remained so 3 months later. BE06 was also treated successfully for 4 weeks and was in remission at the end of this period. The patient was unwilling to take oral prophylaxis, but accepted alternate day mesalazine suppositories, 500mg. RR07 remained in relapse after 6 weeks therapy and despite an improvement in clinical score, required oral prednisolone at the end of the study. YH08 required mesalazine 800mg tds and Predfoam 1 nocte after 8 weeks of therapy because of continued mucosal inflammation. JL09 was neither sigmoidoscopically, nor clinically improved after 4 weeks of therapy and required additional Predfoam enemas 1 nocte. Similarly, HB10 was not improved after 3 weeks of therapy and stared Predfoam enemas 1 nocte, at this time. The patient did not attend until her appointment at 4 weeks, at which time blood was taken but the sigmoidoscopy was not repeated. Apart from the measures of stool appearances and consistency the analysis on HB10 was thus confined to the first 2 weeks.

**Number of Liquid or Soft Stools Passed per Day**

Fig 8.3 shows the significant drop in the average daily number of liquid or soft stools passed during the period of therapy; the data is shown in Table 8.4. There were significant reductions between weeks 0 and 2, P=0.023 and between week 0 and exit from the study, P=0.012.

**Number of Bloody Stools Passed per Day**

Fig 8.4 shows that there was also a significant reduction in the number of stools containing blood between weeks 0 and 2, P=0.048; 1 and 2, P=0.018. The data is shown in Table 8.5. There was also a significant change between week 0 and the exit point of the study, P=0.012.

**Number of Solid Stools Passed/Day**

Fig 8.5 and Table 8.6 shows the number of solid stools passed/day for comparison with the above data. There are no significant changes between the groups.

**Clinical Score**

A clinical score was derived using a modification of a previously described system (Stevens et al 1992), and is shown in Table 5.1. There was a significant reduction between weeks 0 and 2, P=0.014 and between the clinical scores at entry and exit into the study, P=0.031 (Fig 8.6). The data is shown in Table 8.7.
Sigmoidoscopic Score

As shown in Fig 8.7 and Table 8.8 there was a significant improvement in the appearance of the rectal mucosa between weeks 0 and 2, P=0.016 and between weeks 0 and the end of the study, P=0.031. 2 patients underwent sigmoidoscopy a week before the end of the treatment period. RR07 was unable to attend for sigmoidoscopy at the later date. HB10 had been assessed, as per protocol, at week 2 and withdrew from the study at week 3.

Histological Score

The scoring system shown in Table 5.2 was used to assess the degree of mucosal inflammation. The overall results are shown in Table 8.9. As shown in Fig 8.8, there was no significant improvement in histology, though the comparison of scores between week 0 and the last sigmoidoscopy carried during the period of the study gave a P value of 0.078.

Blood Markers of Disease Activity - Erythrocyte Sedimentation Rate, Haemoglobin and Albumin

Fig 8.9 and Table 8.10 and show the results for the ESR. There were no significant changes between weeks 0, 1 and 2; however, there was a significant increase between week 0 and exit from the study, P=0.03. There were no changes in haemoglobin (Table 8.11) or albumin (Table 8.12) levels in the patients studied. These levels were in the normal range except for patient RR06 who was anaemic at the start of therapy. There was a small further drop in her haemoglobin value as therapy continued and she received iron therapy on discontinuation of the study medication.

Thiobarbituric Acid Reactive Substances

Fig 8.10 and Table 8.13 show the TBARS levels in the patients during therapy, adjusted for the total lipid levels (see Chapter 2, p. 56) together with levels for 6 normals (2 female, 4 male, median age 31, range 29-62). There were no significant changes in serum TBARS during therapy; however, the change between week 0 and exit from the study gave a P value of 0.084. There was no significant difference between the normals and the patients with UC at the entry point to the study. Fig 8.11 Table 8.14 show the uncorrected TBARS levels for comparison. There were no significant differences between groups.

Rectal Mucosal Chemiluminescence

There were very variable chemiluminescence responses in the patients studied and no significant differences were noted during therapy (Fig 8.12 and Table 8.15).
Fig 8.3 The average number of liquid or soft stools per day in 10 patients with active UC treated with Se-βCE/Met for 2-8 weeks. There was a significant reduction between weeks 0 and 2, \( P=0.023 \) by the Wilcoxon signed-rank test, with the Bonferroni correction for multiple groups.

