# Immunological Responses Following Surgery in Ulcerative Colitis

by

Rajan Kumar Patel

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The Queen Elizabeth Hospital

Edgbaston

Birmingham B15 2TH

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# Synopsis

The role of serum autoantibodies, soluble adhesion molecules, plasma and mucosal cytokines were studied following surgery for ulcerative colitis (UC).

A high prevalence of ANCA was found in UC but not in Crohn's disease.

ANCA positive UC sera were found to recognise a variety of antigens, namely lactoferrin, cathepsin G, enolase and elastase.

Confocal microscopy revealed maximum immunofluorescence for P-ANCA to emanate from the intranuclear and not the extra-(peri) nuclear portion of the neutrophil, as currently believed.

AECA, like ANCA, are also found in UC, suggesting immunological similarities with systemic vasculitis. Perhaps, UC represents a gut limited vasculitis.

Persistance of ANCA, AECA, anti-EPI and antitropomyosin antibodies inthe sera, several years following total colectomy, suggests that immunological mechanisms are not halted, by total colectomy.

Soluble intercellular cell adhesion molecules, ICAM-1 and E-Selectin, but not VCAM-1, were found to be useful markers of disease activity in UC.

Plasma levels of cytokines do not reflect disease activity, however, there is similar quantitative expression of pro-inflammatory cytokines by isolated mucosal mononuclear cells in both active UC and pouchitis, but not in non-specific proctitis, which seems to suggest that pouchitis is not merely a complication of ileoanal pouch surgery but, that it represents reactivation of UC in ileal mucosa, which has undergone villous atrophy and colonic metaplasia.

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# Statement

**The work on which this dissertation is based is my own independent work except where acknowledged. This work has not been accepted for any other degree, nor is being currently considered in candidature for any other degree.**

**Rajan Patel**

**May 1994**

To my wife,

**Jyoti,**

for **her support and encouragement**

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# List of Abbreviations

| | |
|---|---|
| ANCA | antineutrophil cytoplasmic antibody |
| AECA | antiendothelial cell antibody |
| Anti-EPI | antiepithelial cell antibody |
| Anti-tropo | antitropomyosin antibody |
| IBD | inflammatory bowel disease |
| UC | ulcerative colitis |
| ELISA | enzyme linked immunosorbent assay |
| MPO | myeloperoxidase |
| PR3 | proteinase 3 |
| IIF | indirect immunofluorescence |
| HIV | human immunodeficiency virus |
| HRP | horse radish peroxidase |
| HSP-65 | heat shock protein - 65kDa |
| **SDS** | sodium dodecyl sulphate |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |
| IL- | interleukin- |
| TNF | tumour necrosis factor |
| **G-CSF** | granulocyte colony stimulating factor |
| OD | optical density |
| LPS | lipopolysaccharide |

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## PUBLICATIONS

### Papers

Patel **RT,** Birch D, Stokes R, Ibbotson J, Keighley MRB. The influence of total colectomy on serum antineutrophil cytoplasmic antibodies in inflammatory bowel disease. British Journal of Surgery 1994; 81:724-726.

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**Patel RT,** Bain I, Youngs D, Keighley MRB. Cytokine production in pouchitis is similar to that in ulcerative colitis.

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## Presentations to Learned Societies

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Prize for best presentation].

Influence of total colectomy on serum antineutrophil cytoplasmic antibodies in inflammatory bowel disease. **RT** Patel, D Birch, R Stokes, J Ibbotson, MRB Keighley. The Surgical Research Society, 76th Meeting, Edinburgh, July 1992. ***(One** of 16 **papers selected for competition for Ihe Patey Prize).***

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# CHAPTER 1

# GENERAL INTRODUCTION

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## 1.1 Historical perspective

Chronic ulcerative colitis (UC) is an inflammatory disease of the colon, the chief symptom being diarrhoea with blood and mucus. In the nineteenth century it gradually became more clear that chronic UC was a pathological entity distinct from other known ulcerating diseases observed in the colon and rectum (the most common of which were dysentery and tuberculosis).

The first report of UC has been ascribed to Sir Samuel Wilks (Figure 1.1), a physician and pathologist who lived from 1824 to 1911. In the first part of 1859 he wrote in his *Lectures of Pathological Anatomy* [Wilks, 1859], that the term colitis could not be synonymous with dysentery. He described occasional findings of inflammation of the colon in certain febrile and other disorders without evident cause, *'patches of the colon being red, inflamed, covered with mucus, adherent lymph and ulceration'.*

In September 1859, Wilks presented his classic description of a case with severe UC, 'the morbid appearances in the intestine of Miss Banks',[Wilks, 1859], comprising testimony to court based on examinations a few days after her death for suspected poisoning. He described *'the mucous membrane was ulcerated from end to end, the majority of ulcers being the size of a sixpenny piece, and together occupying more than half the internal surface. The ulcers were of an active and very recent character, most pronounced in the proximal part of the large intestine, with sloughing, and almost detached, with exudation of blood,*

*Figure 1.1. Sir Samuel Wilks. (By kind permission of the Gordon Museum, Guys Hospital, London).*

***mixed with secretions'.*** Wilks believed that the severe changes were of two to three weeks standing and were the cause of death. He discussed the possibility that the ulcerating disease could commence in the mucosa or in the glands beneath. He described having seen two previous cases, out of 3000 autopsies at Guys Hospital, resembling the present one, he then concluded that, although the acute inflammation might be set up by an irritant poison, ***'poison as a primary cause was most unlikely to produce the observed effects'.***

There has been significant advance in the diagnosis and management of patients with UC employing improved diagnostic aids such as endoscopic biopsy, histology and histochemical methods however despite this, the cause of UC after over one hundred and thirty years from the original description still remains to be elucidated.

Over the years a number of theories have been proposed. Popular during the early years was that the disease represented a chronic form of bacillary dysentery or that it represented an allergic response to a dietary antigen. Recent work has focused more on the hypothesis that the defect may lie in the immunological response within the epithelium. Immunological effector mechanisms are thought to account for most of the tissue damage that occurs within the mucosa. However, what triggers these effector mechanisms is unknown. Some theories and pathophysiological mechanisms are considered.

## 1.2 Aetiological Factors

### 1.21 Genetic Factors

It is most likely that there is a genetic component. The observed frequency of the disease in first degree relatives is reported to be about 13% [Gilat, 1986]. This tendency has been shown to extend to cousins, uncles, and grandparents, but with a reduced frequency. As spouses of index cases are rarely affected, this is suggestive of a genetic predisposition. However, unlike Crohn's disease, monozygotic twin studies have not shown such strong concordence for UC. No strong HLA association has emerged either, except in Japan where UC is reported to be associated with HLA-B5 and DR2 [Asakara et al, 1982; Tsuchiya et al, 1977]. After combining all the reported studies of HLA-A and **-B** antigens, an association (albeit weak) of HLA-B27 and -Bw35 with UC has been shown [Biemond et al, 1986; Satsangi et al, 1994].

### 1.22 Environmental Factors

It has been postulated that increased permeability during infancy could lead to sensitization to one or more luminal antigens which could render an individual susceptible to chronic disease later in life. Early weaning [Acheson et al, 1979] and gastroenteritis in infancy [Whorwell et al, 1979] have been suggested as possible risk factors but more recent studies have found no such evidence [Gilat et al,

**1985].**

A number of studies have shown an association between the non-smoking status and **UC** (the converse being the case with Crohn's disease). The mechanism underlying this association is unknown. It has been suggested that there might be a genetic predisposition to inflammatory bowel disease (IBD) and that the smoking habit might determine whether **UC** or Crohn's disease develops [Somerville et al, **1984].**

Another factor which has been investigated is the oral contraceptive pill and this could point to a vascular basis for the disease, but reports have been conflicting [Royal College of General Practitioners, **1974;** Rhodes et al, **1984;** Vessey et al, **1986].**

Investigations into diet and stress factors have not demonstrated any convincing correlations. The role played by free fatty acids is currently being evaluated (Sagar et al, **1994)**

## 1.23 Role of infectious agents

In active **UC,** the mucosa is infiltrated by acute (neutrophils, basophils, eosinophils) and chronic (lymphocytes, plasma cells and macrophages) inflammatory cells.

A number of infectious agents have been implicated in **UC,** but none has satisfied Koch's postulates of i)isolation from all cases, ii) pure growth in culture and iii) passage of cultured organism to an appropriate animal model.

## Bacteria

Many studies have shown changes in the proportions of commensal organisms in the normal gut. However, diarrhoea, irrespective of the primary aetiology, may itself bring about these changes. None of the early studies, which implicated bacteria from the normal flora, have been substantiated. Similarly, it is unlikely that UC is a chronic form of bacillary dysentery [Gorbach, **1986]**.

It appears that the intestinal microflora has a secondary rather than a primary role in UC [Mahida, **1987]**. Bacteria appear to potentiate inflammation following penetration due to increased permeability caused by the initial breach in the epithelial barrier. Their role can perhaps be illustrated, to some extent, in an animal model. Administration of a red seaweed extract, carageenan, to guinea pigs produces a form of colonic inflammation that, in some ways resembles human UC. This inflammation is prevented if the animals are kept germ free or are given oral metronidazole [Onderdonk et al, **1981]**. However, metronidazole does not appear to have a role in the treatment of UC [Chapman et al, **1986]** but it does however form the mainstay treatment of pouchitis.

## Viruses

There is no convincing evidence to date of a specific viral aetiology. Measles virus has been implicated in Crohn's disease, recently, by Wakefield [Wakefield et al, **1992]**.

## 1.3 Immunological factors

Interest in the role of the immune response in UC arose because of a number of observations. These included the nature of the mucosal inflammation, the occasional association with autoimmune disorders and the observation that gammaglobulin concentrations increased during relapse. Many studies have been performed and it has been shown that heightened immune responses, as shown by humoral and cell mediated immunity, to a variety of gut associated antigens occur.

### 1.31 Immune complexes and complement

The idea that antigen-antibody complexes mediate the inflammation in UC is attractive as it could explain not only the colonic inflammation but also its association with extra-intestinal manifestations like arthritis, uveitis and erythema nodosum. Circulating immune complexes have been related to disease activity and extra-intestinal manifestations [Jewell et al, **1983]**. It is presumed that immune complexes in the intestinal mucosa would activate complement which would lead to tissue damage. This has been supported by animal experiments. Deposition of immune complexes within the colonic mucosa of rabbits leads to an acute inflammation which is quite similar to human UC. Morever, if these animals are previously immunized to bacterial antigens, the injection of soluble immune complexes induces chronic colitis [Mee et al, **1979]**.

## 1.32 Autoimmunity

Autoimmunity was suggested by the demonstration of anticolon antibodies which were first described in patients with UC in **1959** [Broberger et al, **1959]**. The antibodies are directed towards an antigenic determinant in the goblet cells of the colonic epithelium, and cross-react with lipopolysaccharide extracts of E.Coli. However, these antibodies are not specific for UC and can be detected in a number of other clinical conditions [Jewell et al, **1985].** Colonocyte specific cytotoxic antibody has also been described [Shorter et al, **1970;** Das et al, **1984;** Auer et al, **1988]**. The relation between these two characterised antibodies is unclear and their importance in the pathogenesis of ulcerative colitis is uncertain.

Recently, a possible target antigen for the anticolon antibody has been described by Das and colleagues to be an isoform of the muscle protein tropomyosin. Anti-tropomyosin antibody has been found to be present in the majority of patients with UC but not in Crohn's disease. Tropomyosin is expressed not only by the colon but also by many other organs, including the gall bladder and skin, which possibly provides an explanation for the biliary and cutaneous manifestations of UC [Das et al, **1990**, Das et al **1992].**

Several other autoantibodies have now been described in IBD. These include the anti-neutrophil cytoplasmic antibody (ANCA) and antiendothelial cell antibody (AECA).

## 1.33 ANCA

A relatively new type of antibody which has received considerable interest recently is the anti-neutrophil cytoplasmic antibody (ANCA). ANCA can be detected by indirect immunofluorescence on alcohol fixed neutrophils. ANCA has been described in detail originally in patients with systemic vasculitides and is currently used to aid diagnosis and monitor disease process in this group of diseases [Davies et al, **1982**; Van der Woude et al, **1985**; Rasmussen et al, **1988**; Falk et al, **1988**; Gross et al, **1990]**. In these diseases the related antigens are enzymes in the granules of neutrophils [Falk et al, **1988**; Ludemann et al, **1990**; Goldschmeding et al, **1989**; Pryzwansky et al, **1978**; Niles et al, **1989**; Lee et al, **1990]**. In essence two types of ANCA have been shown by indirect immunofluorescence:

**i)** C-ANCA: antibody directed against neutrophil cytoplasm in a diffuse manner

ii) P-ANCA: antibody directed against the perinuclear cytoplasm of the neutrophil

Several studies have reported the existence of P-ANCA in the majority of patients **(50-83** %) with ulcerative colitis and 10-30% of patients with Crohn's disease [Saxon et al, **1990**; Rump et al, **1990**; Siebold et al, **1992**; Cambridge et al, **1992]**.

## 1.34 AECA

Anti-endothelial cell antibodies (AECA), were originally implicated as a pathogenic factor in the vascular injury induced in patients with systemic lupus erythematosus [Cines et al, **1984]**. Since then they have been reported to occur in Kawasaki syndrome [Leung et al, **1986**; Leung et al, **1987]**, haemolytic uraemic syndrome [Leung et al, **1988]**, and rheumatoid vasculitis [Heurkens et al, **1989**; Quadros et al, **1990]**. Recently, several studies have reported that sera from **30-60** % of patients with polyarteritis and Wegener's granulomatosis contain antibodies to endothelial cells [Ferraro et al, **1990**; Frampton et al, **1990**; Savage et al, **1991]**.

The role of AECA in the vasculitic disorders and mechanisms by which vascular injury may be brought about is well documented [Cines et al, **1984**; Leung et al, **1986**; Leung et al, **1987**; Leung et al, **1988]**. Although vasculitis has been documented in inflammatory bowel disease very little is known of the mechanisms involved [Murch et al, **1992**; Wakefield et al, **1991]**.

AECA have been recently described in IBD [Romas et al, **1992**; Stevens et al, **1993]** but, as yet, their role has not been clearly defined in this group of diseases.

The problem of determining whether the humoral and cell-mediated immune responses to cell-associated antigens occur as a primary event or are secondary to the inflammation is unresolved.

Immunoregulation

Tissue damage induced by immunological effector mechanisms could imply disordered immunoregulation. This is the process by which immune responses are controlled and include lymphocytes and macrophages. Communication within the microenvironment between the various immune cells is thought to occur via the production of monokines (peptides produced by mononuclear cells) or lymphokines (produced by lymphocytes).

## 1.4 Adhesion molecules

The survival of any complex multicellular organism depends on the ordered and controlled interaction of its various specialised cells with one another and with the surrounding extracellular matrix (ECM). In the case of leucocytes, migration into and within the tissue requires co-ordinated expression and function of cell adhesion molecules (CAM) which mediate adhesion to other cells and to components of ECM. The application of monoclonal antibody and molecular biological techniques has considerably increased our understanding of the mechanisms of leucocyte adhesion.

The classification and detailed description of leucocyte adhesion molecules and their ligands has been the subject of recent reviews [Albelda et al, **1990**; Springer, **1990**; Keelan et al, **19921**.

## 1.41 Role of leucocyte adhesion molecules in inflammation

Direct inspection of the microvasculature shows that circulating leucocytes normally 'roll' along the surface of the vessel wall [Atherton et al, **1972**]. In inflamed tissues the rolling leucocyte becomes tethered to endothelium and subsequently, transmigrates through the vessel wall into the tissues. To a large extent, these different events in leucocyte emigration depend upon i) expression and activation of appropriate adhesion molecules on endothelial cells and ii) leucocytes in response to the mediators of the various forms and stages of inflammation.

### Endothelial cells and other resident cells

The critical mechanism localising inflammatory lesions is probably the activation of adhesion molecule expression on endothelial cells and resident cells within the tissues. Endothelial cells can undergo different phases of activation, each associated with the appearance of different adhesion molecules for leucocytes.

In very early inflammatory lesions translocation of pre-formed **GMP-140** to the endothelial cell surface allows binding of neutrophils, monocytes and probably eosinophils. This is followed, after **4** – 6 hours, by sub-acute phase of endothelial activation, governed by the induction of a number of genes in response to interleukin **1** and/or tumour necrosis factor and involving the de novo synthesis and

expression of ICAM-1, ELAM-1 and VCAM-1 [Pober et al, 1990]. The expression of these three cytokine inducible molecules is differentially regulated by cytokines, potentially resulting in alterations in their relative densities in different forms of immune mediated inflammation and thereby, predisposing to the migration of lymphocytes and monocytes [Thornhill et al, 1991]. The nature of the endothelial adhesion molecules responsible for lymphocyte traffic in established chronic inflammation is not yet clear. New techniques have now been developed to measure not only tissue-bound adhesion molecules, by specific stains using monoclonal antibodies, but soluble forms can also now be measured to assess chronic inflammation.

## Clinical inflammation

In the more complex forms of inflammation presented by clinical pathological material, expression of adhesion molecules can give insight into pathophysiological mechanisms and may even have diagnostic significance.

Cytokines also regulate the expression of adhesion molecules on other resident cells such as epithelial cells, fibroblasts and synovial cells, the optimal stimuli for expression of individual molecules varying with cell type.

## 1.5 Cytokines

The immunology of UC to unravel the intricacies of the inflammatory response has recently received great interest in the hope of finding abnormal regulatory events responsible, if not for triggering, at least for the perpetuation of the local tissue damage. If this less than ideal goal could be achieved a more rational and effective approach to the therapy of IBD could result [Fiocchi, **1993].**

In the strict sense, cytokines are products secreted by activated immune cells. These molecules share certain typical features, such as a multiplicity of cellular sources, activity on essentially all cell types, and functions integrated in a complex, redundant, but coordinated system called the 'cytokine network' [Balkwill et al, **1989].** Their presence in tissues and biological fluids is a physiological phenomenon, a premise that supports the widely held notion that alterations of their levels reflect abnormalities associated with a variety of pathological conditions. This notion explains in part, the tremendous interest all over the world in this 'cytokine explosion' [Durum et al, **1990].** Another reason for this interest is that fundamental cell-to-cell regulatory events are mediated primarily by cytokines [Sporn et al, **1992].** Therefore, the study of these soluble mediators holds the potential for answering basic biological questions while providing clinically valuable markers. Unfortunately,

nothing in science is as good as it seems to be, and the study of cytokines is no exception. When applied to IBD, data gathered from evaluation of cytokines have provided insight into possible pathogenic mechanisms [Fiocchi et al, 1984], novel methods of monitoring disease activity [Mueller et al, 1990], or even the possibility of differentiating Crohn's disease from UC [Gross et al, 1992]. On the other side, there still are many unsolved questions. The seemingly endless number of these molecules continues to lure the investigators into looking at still another, just discovered cytokine with a new irresistible function, distracting and detracting from the study of 'older' mediators, whose spectrum of biological activity has barely been scratched in the labarotory. This results in a practical dilemma: which cytokines are really important in IBD? The 'old' or the 'new' ones? The low level, mostly T cell-derived products with a predominant immunoregulatory function, such as IL-2, IL-4, IL-10, interferon gamma, etc or the abundant, mainly monocyte/macrophage derived molecules with a potent pro-inflammatory effect such as interleukin-1ß (IL-l), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumour necrosis factor ϱ (TNFa)?

This artificial but practical dicotomy poses the question of choosing between the study of primary regulatory events that may reveal basic pathogenic events and the investigation of secondary mechanisms of tissue injury that

may pave the way to a truly efficacious therapy for Crohn's disease and UC. This choice seems unscientific but no one can study all cytokines that may be relevant to these conditions, and each investigator must be content in focusing on selected molecules at the best of his or her ability. To do so it is vital that one studies each molecule with a systematic and thorough approach. Levels of cytokines must be established in the circulation as well as in surgical and biopsy specimens, keeping in mind, however that simple information on serum and mucosal concentrations has limited value without knowing the exact secretory cell, receptor expression, binding characteristics, target cells, and effects [Matsura et al, 1992]. However, without 'going back to basics' further work will be plagued with discrepancies. In Figure 1.2 is shown a possible role of cytokines and inflammatory cells involved in the host response to foreign antigens.

The cytokines studied in this thesis are the pro-inflammatory cytokines:- IL-1ß, IL-6, IL-8 and TNFa.

## 1.51 Interleukin-1

Original terms

Endogenous pyrogen, lymphocyte activating factor (LAF).

Biochemistry

Molecular weight 17 000. IL-1 exists in two forms, a! and ß, coded by two separate genes. IL-la! is predominantly membrane associated, whilst IL-1ß is secreted.

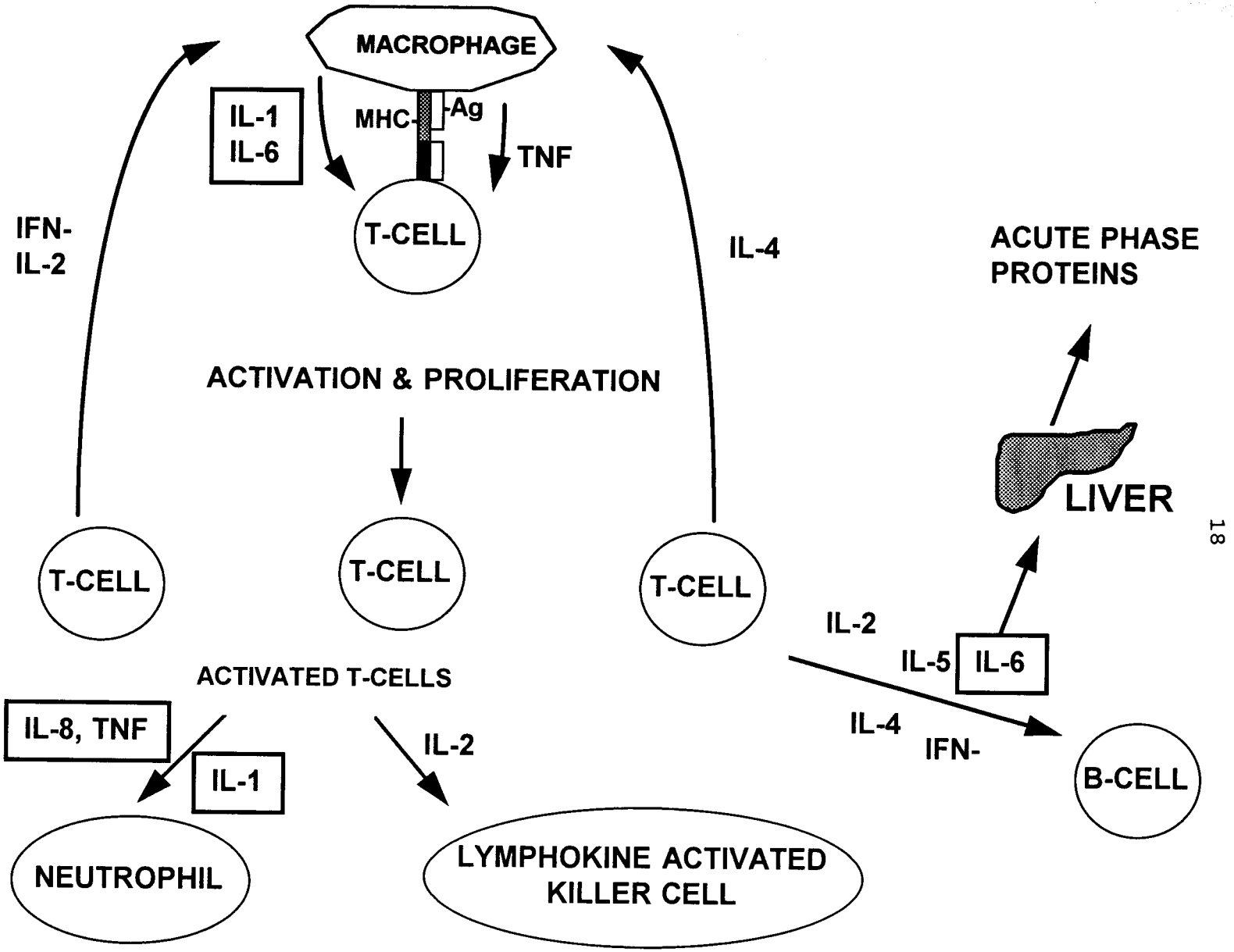



FIGURE 1.2 Cytokines and cells involved in the host response to foreign antigens.

Source.

Although IL-1 is produced by many cells, cells of the mononuclear phagocyte system (monocytes/macrophages) are probably the major source.

Table 1.1 Biological actions of IL-1

| Cell | Actions |
|---|---|
| T cells | Enhances proliferation and cytotoxic activity<br>Induces production of IL-2, IL-6, interferon, CSFs<br>Induces expression of IL-2 receptors<br>Induces chemotaxis |
| B-Cells | Induces proliferation & chemotaxis<br>Augments antibody secretion |
| Monocytes | Induces chemotaxis<br>Increases synthesis of thrombaxane and PGE2<br>Increases production of complement components |
| Neutrophils | Induces release from bone marrow and chemotaxis<br>Induces metabolic activation<br>Increased thrombaxane synthesis and release of enzymes |
| Endothelial cells | Induces expression of adhesion molecules for neutrophils and lymphocytes in endothelial cells<br>Production of procoagulant activity |
| Fibroblasts | Causes proliferation<br>Production of prostaglandin E2 and collagenase |
| Hepatocytes | Synthesis of acute phase proteins<br>Decreased albumin synthesis<br>Decreased plasma iron and zinc levels |
| Hypothalamus | Induces fever via prostaglandin E2<br>Anorexia & somnolence |
| Muscle cells | Prostaglandin mediated proteolysis |
| Epithelial cells | Proliferation<br>Secretion of collagen type IV |
| Osteoblasts | Proliferation<br>Secretion of collagen and prostaglandins |
| Osteoclasts | Resorption of bone<br>Production of prostaglandins and collagenase |

As can be seen in Table 1.1, IL-1 has an effect on a large number of cells to produce a wide range of effects [Malkovsky et al **1988]** and this serves to be a good example

to show the pleiotropic actions exhibited by cytokines. It induces T-cell activation, induces production of a number of cytokines and affects functions of B-cells, monocytes and neutrophils. Endothelial cells are induced to express adhesion molecules for neutrophils and lymhocytes upon stimulation with IL-1. This would allow the latter cells to interact with the endothelial cells and migrate to the site of inflammation.