Table 8.4 Average number of liquid stools/day in 10 patients with active UC treated with Se-βCE/Met for 2-8 weeks.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Weeks of Treatment With Se-βCE/Met</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>HH01</td>
<td>7.6</td>
</tr>
<tr>
<td>BH02</td>
<td>7.0</td>
</tr>
<tr>
<td>AC03</td>
<td>6.3</td>
</tr>
<tr>
<td>MG04</td>
<td>5.0</td>
</tr>
<tr>
<td>KP05</td>
<td>2.0</td>
</tr>
<tr>
<td>BE06</td>
<td>0.0</td>
</tr>
<tr>
<td>RR07</td>
<td>11.0</td>
</tr>
<tr>
<td>YH08</td>
<td>2.1</td>
</tr>
<tr>
<td>JL09</td>
<td>5.9</td>
</tr>
<tr>
<td>HB10</td>
<td>4.9</td>
</tr>
</tbody>
</table>
Fig 8.4 Average number of bloody stools/day in 10 patients with active UC treated with Se-βCE/Met for 2-8 weeks. There were significant reductions in the number of stools containing blood between weeks 0 and 2, P=0.048; 1 and 2, P=0.018 by the Wilcoxon signed-rank test, with the Bonferroni correction for multiple comparisons.

Table 8.5 Average number of bloody stools/day in 10 patients with active UC treated with Se-βCE/Met for 2-8 weeks.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Weeks of Treatment With Se-βCE/Met</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>HH01</td>
<td>9.7</td>
</tr>
<tr>
<td>BH02</td>
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</tr>
<tr>
<td>AC03</td>
<td>6.4</td>
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<tr>
<td>MG04</td>
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<td>KP05</td>
<td>2.0</td>
</tr>
<tr>
<td>BE06</td>
<td>1.0</td>
</tr>
<tr>
<td>RR07</td>
<td>12.0</td>
</tr>
<tr>
<td>YH08</td>
<td>4.7</td>
</tr>
<tr>
<td>JL09</td>
<td>6.1</td>
</tr>
<tr>
<td>HB10</td>
<td>3.0</td>
</tr>
</tbody>
</table>
Fig 8.5  Average number of solid stools/day in 10 patients with active UC treated with Se-βCE/Met for 2-8 weeks. There were no significant changes during the study.

Table 8.6  Average number of solid stools/day in 10 patients with active UC treated with Se-βCE/Met for 2-8 weeks.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Weeks of Treatment With Se-βCE/Met</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>HH01</td>
<td>7.3</td>
</tr>
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<td>BE06</td>
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</tr>
<tr>
<td>RR07</td>
<td>1</td>
</tr>
<tr>
<td>YH08</td>
<td>0</td>
</tr>
<tr>
<td>JL09</td>
<td>0.3</td>
</tr>
<tr>
<td>HB10</td>
<td>1.3</td>
</tr>
</tbody>
</table>
Fig 8.6 Clinical score in 10 patients with active UC treated with Se-βCE/Met for 2-8 weeks. There was a significant reduction between weeks 0 and 2, P=0.014 by the Wilcoxon signed-rank test. Remission was assessed according to a previously described system (Stevens *et al* 1992).

Table 8.7 Clinical score in 10 patients with active UC treated with Se-βCE/Met for 2-8 weeks.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Weeks of Treatment With Se-βCE/Met</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 1 2 3 4 5 6 7 8</td>
</tr>
<tr>
<td>HH01</td>
<td>22.0 8.0 4.7</td>
</tr>
<tr>
<td>BH02</td>
<td>9.8 1.5</td>
</tr>
<tr>
<td>AC03</td>
<td>14.7 3.7 4.9</td>
</tr>
<tr>
<td>MG04</td>
<td>13.5 11.4</td>
</tr>
<tr>
<td>KP05</td>
<td>7.0 3.8 1.3</td>
</tr>
<tr>
<td>BE06</td>
<td>5.0 2.1 1.3</td>
</tr>
<tr>
<td>RR07</td>
<td>27.5 8.9 1.4</td>
</tr>
<tr>
<td>YH08</td>
<td>11.3 8.0 4.2</td>
</tr>
<tr>
<td>JL09</td>
<td>16.0 14.2 11.2</td>
</tr>
<tr>
<td>HB10</td>
<td>12.6 9.7</td>
</tr>
</tbody>
</table>
Fig 8.7  Sigmoidoscopic score in 10 patients with active UC treated with Se-βCE/Met for 2-8 weeks. There were significant reductions in the number of stools containing blood between weeks 0 and 2, \( P=0.016 \); by the Wilcoxon signed-rank test.