***Relevance to gastroenterology***

Increased IL-1 production has been shown in both animal models of colitis [Rachmilewitz et al 1988] and in active human inflammatory bowel disease. Researchers [Satsangi et al, 1987] using a bioassay showed spontaneous production of IL-1 by isolated peripheral blood mononuclear cells from some patients with active Crohn's disease.

In vitro synthesis of IL-1ß by isolated intestinal mononuclear cells has also been studied [Mahida et al, 1989a]. There was enhanced production of the cytokine by mononuclear cells from mucosa with active inflammatory bowel disease compared with cells from control mucosa. Depletion of the mononuclear cells of macrophages reduced the amount of IL1-ß produced suggesting that these cells are the predominant producers of these cytokines in the intestinal mucosa. These results have recently been confirmed using a bioassay [Simon et al, 1989]. High levels of IL-1 have also been shown to be present in mucosal

biopsy specimens obtained from patients with active inflammatory bowel disease. %-aminosalicylic acid, at concentrations much lower than those found in the colonic lumen of patients taking this drug, inhibited synthesis of IL-1 in organ cultures of inflamed biopsies [Lamming et al, 1989]. Dexamethasone but not sulphapyridine also inhibited synthesis of this cytokine.

It is likely that interleukin-1 plays a major role in the pathogenesis of inflammatory bowel disease as it is a primary Stimulator of diarrhoea, the major symptom of intestinal inflammation [Sartor, 1994].

## 1.52 Interleukin-6 (IL6)

Original terms. B-cell stimulating factor 2, B-cell differentiation factor.

Biochemistry. Molecular weight 25 000.

Source. Monocytes, T-cells, fibroblasts, endothelial cells.

Biological actions. IL-6 has been shown to stimulate immunoglobulin synthesis by B-cells. Its actions on T-cells include acting as a Co-Stimulator in proliferation and also as cytolytic T-cell differentiation factor. Like IL-1 it induces synthesis of acute-phase proteins by hepatocytes [O'Garra, 1989b].

***Relevance to gastroenterology***

IL-6 is known to regulate immune responses, haematopoiesis and perhaps more importantly in IBD, the acute phase

response [Thomson, **1991]**. The latter is a systemic response to inflammation and tissue injury which is characterised by leucocytosis, fever, increased vascular permeability, alterations in plasma metal and steroid concentrations together with increased levels of acute phase proteins. This symptom complex is not uncommon in IBD.

High circulating levels of **IL-6** have been shown in acute pancreatitis [Leser et al, **1989;** Heath et al, **1993]** and in active inflammatory bowel disease [Mahida et al, **1990,** Hyams et al, **1993]**. where they correlated with severity of disease.

## 1.53 Interleukin 8 (IL8)

Synonyms. Monocyte derived neutrophil chemotactic factor (MNCF)**,** neutrophil activating peptide-1 (NAP-1)**,** neutrophil activating factor (NAF)**.**

Biochemistry. Molecular weight **10** 000.

Source. Cells of the mononuclear/macrophage system are probably the main source. **IL-8** can also be produced by fibroblasts, epithelial cells, endothelial cells and hepatocytes.

Biological actions.

**IL-8** is a potent chemotactic factor for neutrophils and could well represent the first signalling mechanism from activated macrophages to attract neutrophils to the site of an early inflammatory lesion. It has other effects on activated neutrophils, which include release of stored

enzymes, production of reactive oxygen metabolites and expression of cell-surface adhesion molecules. It is also chemotactic for T-cells [Baggiolini et al, 1989].

Neutrophil accumulation in the inflamed intestinal mucosa is a prominent feature in ulcerative colitis. Activated neutrophils may contribute to tissue damage at sites of inflammation. The factor(s) responsible for the mucosal recruitment of neutrophils in ulcerative colitis is not certain. IL-8 was originally isolated from endotoxin treated monocytes but can also be produced by a variety of other cells upon stimulation with a number of cytokines including IL-1 and TNFa [Striter et al, 1988; Striter et al, 1989, Watson et al, 1988].

***Relevance to gastroenterology***

There are, as yet, no conclusive data but it is highly likely that IL-8 could be pivotal in stimulating early neutrophil involvement in inflammatory diseases such as ulcerative colitis. Adhesion molecules induced on endothelial cells by IL-1 and TNF would allow neutrophils to interact with these cells. The neutrophils could then migrate under the influence of IL-8 into the mucosa. Subsequent production of leukotrienes would then lead to further Chemotaxis of neutrophils into the mucosa. IL-8 could thus be a therapeutic target. Inhibitors of synthesis or release of antagonists to its receptor could have a role in the treatment of inflammatory bowel disease.

## 1.54 Tumour Necrosis Factor *a*

Synonym. Cachectin

Biochemistry. Molecular weight (TNF*a*) 17 000.

Source. Principally cells of the mononuclear phagocyte system.

Biological actions. TNFa has a wide range of effects, which induce killing, stimulation of growth and induction of differentiation of cells [Tracey et al 1989]. Acute systemic release of this cytokine induces shock. It has also been implicated in cachexia of malignancy and chronic infection. A wide range of biological properties have also been implicated in inflammation. It shares many of these with **IL1**, synthesis of which it can induce in cells of the mononuclear phagocyte system. Properties it shares with **IL-1** include induction of adhesion molecules for neutrophils and lymphocytes and induction of pro-coagulant activity on endothelial cells, proliferation of fibroblasts, activation of neutrophils, induction of fever, elevation of acute-phase proteins and decreased albumin synthesis by hepatocytes.

***Relevance to gastroenterology***

The role of TNFa in gastrointestinal diseases is currently unknown. TNFa could be implicated in cachexia of gastrointestinal malignancy and active inflammatory bowel disease, especially Crohn's disease. Elevated plasma levels have been reported in patients with Crohn's disease

[Sartour, 1994]. Preliminary studies have shown no significant difference in the amount of TNFa produced by mononuclear cells isolated from histologically normal colonic mucosa and those cells isolated from mucosa with active inflammatory bowel disease. This is in contrast to the enhanced production of IL-1 by mononuclear cells from inflamed mucosa [Mahida et al 1989c]. Expression of TNF-specific messenger RNA has been demonstrated in mucosal mononuclear cells in cytomegalovirus enteritis [Lamerson et al 1989].

Intravenous infusion of high doses of TNFa have been shown to produce shock, diarrhoea, necrosis of epithelial and endothelial cells, degranulation of Paneth cells, luminal extravasation of neutrophils and red blood cells in the colon and small intestine. Infusion of TNFa over a period of 10 days has been shown to cause acute and chronic inflammation of the intestinal lamina propria and submucosa with focal mucosal ulceration, weight loss, bile duct proliferation, periportal inflammation, anaemia and leucocytosis [Sartour, 1994]. These are not unlike the symptoms of severe UC.

Pro-inflammatory cytokines also induce epithelial expression of other cytokines such as IL-2, IL-4, IL-10, TGFB, Class I and II major histocompatibility complex antigens and manganese dependant superoxide dismutase.

Early reports that raise the possibility that tissue cytokine concentrations can assist in the differential diagnosis of intestinal inflammation such as :-

i)IL-2 and interferon y are increased in mucosal biopsy results from patients with Crohn's disease but not in UC [Mullin et al, **1992,** Breese et al, **1993]** and

ii)IL-10 is increased in UC but not in Crohn's disease need further verification.

**A** distinct disorder of intestinal inflammation which may occur after restorative proctocolectomy **is** broadly known as pouchitis. This occurs in the neorectum created from the terminal ileum after an ileoanal pull-through procedure in the management of ulcerative colitis. Although the actual relation of this disorder to IBD remains uncertain, it may offer in vivo experimental models that could provide insight into the pathophysiologic processes contributing to ulcerative colitis. Pouchitis is found predominantly in patients with pre-existing ulcerative colitis and only infrequently in patients undergoing the same operation for familial polyposis coli [Lohmuller et al, **1990]** and the simultaneous study of patients with IBD and pouchitis forms the basis of this study.

## 1.60 AIMS

1.To assess the prevalence and titres of ANCA following surgery for UC looking especially at patients who had undergone restorative proctocolectomy.

2.Determination of the antigen specificity of ANCA.

3.To determine the target site of ANCA ie the exact morphological site of antigen-antibody binding which gives rise to the P-ANCA and C-ANCA immunofluorescence patterns in **IBD** using confocal scanning laser microscopy.

4.To assess the prevalence of AECA following surgery for UC looking especially at patients who had undergone restorative proctocolectomy.

5.To assess the prevalence of anti-EPI antibodies in UC. To also assess whether the autoantibodies ANCA, AECA and Anti-EPI correlated with disease activity or treatment and whether they were cross-reactive with each other.

6.The aims of this study were to determine levels of the soluble cell adhesion molecules in patients with chronic **IBD** in relation to prevalence, treatment and disease activity.

7.The aims of this study were to investigate the role of the pro-inflammatory cytokines in **IBD**. Plasma levels of these cytokines were determined at the same time as trying to establish the local production in the colon by means of colonic and ileal tissue biopsies.

8.The aims of this particular study were to assess whether there was increased cytokine production in pouchitis and if

***so,*** **are the levels of these cytokines comparable to UC. This hypothesis would put further weight on the belief that pouchitis did indeed represent reactivation of UC.**

# Chapter 2

## The influence of total colectomy on serum anti-neutrophil cytoplasmic antibodies in inflammatory bowel disease

## 2.1 Introduction

The causes and pathogenesis of inflammatory bowel disease are still unclear. A relatively new type of antibody which has received considerable interest recently is the anti-neutrophil cytoplasmic antibody (ANCA). ANCA can be detected by indirect immunofluorescence on alcohol fixed neutrophils (Figures **2.1 & 2.2**). ANCA has been described in detail originally in patients with systemic vasculitides and is currently used to aid diagnosis and monitor disease process in this group of diseases [Davies et al, **1982**; Van der Woude et al, **1985**; Rasmussen et al, **1988**; Falk et al, **1988**; Gross et al, **1990**]. In these diseases the related antigens are enzymes in the granules of neutrophils [Falk et al, **1988**; Ludemann et al, **1990**; Goldschmeding et al, **1989**; Pryzwansky et al, **1978**; Niles et al, **1989**; Lee et al, **1990**]. In essence, two types of ANCA have been shown by indirect immunofluorescence:

i) P-ANCA: antibody directed against the perinuclear cytoplasm of the neutrophil (Figure **2.1**)

ii) C-ANCA: antibody directed against neutrophil cytoplasm in a diffuse manner (Figure **2.2**)

**Figure 2.1** Antineutrophil cytoplasmic antibody staining seen on indirect immunofluorescence on ethanol fixed neutrophils: perinuclear pattern (P-ANCA)

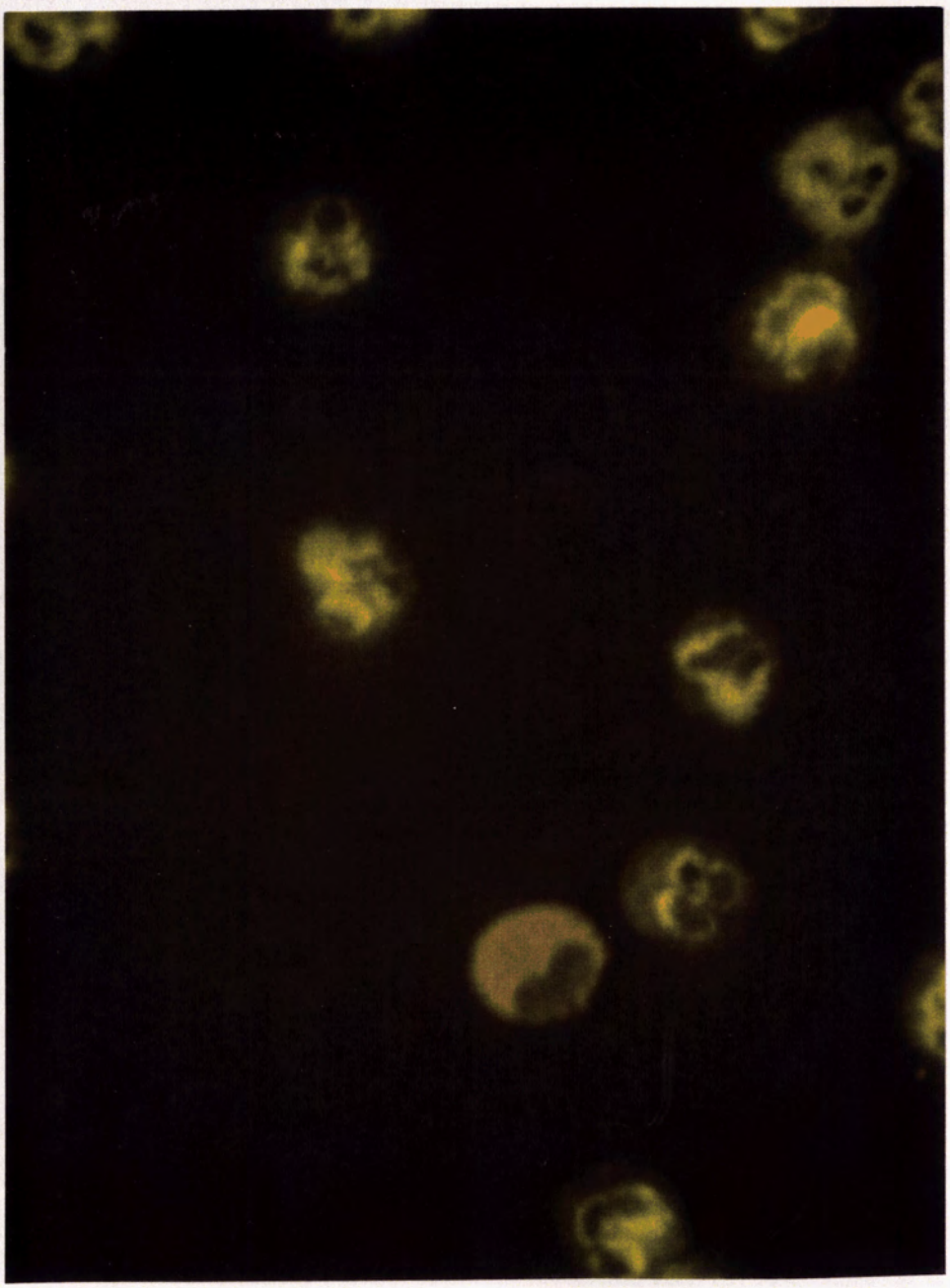
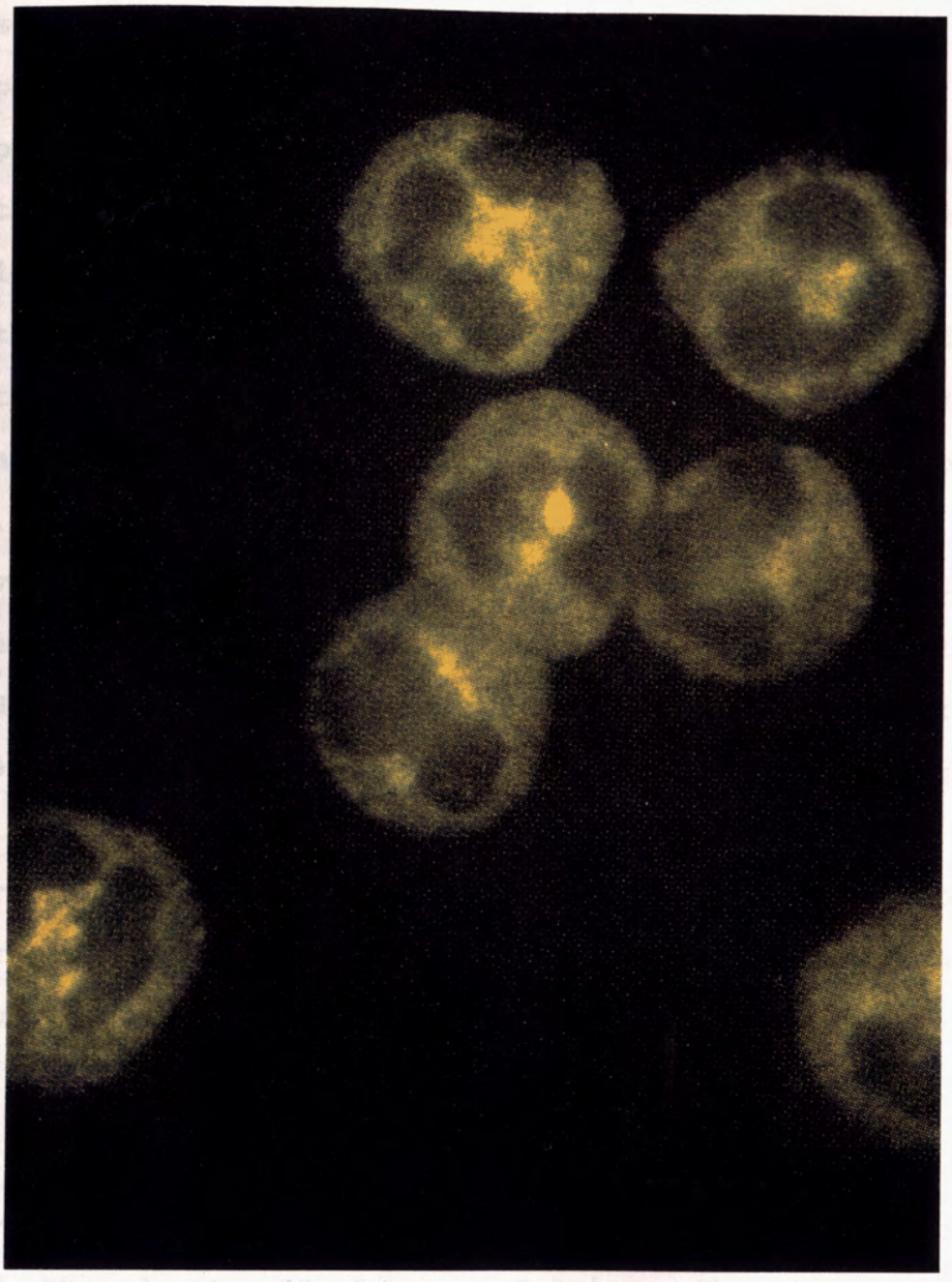


**Figure 2.2** Neutrophils with immunofluorescence extending to the edge of the cytoplasm (C-ANCA).



Granulocyte specific anti-nuclear antibodies (GS-ANAS) were reported even earlier than ANCA in **1966** [Farber et al, **1966],** primarily in patients with rheumatoid arthritis, although there are now reports of their being found in diseases such as SLE, drug induced lupus, chronic active hepatitis and myasthenia gravis [Wiik, **1980].** GS-ANAS were reported in a preliminary study in the sera of patients with IBD in **1983, (25%** in UC and **3%** in Crohn's disease) [Nielson et al, **1983].** It is likely that what was interpreted as nuclear staining was, in fact, the diffuse cytoplasmic staining with perinuclear highlighting that has since been reported in the sera of IBD patients [Saxon et al, **1990].**

Several studies have reported the existence of P-ANCA in the majority of patients **(50-83** %) with ulcerative colitis and **10-30%** of patients with Crohn's disease [Saxon et al, **1990;** Rump et al, **1990;** Siebold et al, **1992;** Cambridge et al, **1992].**

Our study focuses on the prevalence of ANCA following surgery and especially total colectomy in inflammatory bowel disease.

## 2.2 Patients and methods

Sera taken from patients and healthy controls were coded and stored at **-80** degrees Celsius. The diagnosis of inflammatory bowel disease in the study group had been made by prior clinical, radiological, endoscopic and

histopathological methods.

## Ulcerative Colitis

101 patients with ulcerative colitis, median age **40**, range **16-72** years, and **55** were males. These patients were graded into **3** groups (mild to severe) according to criteria, as previously described [Kodner et al, **1990**], in a modified form including endoscopic findings (Table 1), and in two further groups who had surgical intervention (Table 2). Two patients who had undergone restorative proctocolectomy had also undergone prior liver transplantation for sclerosing cholangitis.

Table 3 shows 12 patients who had blood samples taken before, 6-8 weeks and 6 months after total colectomy for severe ulcerative colitis. 5 patients who had undergone restorative proctocolectomy had clinical evidence of pouchitis when they were tested for ANCA. There were also **5** patients with indeterminate colitis who had had colectomy but there was doubt on histopathological grounds as to whether the diagnosis was Crohn's or ulcerative colitis.

## Crohn's disease

There were **40** patients with Crohn's disease, median age **30**, range **18-65**, and 22 were males. Disease activity was assessed using the Crohn's disease activity index [Harvey et al, **1980**]. The median disease duration was 6 years

(range 1-15 years). 8 patients had disease confined to the colon/rectum, 12 confined to small bowel alone and 20 affecting both large and small bowel. 4 patients included in the above groups had an ileostomy after undergoing proctocolectomy for Crohn's colitis.

## Controls

There were 2 control groups of patients:- 24 healthy volunteers whose median age was 34.1 years, range 21-41 years (Table 4) and 24 non-inflammatory bowel disease whose median age was 37.1 years and range 21- 58 years (Table 5).

## Preparation of Human Neutrophils

Blood was taken from healthy group O+ve individuals, in endotoxin-free tubes, to prevent activation of neutrophils. The neutrophils were separated using Percoll gradients. The cytocentrifuge preparation of neutrophils was then applied to slides and fixed with ethanol. Commercially produced precoated neutrophil slides were also used to standardise indirect immunofluorescence readings (Inova, Minneapolis USA). The slides were stored at 4° C and brought to room temperature before use.

## Indirect Immunofluorescence

Test sera diluted 1 in 20 with phosphate buffered saline (PBS) were incubated on the neutrophil coated slides for 30

minutes. After washing in a water bath for 30 minutes, FITC anti-human IgG (The Binding Site, Birmingham) was then placed on the slides ( 1 in 80 dilution) and incubated for 30 minutes. After washing and fixing with DABCO (fixing reagent) the slides were read on the Leitz Orthoplan immunofluorescence microscope by two independent observers at 50X magnification.

### Anti-nuclear Antibodies

Five sera, which suggested the presence of anti-nuclear antibody on indirect immunofluorescence, were tested using pre-coated slides with Hep-2 cells (The Binding Site, Birmingham).

## Statistics

Results were not normally distributed and are expressed as medians with ranges. Comparisons of the prevalence and titres of ANCA, in different subjects, have been made using the Chi square test and the Mann-Whitney U test.

## 2.3 Results

The predominant pattern was P-ANCA on indirect immunofluorescence. Inter-observer variation in the interpretation of indirect immunofluorescence readings was < 2 %. Overall 71/101 (70.3%) of the sera from patients with UC had P-ANCA and 5/101 (4.9 %) had C-ANCA (Table 1 &

2). 63.2 % of sera of patients having had subtotal colectomy who still had a rectum had P-ANCA. What seems very interesting is that 29/39 (74.4%) of the sera of patients, who had undergone restorative proctocolectomy, had P-ANCA. These patients had had removal of the primary target of the inflammatory process (the colon, rectum and anal mucosa) and yet still have high titres of ANCA. I have also noted that the titres of ANCA do not fall with time in the patients who have total colectomy as long as 4 years earlier.

Table 2.1. Characteristics of ulcerative colitis patients by disease severity and ANCA distribution

| UC | n | sex (m:f) | age (yr) | disease duration (yrs) | C-ANCA (%) | P-ANCA (%) |
|---|---|---|---|---|---|---|
| Mild | 15 | 7:8 | 48.3 | 5.5 | 0 (0) | 9 (60.0) |
| Moderate | 13 | 8:5 | 44.7 | 2.9 | 1 (6.6) | 10 (76.9) |
| Severe | 15 | 9:6 | 34.7 | 5.8 | 2 (13.3) | 11 (73.3) |

Table 2.2 Characteristics of patients with ulcerative colitis who had undergone surgery and ANCA distribution.

| UC | n | sex | age yrs | Disease duration yrs | Colectomy median yrs | Pouch median yrs | C-ANCA (%) | P-ANCA (%) |
|---|---|---|---|---|---|---|---|---|
| STC | 39 | 10:9 | 31.5 | 5.3 | <1 0-3 | - | 1 (5.2) | 12 (63.2) |
| RPC | 39 | 21:18 | 39.7 | 10.4 | 2.8 | 1.4 (0-4) | 1 (2.5) | 29 (74.4) |
| TOTAL | 101 | 55:46 | 40 | 5.0 | | | 5 (5.0) | 72 (71.3) |

STC = Subtotal colectomy, RPC = Restorative proctocolectomy

The median serum ANCA titre of 12 patients, who underwent total colectomy for ulcerative colitis, all of whom were known to have P-ANCA prior to surgery, doubled 6-8 weeks after surgery, and after 6 months the titre reverted to the preoperative titre (Table 3). Five patients with clinical pouchitis all had P-ANCA. 2 patients with ulcerative colitis, who had undergone liver transplantation for primary sclerosing cholangitis, prior to total colectomy, also had P-ANCA detectable in their sera.

Table 2.3. Serial median ANCA titres over time in patients with ulcerative colitis following surgery.

| n | P-ANCA | Before colectomy | After colectomy | |
|---|---|---|---|---|
| | | | 2-3 mths | 6 mths |
| 12 | 12 | 1/64 | 1/128 | 1/64 |

In patients with Crohn's disease 10/40 (25%) were found to have P-ANCA and 1/40 (2.5%) C-ANCA. I found that from the 10 out of the 40 patients with P-ANCA, 5 patients had undergone subtotal/total colectomy and 4 had evidence of Crohn's colitis. Only one patient with small bowel Crohn's has no evidence of the disease affecting the colon, as yet.

Table 2.4.Characteristics of Crohn's disease, indeterminate colitis and control groups.

| Disease | n | sex m:f | age yrs | Disease duration yrs | C-ANCA (%) | P-ANCA (%) |
|---|---|---|---|---|---|---|
| Crohn's | 40 | 22:18 | 30.0 | 9.2 | 0 | 10 (25) |
| Indeterminate colitis | 5 | 1:4 | 34.8 | 6.6 | 0 | 3 (60) |
| Healthy volunteers | 24 | 12:12 | 37.1 | - | 0 | 0 |
| Non-IBD bowel disorder | 24 | 12:12 | 34.1 | - | 0 | 0 |

**ANCA** was not demonstrated in any of the 24 healthy, or 24 non-IBD sera, which were used as controls.

Table 2.5. Distribution of non-inflammatory bowel disease disorders.

| | n | P-ANCA |
|---|---|---|
| Familial adenomatous polyposis (pouch) | 5 | 0 |
| Non-specific proctitis | 5 | 0 |
| Constipation | 9 | 0 |
| Irritable bowel syndrome | 6 | 0 |
| Total | 24 | 0 |

In our study group of patients, the effect of steroid and/or salazopyrine therapy, did not seem in any way to affect the prevalence or titre of ANCA. There were no positive anti-nuclear antibodies detected on the five suspected sera using Hep2 cells.