Table 8.8  Sigmoidoscopic Score in 10 patients with active UC treated with Se-βCE/Met for 2-8 weeks.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Weeks of Treatment With Se-βCE/Met</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>HH01</td>
<td>3</td>
</tr>
<tr>
<td>BH02</td>
<td>2</td>
</tr>
<tr>
<td>AC03</td>
<td>2</td>
</tr>
<tr>
<td>MG04</td>
<td>2</td>
</tr>
<tr>
<td>KP05</td>
<td>2</td>
</tr>
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<td>BE06</td>
<td>2</td>
</tr>
<tr>
<td>RR07</td>
<td>2</td>
</tr>
<tr>
<td>YH08</td>
<td>3</td>
</tr>
<tr>
<td>JL09</td>
<td>3</td>
</tr>
<tr>
<td>HB10</td>
<td>2</td>
</tr>
</tbody>
</table>
Fig 8.8  Histological score in 10 patients with active UC treated with Se-βCE/Met for 2-8 weeks. There was no significant change during the period of the study.

Table 8.9  Histological score in 10 patients with active UC treated with Se-βCE/Met for 2-8 weeks.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Weeks of Treatment With Se-βCE/Met</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>HH01</td>
<td>10</td>
</tr>
<tr>
<td>BH02</td>
<td>3</td>
</tr>
<tr>
<td>AC03</td>
<td>6</td>
</tr>
<tr>
<td>MG04</td>
<td>9</td>
</tr>
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<td>KP05</td>
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<td>RR07</td>
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<td>YH08</td>
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<tr>
<td>JL09</td>
<td>11</td>
</tr>
<tr>
<td>HB10</td>
<td>8</td>
</tr>
</tbody>
</table>
Fig 8.9  ESR in 10 patients with active UC treated with Se-βCE/Met for 2-8 weeks. There was no significant change between individual weeks. However there was a significant increase between the ESR at entry and exit points of the study, P=0.03).

Table 8.10 ESR (mm/hr) in 10 patients with active UC treated with Se-βCE/Met for 2-8 weeks.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Weeks of Treatment With Se-βCE/Met</th>
</tr>
</thead>
<tbody>
<tr>
<td>HH01</td>
<td>32 48 41 46 44 41</td>
</tr>
<tr>
<td>BH02</td>
<td>22 20 24</td>
</tr>
<tr>
<td>AC03</td>
<td>8 14 12 10</td>
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<tr>
<td>MG04</td>
<td>6 14 19</td>
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<td>KP05</td>
<td>10 8 6 10</td>
</tr>
<tr>
<td>BE06</td>
<td>21 22 30</td>
</tr>
<tr>
<td>RR07</td>
<td>17 26 24 42</td>
</tr>
<tr>
<td>YH08</td>
<td>7 7 7 5 5 6</td>
</tr>
<tr>
<td>JL09</td>
<td>10 10 11</td>
</tr>
<tr>
<td>HB10</td>
<td>10 12 13 15</td>
</tr>
</tbody>
</table>
Table 8.11  Haemoglobin (g/dl) in 10 patients with active ulcerative colitis treated with Se-βCE/Met for 2-8 weeks.

<table>
<thead>
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<th>Weeks of Treatment With Se-βCE/Met</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0</td>
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<tr>
<td>BH02</td>
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<tr>
<td>AC03</td>
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<tr>
<td>MG04</td>
<td>13.8</td>
</tr>
<tr>
<td>KP05</td>
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<tr>
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<td>10.9</td>
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<tr>
<td>YH08</td>
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<tr>
<td>JL09</td>
<td>14.5</td>
</tr>
<tr>
<td>HB10</td>
<td>11.9</td>
</tr>
</tbody>
</table>

Table 8.12  Serum albumin (g/l) in 10 patients with active UC treated with Se-βCE/Met for 2-8 weeks.