## 2.4 Discussion

ANCA were originally described in the sera of patients with systemic vasculitis [Davies et al, 1982]. In this study it was shown that patients with idiopathic segmental necrotizing glomerulonephritis had detectable ANCA, which disappeared after treatment. Van der Woude then showed that ANCA were associated with active Wegener's granulomatosis and, since then, many reports of its existence in various vasculitic conditions have been made [Van der Woude et al, 1985; Rasmussen et al, 1988; Falk et al, 1988; Lee et al,

1990].

In 1990 ANCA were described in the sera of patients with IBD [Saxon et al, 1990; Rump et al, 1990] and these findings have been confirmed by others [Siebold et al, 1992; Cambridge et al, 1992], including this study. In addition, this study has shown that the prevalence of ANCA is unaffected by surgery, especially total colectomy. ANCA detection in patients with systemic vasculitis has been found to aid in the clinical diagnosis and monitoring of disease activity following treatment [Van der Woude et al, 1985; Lee et al, 1990]. However, the clinical value of ANCA in inflammatory bowel disease still needs further evaluation, since this study has found no correlation between the prevalence and titres of ANCA and medical treatment.

In the 12 patients who had ANCA levels measured before and after total colectomy, it does not seem unusual that the ANCA titres temporarily doubled after 6-8 weeks, as this can be explained by the fact that these patients had prior severe colitis and the trauma induced by surgery may cause specific and non-specific increases in circulating immunoglobulins. However, what seems unusual, is the prevalence and high titres of ANCA for as long as 4 years after restorative proctocolectomy, in this series in asymptomatic patients. I believe that immunological mechanisms that resulted in UC are not halted by total colectomy. A possible reason for the persistence of ANCA

could be that chronic pouchitis may continually fuel the immunological process keeping levels of ANCA at detectable levels. ANCA have also been demonstrated in rheumatoid arthritis [Savige et al, 1991], and in a small percentage of patients with acquired immune deficiency syndrome with vascultic features [Klaassen et al, 1992]. Histological evidence of vasculitis has been demonstrated, not only in the above mentioned diseases, but also in Crohn's [Wakefield et al, 1989; Wakefield et al, 1991] and, to a lesser extent in UC [Murch et al, 1992], thus, suggesting an immunological link between these groups of diseases.

The systemic vasculitides and inflammatory bowel diseases share common features, in that both groups are of unknown aetiology, have autoimmune features, have ANCA and anti-endothelial cell antibodies [Romas et al, 1992] in the sera, and can be primarily treated by immunosuppressive steroid therapy. The usefulness of P-ANCA, as a diagnostic test for Crohn's disease, is doubtful, due to its low prevalence. However, these findings seem to suggest that prevalence of P-ANCA in Crohn's may be related to colitis as 90% of the Crohn's patients who were found to have P-ANCA had colitis. In the systemic vasculitides ANCA have been postulated to cause vascular injury mediated by respiratory bursts and degranulation of cytokine primed neutrophils [Falk et al, 1990].

ANCA have been shown, not only, to activate monocytes but also to play a role in their growth regulation [Ewert et

al, 1991]. Such mechanisms may be in operation in inflammatory bowel disease, and the study of patients who have undergone restorative proctocolectomy may help to further investigate the role of these autoantibodies.

## *2.5* Summary

Perinuclear anti-neutrophil cytoplasmic antibodies **(P-ANCA)** have been previously demonstrated in patients with various forms of vasculitis, and more recently, in IBD by an indirect immunofluorescence technique.

The sera of 194 patients were tested for **P-ANCA** :– 101 with **UC** {43 with varying grades of **UC,** 19 following subtotal colectomy and 39 following restorative proctocolectomy **(RPC)** for **UC}, 40** with Crohn's disease **(CD),** 5 with indeterminate colitis, 24 non-IBD and 24 healthy volunteers (controls).

Overall the prevalence of **P-ANCA** in **UC** was 70% (71/101). **P-ANCA** was still present in the majority of patients following restorative proctocolectomy, 74% (29/39) where the mean follow-up after surgery was 1.3 years. **All** 5 patients who had pouchitis following **RPC** were **P-ANCA** positive. By contrast only 25% (10/40) of Crohn's disease patients had **P-ANCA,** 9 of the 10 had Crohn's colitis. **P-ANCA** was not detected in controls. These results show that **P-ANCA** is more prevalent in colonic inflammatory bowel disease, especially **UC.**

The persistence of **P-ANCA** in the sera over one year after **RPC** suggests that the antigen(s) is (are) not fully eradicated and therefore, that it is not just the colon that is targeted immunologically in **UC.**

# CHAPTER 3

# DETERMINATION OF THE NEUTROPHIL ANTIGEN(S)

## 3.1 Introduction

As described in the previous chapter, autoantibodies against the neutrophil cytoplasm can be demonstrated by indirect immunofluorescence in the serum of most patients with ulcerative colitis, and in about one quarter of patients with Crohn's disease. However, the origin and nature of the antigen or antigens has yet to be fully established. ANCA can be distinguished by distinct immunofluorescence patterns, namely granular cytoplasmic ANCA (C-ANCA) and perinuclear ANCA (P-ANCA).

In sytemic vasculitis the majority of C-ANCA are specific for proteinase 3 (PR3) [Goldschmeding et al, 1989; Goldschmeding et al, 1990; Ludemann et al, 1990] and a substantial proporton of P-ANCA specific to myeloperoxidase [Falk et al, 1988; Falk et al, 1990A; Varagunam et al, 1991].

The strong association of ANCA with systemic vasculitis has been largely established [Frampton et al, 1990; Garrett et al, 1992; Savage et al, 1990; Stupp et al, 1991]. Proteinase3-ANCA are more commonly found in patients with biopsy proven Wegener's granulomatosis [Van der Woude et al, 1985], and myeloperoxidase-ANCA are more commonly found in patients with a vasculitis limited to the kidney [Falk et al, 1988]. These associations have been confirmed by other investigators [Noel et al, 1993].

Conversely, ANCA directed against neutrophil cytoplasmic antigens, other than myeloperoxidase and proteinase 3, have

been reported rarely in vasculitis patients [Lesavre, **1991],** but more frequently, in patients with various chronic conditions such as inflammatory bowel disease [Saxon et al, **1990**; Rump et al, **1990],** rheumatoid arthritis [Savige et al, **1991],** chronic hepatic diseases [Kallenberg et al, **1992**; Hardarson et al, **1993],** several infections including HIV infection [Klaassen et al, **1992**; Davenport, **19923**. Rare ANCA antigen specificities to elastase, lactoferrin and CAP 57 have been observed in a few patients with different forms of vasculitis as recently reviewed [Lesavre, **1991].**

Several independant investigators recently, have reported that the neutrophil antigens in inflammatory bowel disease are not myeloperoxidase or proteinase **3** [Saxon et al, **1990**; Cambridge et al, **1992],** and that inflammatory bowel disease ANCA are directed against different, as yet unknown, antigens. This prompted me to carry out the following studies, in order to determine antigen specificity of ANCA found in inflammatory bowel disease.

## 3.2 Aims

The aims of the following study were to determine the specificity of ANCA using:-

**1)** purified neutrophil antigens (enzymes)

**2)** using sonicated neutrophil protein.

## 3.3 Methods

### 3.31. Neutrophil Antigen determination using enzyme linked immunosorbent assay **(ELISA)**

The method for this ELISA previously had been validated by the Dept. of Renal Medicine, University of Birmingham, using test sera in patients with systemic vasculitis [Varagunam et al, **1991**]. The following purified neutrophil granule enzymatic components were obtained commercially:

1. lactoferrin (Sigma - human milk)
2. cathepsin G (BDH - human neutrophil)
3. myeloperoxidase (Sigma - human neutrophil)
4. elastase (BDH - human neutrophil)
5. lysosyme (Calbiochem - human neutrophil)
6. enolase (BDH - human neutrophil)
7. ß-glucoronidase (Sigma - bovine liver)

The immunoplates were coated with the antigen at a concentration of 10 µg/l overnight at **4°C** for 16 hours. The plates were washed, then blocked with 2% bovine serum albumin in PBS-Tween for one hour at **37°C**. All washes subsequently were carried out with saline-Tween. Test sera diluted **1** in 10, were incubated on the plate for one hour after washing. Alkaline phosphatase conjugated anti-human IgG was incubated for one hour at **37° C**. After washing

alkaline phosphatase Substrate was placed on the plate and incubated for 15 minutes at 37°C, before reading the plate at 405nm.

Optical density (OD) readings were plotted against the numbers of control and test sera for the various antigens.

## 3.32 SDS-Page Electrophoresis, Western Blotting with immunodetection

### 3.321 Preparation of neutrophils

Neutrophils for all studies were isolated from fresh whole blood using discontinuous density gradients of Percoll (Pharmacia, UK). Density gradient centrifugation is an established method for the separation and purification of cells, making use of differences in the size and density of blood cells, to separate them under reasonably mild conditions.

Percoll is a silica sol coated with polyvinyl-pyrrolidine (PVP), and is virtually free from unbound PVP [Pertoft et al, 1978]. It has a very low osmolality (<23mOs/kg $H_2O$), is thought to be non-toxic to cells [Kurnick et al, 1979; Pertoft et al, 1977] and does not affect cell morphology [Schumacher et al, 1978]. Neutrophil function may be influenced by cell isolation procedures [Fearon et al, 1983; Haslett et al, 1985]. In comparison to neutrophils

separated on Percoll gradients, those separated on, for example, Ficol-Hypaque gradients show spontaneous change of cell shape, reduced chemotactic responsiveness and increased release of Superoxide anion and lysosomal enzymes [Haslett et al, **1985].**

### *3.322* **Cell** isolation

Fresh venous blood was collected using a sterile **21** gauge needle and syringe into tubes containing **20** units of heparin per ml.

### *Gradient*

An isoosmotic gradient stock solution of Percoll was made by adding **1** part **1.5M** saline (density **1.058** g/ml) to **9** parts Percoll (density **1.130** g/ml, manufacturer's data). The density of the stock Percoll was calculated from the formula:

$$\frac{\text{(volume} \times \text{density of Percoll)} + \text{(volume} \times \text{density of diluting medium}}{\text{(volume of Percoll)} + \text{(volume of diluting medium)}} = 1.123 \text{ g/ml}$$

Stock Percoll was diluted further with **0.15M** saline (density **1.009g/ml)** to densities of **1.070** g/ml [Giddings et al, **1980]** and **1.097** g/ml [Jepsen et al, **1982]** for the separation of white cells. This was done using the formula:

$$\text{Volume diluting medium} = \text{volume stock Percoll} \times \frac{\text{(density stock Percoll)} - \text{required density)}}{\text{(required density)} - \text{(density diluting medium)}}$$

## *Cell isolation*

six mls of Percoll, density 1.097 g/ml, were placed into a sterile universal container, then 4 mls Percoll, density 1.070 g/ml, were layered carefully on top, taking care not to cause mixing at the interphase. This was done with a sterile syringe fitted with a wide bore needle, keeping the tip against the wall of the tube just above the surface of the liquid. Five mls of blood were diluted with an equal volume of phosphate buffered saline, and carefully layered on top of the Percoll in the same way. The tube was centrifuged at 200g for 25 minutes, resulting in the isopycnic banding of cells at the relevant interphases.

The pellet at the bottom of the tube consisted of red cells; granulocytes were suspended between the two layers of Percoll; mononuclear cells (monocytes and lymphocytes) were suspended above the 1.070 g/ml density Percoll layer. The granulocytes were removed with a pipette and washed twice with Dulbecco's B phosphate buffered saline (PBS), centrifuging at 1500g for 10 minutes after each wash. Remaining erythrocytes were lysed by hypotonic shock treatment, by adding 9 parts sterile distilled water, followed immediately by 1 part 10x PBS, and then centrifuging for 10 minutes.

Neutrophils were counted with an equal volume of white cell staining fluid (appendix A) and counted in an improved Neubauer chamber. The cells were resuspended in PBS and all

neutrophil suspensions were, at least, 95% pure, the remaining cells being contaminating eosinophils, basophils and, rarely, monocytes.

### *Preparation of sera*

All test sera used were obtained fresh from venous blood, which was allowed to clot prior to centrifugation at 1500g for 10 minutes. The serum supernatant was transferred to labelled sterile tubes.

### **3.323** Neutrophil sonication

Neutrophils were resuspended in sodium acetate (0.2 M, pH 4.2), in a 2 ml glass bijoux.

The sonicator machine in the Cancer Research Laboratories, Birmingham was employed for the next step. Sonication was performed for 10 minutes, in bursts of 1 minute with 15 second intervals, with the sample sitting in ice at 0°C. The suspension was made up to 5ml with sodium acetate buffer, and centrifuged at 8000g for 10 minutes at 4°C.

The supernatant containing mainly the neutrophil cytoplasmic components was removed using a pipette. The protein concentration of the supernatant was assessed using the Lowry technique, after which it was aliquoted into ependorfs and stored at -80°C. The remaining pellet was resuspended in sodium acetate buffer, and examined under the microscope to assess the efficacy of sonication.

A test ELISA was carried out on all neutrophil sonicated suspensions (coating plate at 10 μg/ml), using known P-ANCA sera, to ensure presence of neutrophil antigens in the sonicate.

### **3.324** Gel Electrophoresis

In electrophoresis, the migration of proteins is dependant largely upon the charge, size and shape of the molecules. However, in the presence of sodium dodecyl sulphate (SDS), proteins bind the SDS, become negatively charged and have a similar charge:weight ratio. When SDS coated proteins are placed in an eletric field, their spatial separation will depend only upon their size and shape. By varying the concentration of the polyacrylamide gel, used as the medium for the electrophoretic separation, different resolution ranges of molecular weight may be obtained. Proteins may be fractionated in the native state, but more information is usually obtained if the disulphide bonds are first reduced, allowing separation of the individual peptide chains. After heating to 100°C, in the presence of reducing agents and SDS, the proteins unfold and bind about 1.4 g SDS $g^{-1}$ protein. The strong negative charge on the proteins thus means that their electrophoretic mobility will be inversely proportional to the logarithm of their molecular weight [Hudson et al, 1989].

In practical terms, a stacking gel is cast on the top of the separating gel, this has both a lower percentage polyacrylamide concentration (typically between 3 – 5 %), and is prepared using a buffer with a slightly different composition. The different mobilities of chloride and glycine and the slow rate of entry into the separating gel, relative to the rate of progression through the stacking gel, are exploited to concentrate the proteins in a narrow band at the interface between the stacking and separating gels. This allows the variable loading of sample. Originally, the technique was performed in gel tubes, but these have almost entirely been replaced by flat gel slabs. Polyacrylamide gel electrophoresis (PAGE) has become a standard tool for the separation and purification of proteins. In this study, the separation of proteins in the neutrophil sonicate was carried out using sodium dodecyl sulphate Polyacrylamide gel electrophoresis (SDS-PAGE).

The method for PAGE was carried out, as previously described [Laemmli, 1970; Towbin et al, 1979], using the Biorad Protean II xi 16cm gel tank and Biorad gel former (Figure 3.1).

Preparation of the gels was carried out using the formulae given in the Biorad, UK manual:

Glass plates were thoroughly cleaned with ethanol. Two glass plates were clamped together with 1mm spacers and placed into the gel stand. The plates were pressed firmly down onto the rubber base, and side screws tightened to

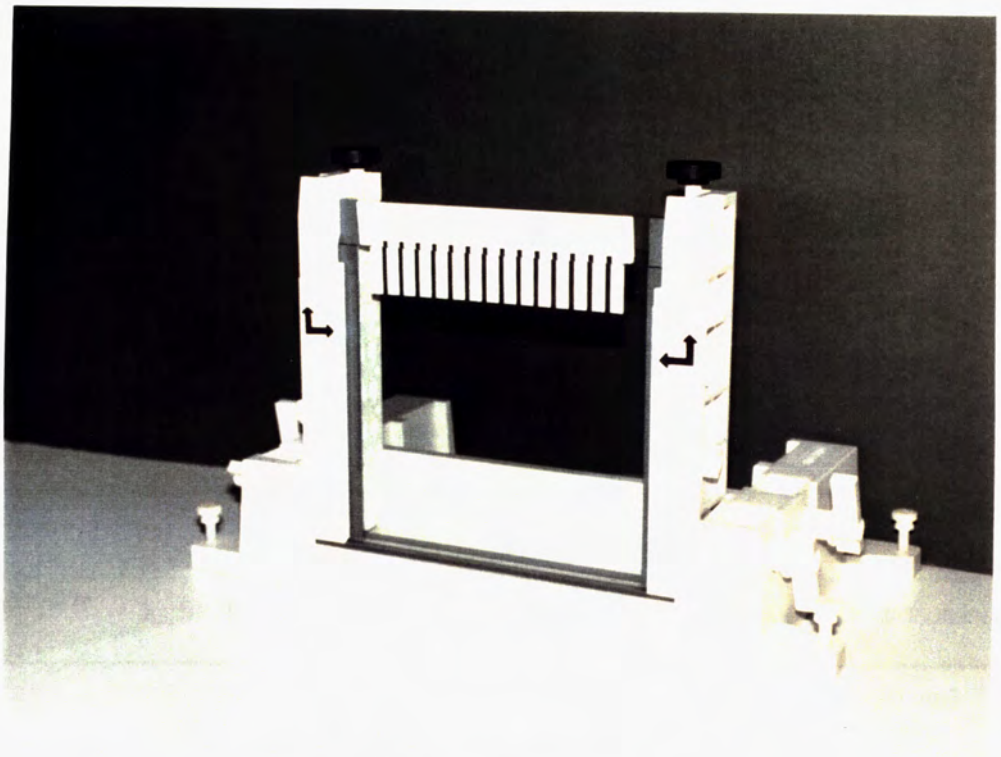


Figure 3.1a The Biorad gel former

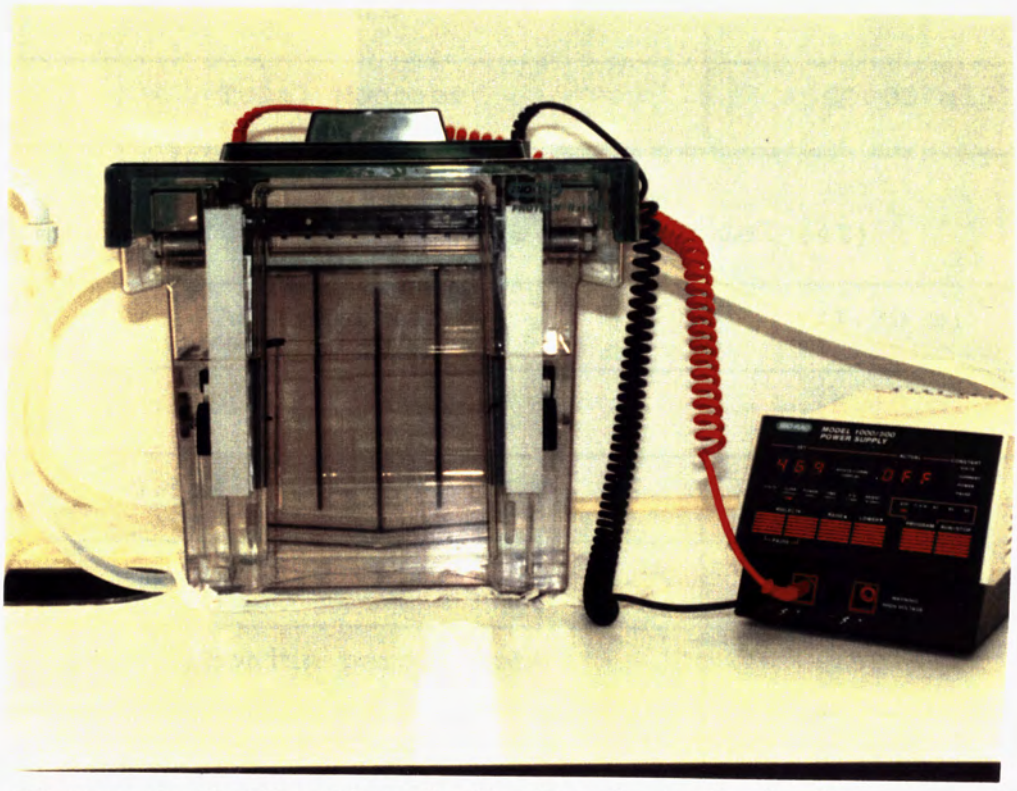


Figure 3.1b The Biorad electrophoresis tank

obtain a water tight seal in which to pour the gel. Next, the separating gel (12%) (Table 3.1) and stacking gels (4.5%) (Table 3.2) were poured according to the following recipes:-

Table 3.1. Constituents of the Separating Gel

| | |
|---|---|
| (12%)Acrylamide bis | 20.0 ml |
| Distilled water | 16.75 ml |
| 1.5 M Tris-HCL, pH 8.8 | 12.50 ml |
| 10 % (w/v) SDS | 0.50 ml |
| 10 % ammonium persulphate | 250 $\mu$l |
| TEMED | 25 $\mu$l |
| Total Monomer | 50.00 ml |

Table 2. Constituents of the Stacking Gel (4%)

| | |
|---|---|
| Acrylamide/bis | 1.30 ml |
| Distilled water | 6.10 ml |
| 0.5 M Tris-HCL, pH 6.8 | 2.50 ml |
| 10 % (w/v) SDS | 100 $\mu$l |
| 10 % ammonium persulphate | 50 $\mu$l |
| TEMED | 0 $\mu$l |
| Total Monomer | 10 ml |

## *TEMED*

TEMED initiates the setting process, hence, this was added just before the gel was poured in between the glass plates. After the separating gel had set, the stacking gel was poured and allowed to set with a 16 well comb. When the stacking gel had set, the comb was removed, and the wells were washed twice with distilled water.

The neutrophil sonicate samples, which previously had been added to the sample buffer (Appendix A), were brought to boil to 100°C for **30** seconds, and allowed to cool for 5-10 minutes.

100 µl of the sonicate was loaded onto the wells using a one ml syringe with a **23** gauge needle, followed by electrode buffer. The maximum capacity of each well was 135 µl. 100 µl of prestained molecular weight standard was placed in the first and last well of each gel. The gel was removed from the tank and placed into the gel tank, which was filled two thirds with electrode buffer. After filling the top tank with approximately 300 ml of electrode buffer, the electrodes were connected, and the gel was allowed to run until the dye front reached the bottom.

Initially, the gel was run at **24** mA constant current usually for **2** hours, or until the samples reached the separating gel. After this, the current was increased to **30** mA per gel constant current for approximately 5 hours, or until the dye front reached the bottom. Generally, with

fresh electrode buffer, running time was faster.

### *Coomassie Blue Staining*

In order to assess the initial results of electrophoresis of the sonicate, the gels were stained with Coomassie Blue (Appendix A). The gel was stained for 3 hours, usually to assess both the quality and quantity of protein separation before each new batch of neutrophil sonicate was tested any further Figure 3.2.

### **3.325** Western Blotting (Transfer of protein to nitrocellulose membrane)

As soon as the gel had run, having marked the left hand bottom side of the gel, it was removed from the glass plates whilst "under water" in the blotting buffer, to prevent cracking of the gel. Filter papers, cut to size, soaked in blot buffer, were placed on either side of the gel and presoaked nitrocellulose membrane. The whole slab was placed in between wet pads and placed in the caskets. The blotting tank (Figure 3.3) was filled with blotting buffer (Appendix A) and the caskets were placed in them. The slab was placed so that the nitrocellulose membrane was facing the anode side. The electrodes were connected and a current of 500 mA and a voltage 900 V was passed for 3 hours, for transfer of proteins to occur (Figure 3.4).