<table>
<thead>
<tr>
<th>Patient</th>
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</tr>
</thead>
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<td>BH02</td>
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<tr>
<td>AC03</td>
<td>44</td>
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<tr>
<td>MG04</td>
<td>42</td>
</tr>
<tr>
<td>KP05</td>
<td>41</td>
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<td>BE06</td>
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<tr>
<td>RR07</td>
<td>37</td>
</tr>
<tr>
<td>YH08</td>
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</table>
Fig 8.10  Lipid-adjusted TBARS in 10 patients with active UC treated with Se-βCE/Met for 2-8 weeks. TBARS in 6 normal subjects are shown for comparison. There was no significant change during the period of the study.

Table 8.13  TBARS (nmol/l/mmol serum lipid) in 10 patients with active UC treated with Se-βCE/Met for 2-8 weeks.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Weeks of Treatment With Se-βCE/Met</th>
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<tbody>
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<td>MG04</td>
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</table>
Fig 8.11 Uncorrected TBARS in 10 patients with active UC treated with Se-βCE/Met for 2-8 weeks. TBARS in 6 normal subjects are shown for comparison. There was no significant change during the period of the study.

Table 8.14 Uncorrected TBARS (μmol/l) in 10 patients with active UC treated with Se-βCE/Met for 2-8 weeks.

<table>
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<th>Weeks of Treatment With Se-βCE/Met</th>
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<td>0.24</td>
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<tr>
<td>HB10</td>
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</table>
Fig 8.12  \( \log_{10} \) of the mean of the luminol-amplified chemiluminescence response from two biopsies from 10 patients with active UC treated with Se-βCE/Met for 2-8 weeks. There was no significant change during the period of the study.

Table 8.15  Mean chemiluminescence response (photons/min/mg wet weight of tissue) of two biopsies taken from 10 patients with active UC treated with Se-βCE/Met for 2-8 weeks.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Weeks of Treatment With Se-βCE/Met</th>
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Adverse Effects

No adverse effects which could be definitely linked to the study medication occurred during this study. Patient BH02 had a headache on day 2-4 of starting the medication, and felt slightly dizzy on day 2 for 2-3 hours. These symptoms were mild and did not prevent her carrying out her normal activities. They were not persistent on continuing the medication and were described as similar to symptoms she normally experienced with migraine.

Patients Compliance

Compliance with the study medication was generally very high. Three patients BH02, RR07 and YH08 missed 1-2 doses of the study medication at weeks 2, 5 and 7 respectively.

SUMMARY OF MAIN FINDINGS

10 patients with mild-moderate left-sided/distal UC were treated with 2-8 weeks antioxidant nutrient (SeβCE/Met) therapy:

• 4/10 patients achieved remission.
• There were no adverse effects from the study medication
• There was a significant reduction in number of liquid/soft stools and bloody stools and in the overall clinical score.
• There was a significant reduction in the sigmoidoscopic appearances.
• There was no significant improvement in histological appearances.
• There were no significant changes in haemoglobin or albumin and a small significant increase in ESR.
• There was no improvement in serum TBARS or in rectal mucosal chemiluminescence

DISCUSSION

The aim of this small pilot study of antioxidant nutrient therapy in active UC was primarily to ensure the safety of this therapy: it has shown that there were no adverse effects to the study medication. All patients experienced an improvement in clinical measures of disease activity and 7/10 in sigmoidoscopic score. These changes could have been due, at least in part, to a combination of placebo effect and observer bias. As the clinical data at week 0 (entry into the study) were obtained retrospectively, significant improvements between week 0 and week 1 must be viewed with caution. However, the number of bloody stools reduced significantly between weeks 1 and 2 as well as between 0 and 1, suggesting that recall bias at week 0 was not an important factor, at least for this variable. 4/10 patients achieved
remission, which is higher than the published placebo rate of response in placebo-controlled trials of mild-moderately active UC carried out for 6-12 weeks. Rates of remission in a representative sample of these trials were 5% (Ginsberg et al 1992), 9% (Sninsky et al 1991), 20% (Feurle et al 1989) and 29% (Sutherland et al 1987).

There were no improvements in more objective measures of inflammation, nor improvements in measures of ROS production in either blood or the rectal mucosa. There was, if anything, a small increase in the ESR.

Antioxidant nutrient therapy appeared particularly effective in reducing the number of liquid or soft stools. This may have been due to an effect on colonic secretion mediated by ROS. Hydrogen peroxide stimulates chloride secretion in the isolated rat colon, via prostaglandin secretion (Karayalcin et al 1990) and the isolated jejunum from vitamin E deficient rats shows increased basal short circuit current in Ussing chamber experiments and an increased response to aminophylline (Lindley et al 1994). High doses of vitamin A and E supplements reduced the incidence of diarrhoea in a placebo controlled trial in children in China, though a concomitant reduction in respiratory disease indicates that these results may reflect enhanced activity of the immune system (Lie et al 1993). Vitamin E therapy converts the secretory state induced by radiation-induced enteritis into an absorptive one, without affecting the histological appearances (Empey et al 1992a). It is possible, therefore, that one of the main effects of antioxidant therapy is to reduce the colonic secretions, and thus diarrhoea, which is one of the most disabling features of UC.

The lack of a significant response in mucosal chemiluminescence or serum TBARS to antioxidant nutrient therapy suggests that either the dose, or route of administration, was ineffective or that these measures are inappropriate for assessing rectal and serum oxidant activity.

There is good evidence that luminol-amplified chemiluminescence of rectal mucosal biopsies correlates with mucosal inflammation (Simmonds et al 1992a; Keshavarzian et al 1992b), and clinical disease activity (Chapter 5). However, it is a technique which shows considerable variability in individual patients (see Chapter 5, p. 120). It may therefore be inappropriate for follow-up assessments of oxidant stress in individual patients. Furthermore, as luminol-amplified chemiluminescence is largely dependent on activated neutrophils (De Chatelet et al 1982) (see Chapter 5, p. 124), it is likely that significant improvements will depend upon histological improvement, which we did not observe during this study.

The levels of mucosal ROS were lower for equivalent sigmoidoscopic score than described in Chapter 5. The reasons for this are not clear, but may have been to delay in chemiluminescence assessments of study biopsies due to clinical commitments by the investigator (the author). Chemiluminescence was carried out within 2.5 hours, as previously defined (Simmonds et al 1992a); however it is
possible that there is a small reduction in chemiluminescence over this time. The trial patients in the present study were derived from a busy gastroenterology clinic and the biopsies taken were not analysed until the clinical work load was completed, whereas the majority of the biopsies analysed in Chapter 5 were derived from colonoscopy lists and were thus analysed soon after they had been retrieved.

The level of serum TBARS is similar to those found in previous studies, if at the lower end of the range (see Chapter 2, p. 55). The data presented here suggest that serum TBARS are not raised in active left-sided/distal UC, but a more extensive study would be required to prove this. It remains to be tested if patients with more aggressive and/or extensive disease have raised levels of serum TBARS.

Serum TBARS did not alter significantly during the period of antioxidant therapy, but were not different from normals at entry to the study; it is possible that mucosal oxidant stress may be present without significant alterations in systemic oxidant status, at least in patients with disease limited to left-sided and distal colitis. Colorectal mucosal TBARS are increased in IBD and improve with decreased disease activity (Ahnfelt-Ronne et al 1990), and it is possible that lipid peroxidation in IBD occurs in the mucosa but not serum in UC.

The oral route for antioxidant nutrient therapy was chosen because a similar medication at this dosage was effective in recurrent pancreatitis (Uden et al 1992). It is possible that, in UC, high concentrations of intraluminal antioxidants may be required for an anti-inflammatory response. Effective doses of 5-ASA produce luminal concentrations of aminosalicylates in the millimolar range (Staerk-Laursen et al 1990), and are similar to those required for an antioxidant effect with biopsies from patients with UC, in vitro (Simmonds et al 1992c). Future studies should consider using rectal administration to provide higher concentrations of specific antioxidant compounds in patients with active UC.