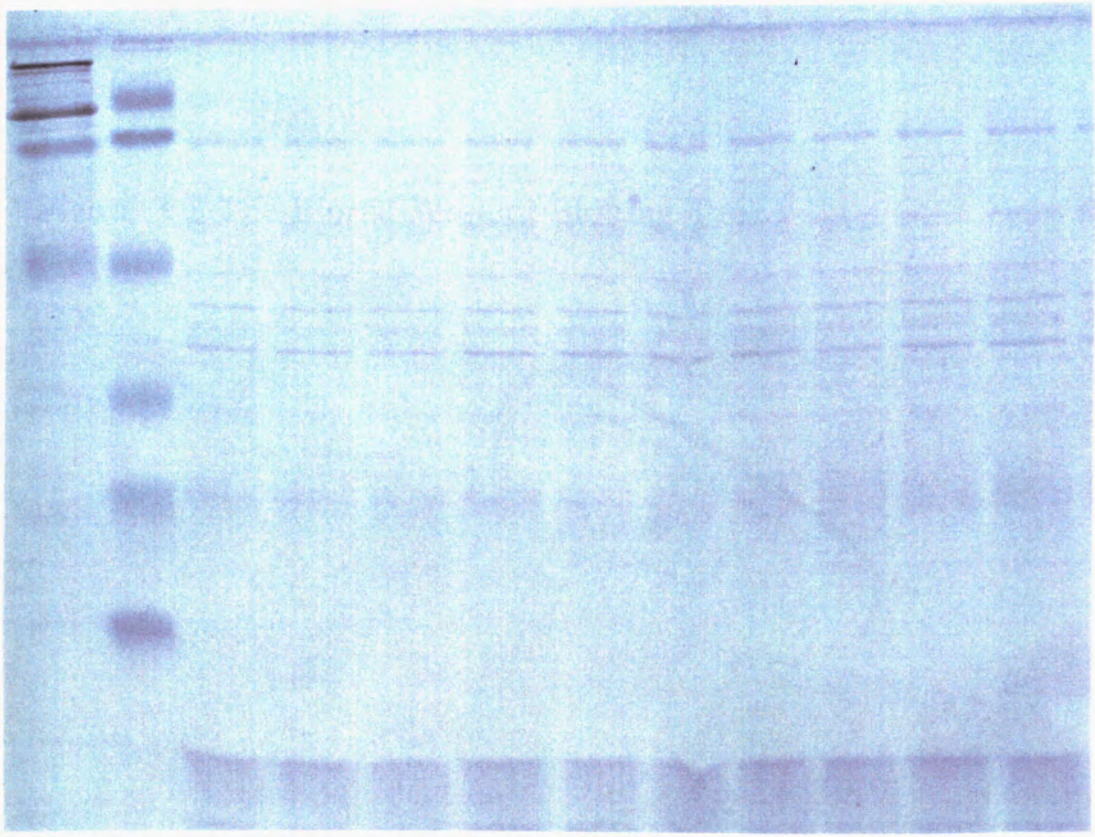


Figure 3.2a SDS PAGE gel stained with Coomassie blue

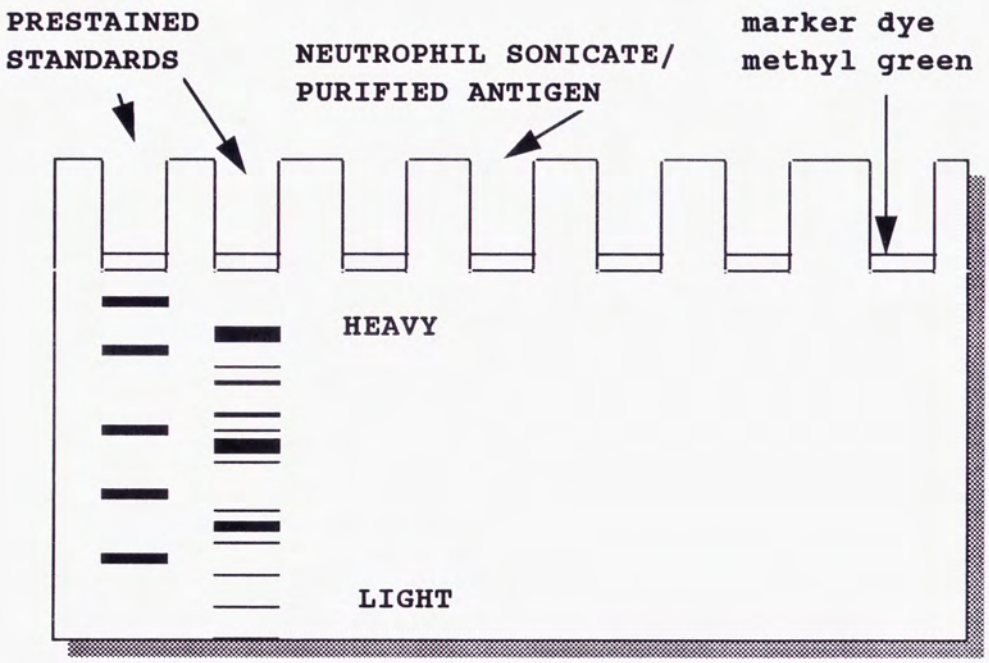
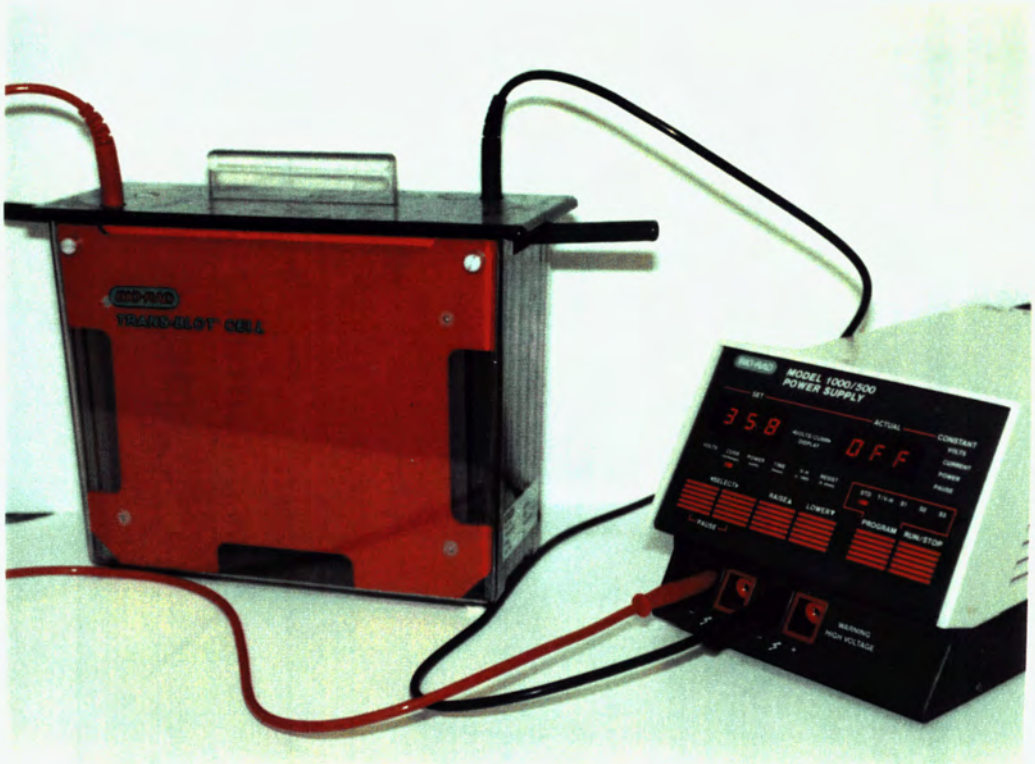



Figure 3.2b The SDS PAGE gel format

Figure 3.3 The Biorad Western Blotting Tank



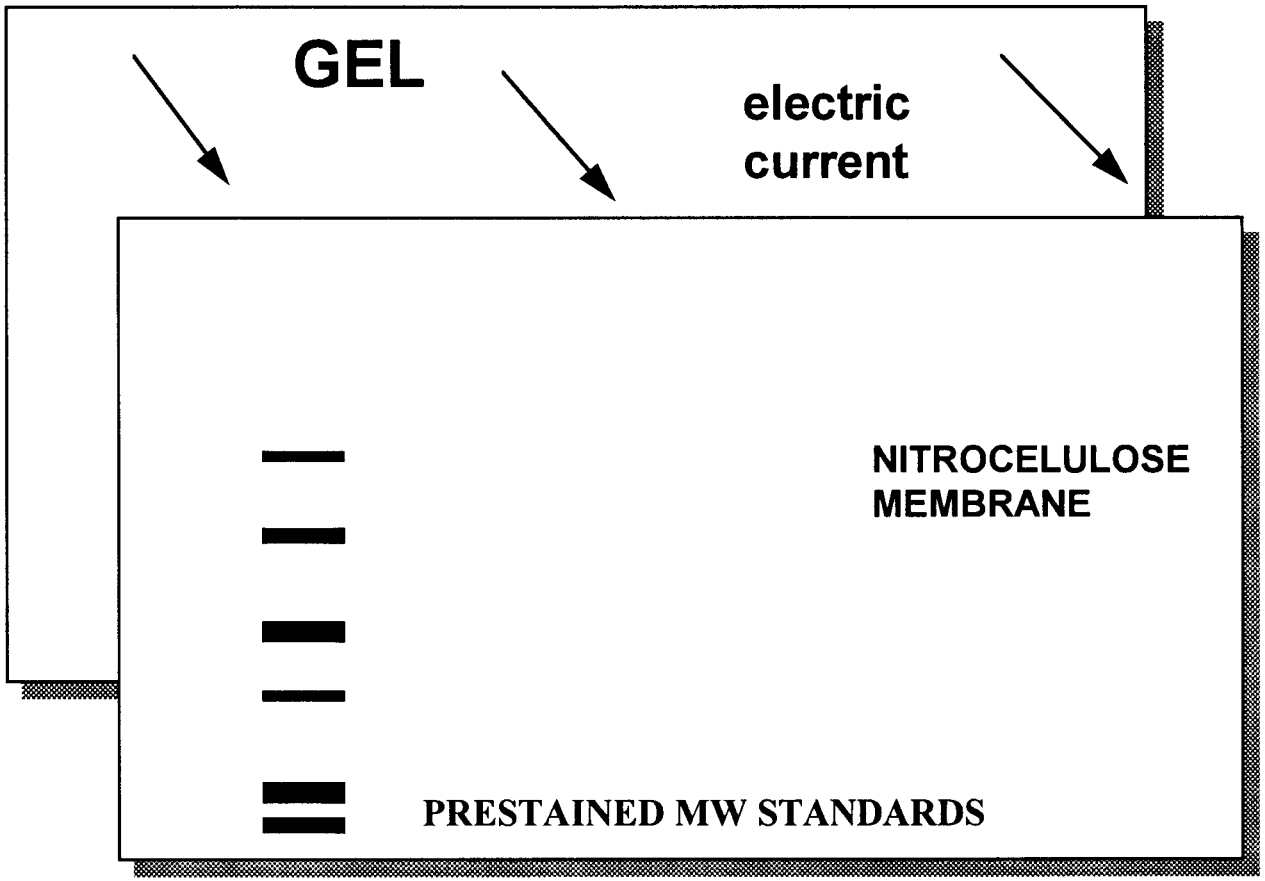



*Figure 3.4* Western Blotting:Transfer of proteins from gel to nitrocellulose membrane

## **3.326** Immunodetection

After the proteins were transfered (as judged by the transfer of the prestained molecular weight markers), the blot was blocked overnight in 5% non-fat milk. The next day the blot was cut into strips and subjected to immunostaining. The principle is the same as the ELISA method. The strips were incubated in a specially drilled plastic tray (Department of Physiology, University of Birmingham) containing 15 lanes each 1cm in width, 10 cm long and 1cm in depth (Figure 3.5).

Test sera or controls were diluted 1 in 50 in 3% BSA/Tris saline Tween, and incubated overnight with the individual strips.

The following morning, the serum sample was tipped out and the strip was washed 3x with PBS on an automatic rocking platform in 5 minute pulses.

The strips were incubated with conjugate - ( horse radish Peroxidase - antihuman IgG ) 1 in 100 dilution in 3% BSA /Tris saline-Tween for 3 hours.

Washing was carried out as described above. The Substrate - AEC (brick red colour) was added and bands, if present, would usually appear in 5 minutes. The reaction would be stopped with distilled water after a maximum of 20 minutes.

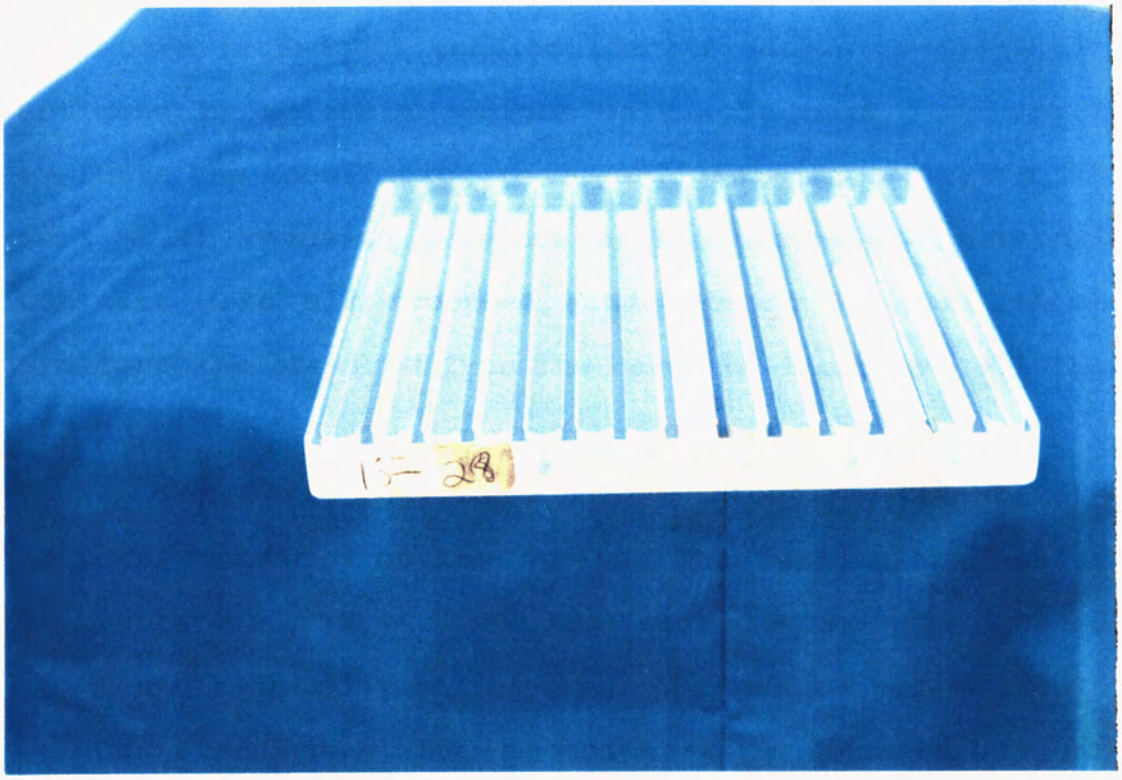


Figure 3.5 The nitrocellulose strip incubator tray

### **3.327** Specificity of antigenic determinants

The above techniques enabled identification of particular antigens by molecular weight only. The next step was to evaluate the specificity of the antigenic determinants. The sera which revealed evidence of antibodies to neutrophil sonicate were all recorded, and batched together by the similarities obtained from the bands on the nitrocellulose strips.

These sera were tested again to confirm the banding patterns obtained on the strips. The particular antigens (ie.lactoferrin MW = 77,000 kDa) were then run down the gel at 1 μg/ml in conjunction with the prestained molecular weight standards. The proteins were transfered onto nitrocellulose membrane by Western blotting, blocked with milk and cut into strips, as described above.