Although a wide range of antioxidants was employed in this study, it remains possible that alternative antioxidant oxidant pathways need to be targeted to achieve a therapeutic effect. It has been suggested that the iron chelating properties of aminosalicylates may explain their efficacy (Grisham 1990a). It is possible that to have a potent antioxidant effect iron chelation is necessary (see Chapter 7).

In conclusion, antioxidant nutrient therapy improved clinical and sigmoidoscopic measures of disease activity, and produced clinical remission in 4 out of 10 patients. However, neither objective measures of inflammation nor oxidant activity were improved suggesting that further trials with larger oral doses, rectal administration or alternative antioxidant nutrients, are required.
GENERAL DISCUSSION AND FUTURE WORK

ROS are now well established in the extensive ranks of potentially pathogenic inflammatory mediators produced in excess by the inflamed mucosa in IBD. However, the relative importance of these mediators remains to be established. Two important questions are whether the levels of the proposed mediator correlate with disease activity, and whether specific antagonist therapy is anti-inflammatory.

The results presented in Chapter 5 show that ROS production by the mucosa in UC correlates with sigmoidoscopic and clinical disease activity scores, as well as the intensity of the mucosal neutrophil infiltrate; these results confirm and extend previous data (Simmonds et al 1992a; Keshavarzian et al 1992b) and provide an answer to the question raised above.

The second problem facing those seeking effective antioxidant therapies is the wide range of potential candidate compounds. Previous work in this laboratory demonstrated that inflamed colorectal biopsies from patients with UC could be used to detect antioxidant activity using standard antioxidants and current therapeutic agents (Simmonds et al 1992a). This technique thus appeared to be useful in detecting potential antioxidants that could be considered for therapeutic use in IBD, and in investigating the antioxidant activity of the many other proposed therapies for IBD whose antioxidant properties are unknown. However, the time-scale of the study of Simmonds et al, 1992 was limited by the supply of inflamed colorectal biopsies from patients with UC. Thus in this thesis a model has been developed to provide greater quantities of inflamed colonic tissue for in vitro testing of antioxidants, based on acetic acid-induced colitis. The model was validated by demonstrating that ROS production in this model correlates with the degree of inflammation (Chapter 3) and by the similarity of the response between colonic biopsies from acetic acid-induced colitis and UC to standard antioxidants and conventional therapies for IBD (Chapter 4).

The acetic acid-induced colitis model in rats was used to show that the novel antioxidant compounds, LY231617 and amflutizole, have antioxidant activity which is approximately equivalent to that of 5-ASA. Further research into the potential role of these compounds is now being carried out by the pharmaceutical company with respect to toxicity and pharmacokinetics. If these are successful, phase II clinical studies, followed by trials in IBD, will be worthwhile.
The results of studies into the antioxidant and anti-inflammatory effects of rh-MnSOD were disappointing. As with CuZnSOD, the size of this tetrameric enzyme, preventing adequate tissue concentrations, is likely to underlie this poor performance. Further difficulties for a potential use in humans lie in the bell-shaped response noted in in vivo models, as discussed in Chapter 6. On the basis of this data rh-MnSOD cannot be recommended as a potential therapeutic agent in IBD.

An antioxidant effect with the iron chelators, desferrioxamine and 1,10-phenanthroline, was only detected in biopsies from patients with UC. It is hypothesised that iron-dependent mechanisms are important in UC, but not acetic acid-induced colitis. To explore this further, the effect of iron chelators on inflammation and ROS production in more chronic models of inflammation, such as those induced by TNBS and carrageenan, should be investigated using iron-loaded and iron-depleted animals. Nevertheless, the results presented here are sufficiently promising that clinical studies of topical iron chelators in active UC should be considered.

One of the principal determinants of the importance of ROS in IBD is the effect of specific antioxidant therapy on disease activity. In Chapter 8, the results of an open-label, pilot study, primarily designed to assess safety, are presented.

In this study, a combination of selenium, β-carotene, ascorbate, α-tocopherol and methionine, improved clinical scores and induced remission in 4 out of the 10 patients studied. There was no significant improvement in mucosal histology. Luminol-amplified chemiluminescence also failed to improve but was found to have great variability and therefore may not be a useful way of following mucosal ROS production over time. Serum TBARS did not alter, but were not different from normals at entry to the study: it is possible that mucosal oxidant stress may be present without significant alterations in systemic oxidant status, at least in patients with disease limited to the left colon. Further studies of lipid peroxidation in the blood of patients with more active and extensive disease would help to test this hypothesis.

A controlled trial of specific antioxidant therapy is warranted and this pilot study suggests that antioxidant nutrients are a valid pharmaceutical intervention for this purpose. As discussed in the Chapter 8, the regime studied was largely chosen for its convenience and because a similar regime had shown efficacy in recurrent pancreatitis (Uden et al 1992). For further studies, certain changes should be made to this combination of antioxidant nutrients. An increased dose of α-tocopherol would be logical, given its lack of toxicity and antioxidant effect: it would be useful in the future to examine the latter in vitro using the system described in Chapter 4.

The theoretical risk of increasing faecal concentrations of the ascorbate radical by oxidation of large oral doses of ascorbate remains untested. It remains possible
that larger doses of oral ascorbate than used in the present studies may be beneficial in active UC. An interesting study, therefore, would be to observe the effect of ascorbate enemas in patients with UC and controls on the extent and rapidity of formation of the ascorbate radical in the colonic lumen, its absorption into the colonic mucosa and the effect on mucosal ROS production.

Recent long-term studies of β-carotene supplementation have suggested that it may be associated with mutagenicity. Given that its role as an antioxidant in vivo has also been questioned, a future controlled trial in UC should consider leaving β-carotene out of the combination of therapy.

Previous studies have shown a limited role for reduced glutathione in reducing ROS production by the inflamed mucosa in UC (Simmonds et al 1992a). No antioxidant effect for N-acetylcysteine was demonstrated in the latter studies or with colonic biopsies from acetic acid-induced colitis, as shown in Chapter 4. The use of exogenous substrate for glutathione peroxidase in future trials of antioxidant therapy in UC may therefore be unnecessary. Furthermore, methionine has a distinctive odour which would complicate the implementation of double-blind controlled trials.

One of the difficulties of the current research relates to the equipment used to assess ROS production. The Tri-Carb Scintillation counter has no temperature-control facilities and has no injector ports to allow addition of compounds whilst measuring the light production. Compounds must be added prior to placing the vial in the analyser. These problems can be overcome by newer, dedicated, chemiluminometers. Such equipment, the Bertol LKB luminometer, has recently been purchased for use in this laboratory and will enable studies of ROS production to performed at physiological temperature and with greater control over the time-course of the study. In addition, study of the effects of combinations of antioxidants will help tailor antioxidant regimes prior to further clinical investigations.

The above discussion concerns the work in this thesis which concentrates on the use of antioxidant therapy in IBD. A complementary approach is to examine the mechanisms by which ROS may cause tissue damage. As discussed in the Introduction to this thesis, ROS may have an important role in the regulation of the inflammatory response, particularly with regard to the activation of nuclear transcription factors such as NFκB. A valuable new approach will be investigations of NFκB activation in IBD. Such research has commenced in this laboratory using immunohistochemistry, based on a monoclonal antibody to the P65 active moiety of NFκB; the relation of NFκB activation to disease activity, and its response to in vitro oxidant stress, in peripheral blood lymphocytes, is being assessed.

In conclusion, this thesis has provided further evidence for a possible pathogenic role for ROS in IBD, and a number of new therapies have been suggested,
such as LY231617, amflutizole, iron chelation and antioxidant nutrient therapy. The importance of ROS amongst the panoply of mediators in inflammation is thus gaining ground; as George Orwell might have said, "All mediators are equal, but some mediators are more equal than others". If ROS prove to be the pigs in the farm, the usefulness and direction of antioxidant therapy will depend on whether they wreak destruction in the fields or, as in ‘Animal Farm’, orchestrate it from the ‘nucleus’ of the farm kitchen. ROS may be generals, warriors or refugees in the inflammatory war, but they appear to be poor diplomats.
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APPENDIX

Some of the material presented in this thesis has already been published in paper or abstract form. The references to these publications are given below:


ADDENDUM OF ERRATA

The following corrections apply to the thesis.


Page 77, line 2 - ‘formol saline’ should read ‘0.9% NaCl containing 10% formalin’.