These strips were incubated with IgG (H + L) sheep anti-lactoferrin antibodies, followed by washing x3 with PBS, followed by addition of IgG (H+L) pig anti-sheep HRP antibody. The Substrate AEC was added to obtain the colour precipitate. The antigens determined from neutrophil sonicate by molecular weight were compared, and confirmed to be particular neutrophil enzymes ie. lactoferrin [Peen et al, 1993].

Sheep anti-cathepsin G antibody (H + L), anti-enolase (H + L), anti-elastase (H + L), anti-myeloperoxidase (H + L), and anti-lysozyme were used similarly to confirm presence of the detected neutrophil enzymes (antigens).

Summary illustrating the two procedures

1.neutrophil sonicate (containing antigen) on nitrocellulose membrane

test or control serum

HRP conjugated sheep antihuman IgG

AEC (red precipitate)

2.pure antigen (ie lactoferrin)

sheep anti-lactoferrin antibody

HRP conjugated pig anti-sheep IgG

AEC (red precipitate)

All the antibodies described in this chapter, were kindly donated from The Binding Site (BDH), Birmingham.

## 3.4 Statistics

Comparisons of the prevalence and titres of ANCA in different subjects were made using the Fisher's exact test, Chi squared analysis and student's t-test. The Wilcoxon - Rank test was used to determine statistical significance of the optical density values for the neutrophil purified antigen ELISA. Optical density values, greater than the 95th percentile above the control mean, were taken as significantly different.

## 3.5 Results

In total, there were 71 out of 101 (70.2 %) patients with **P-ANCA** detected by indirect immunofluorescence. None of **ANCA** negative sera gave a positive value using the ELISA method.

The optical density values obtained for the individual commercially obtained antigens are shown in Figures **3.6 - 3.12**. Tables **3.3** and **3.4** show a quantitative figure for the antigens using the 95th percentile of healthy controls as a cut-off.

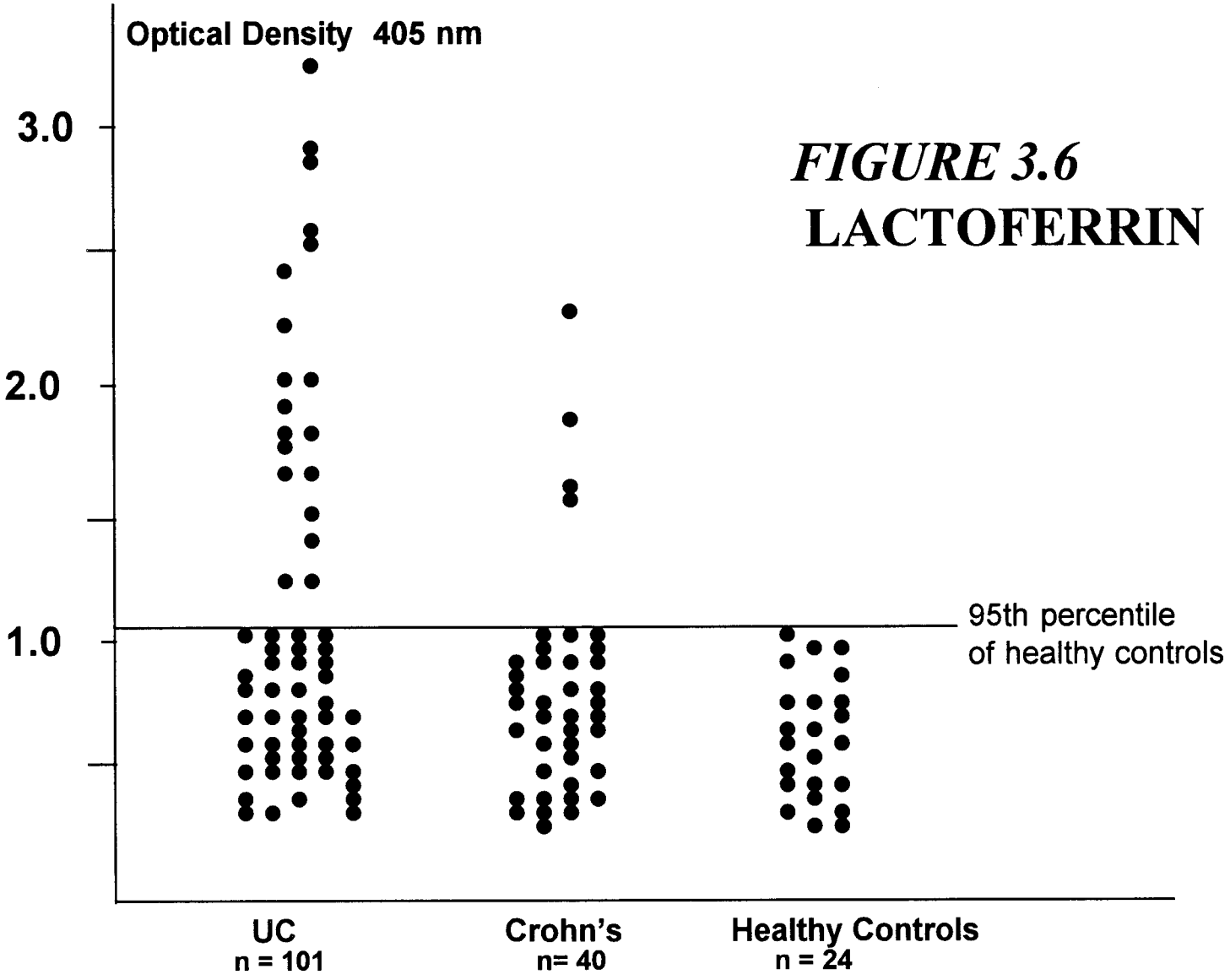



*FIGURE 3.6*
**LACTOFERRIN**

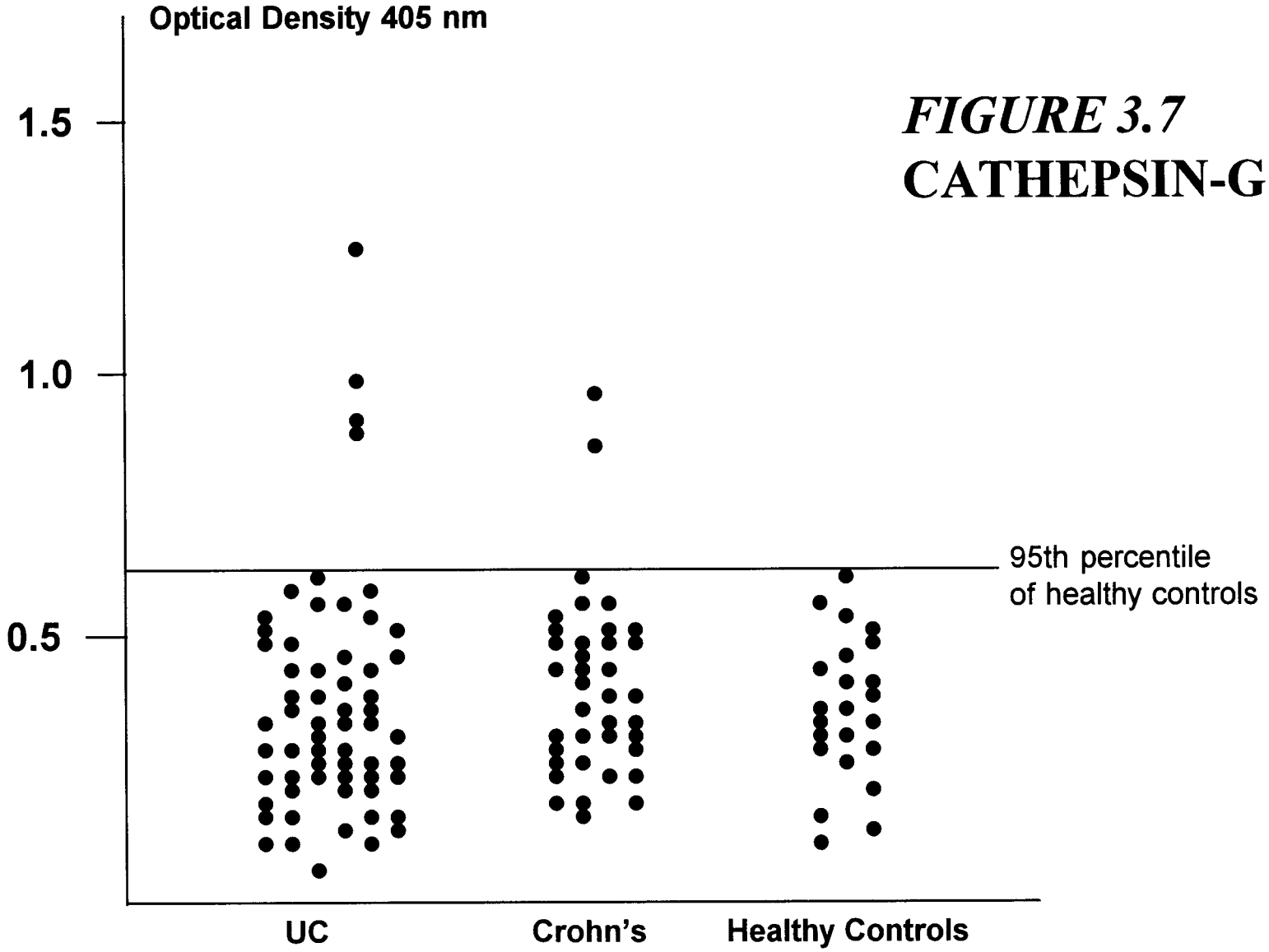



***FIGURE 3.7***
**CATHEPSIN-G**

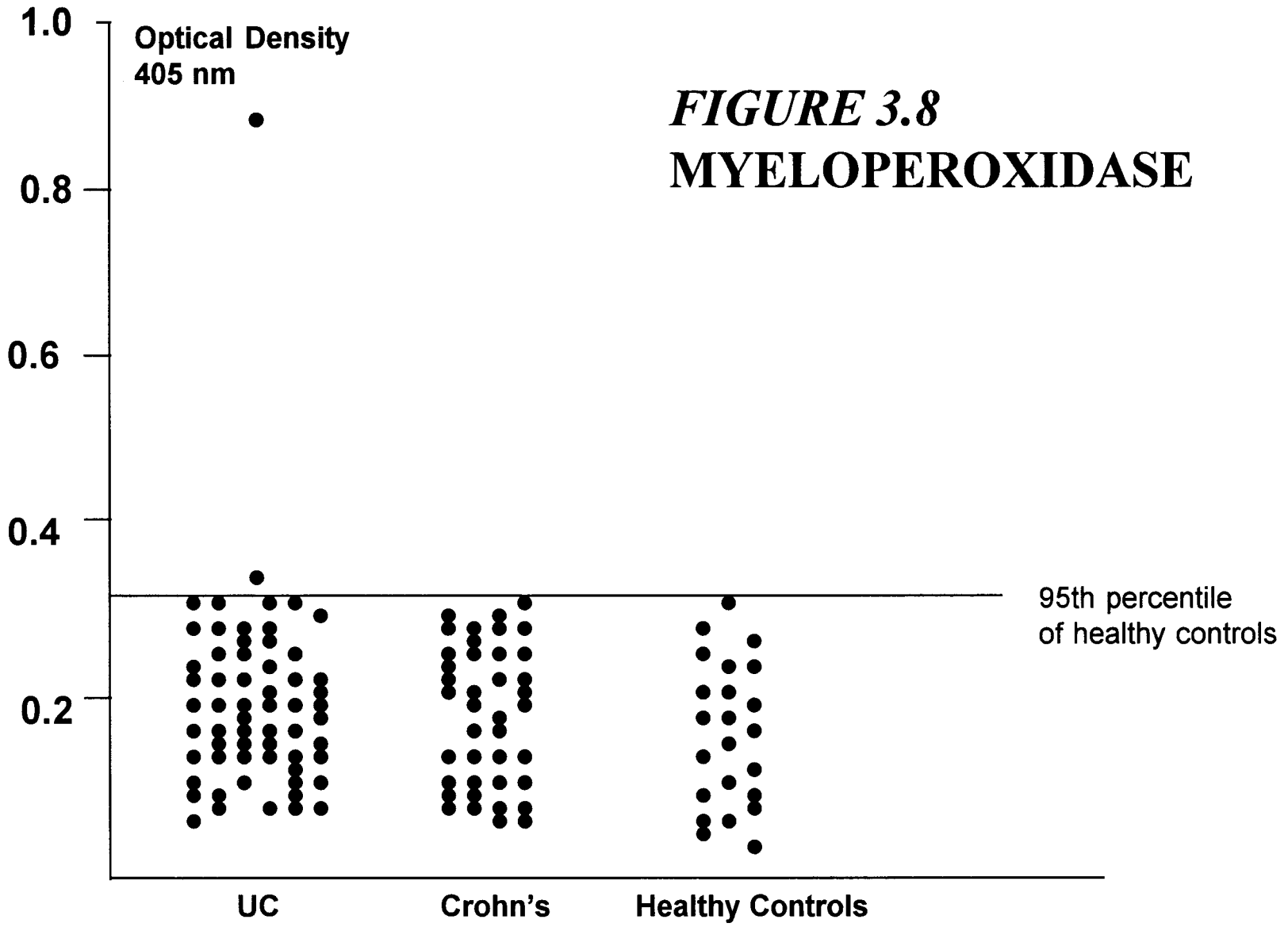



*FIGURE 3.8*
**MYELOPEROXIDASE**

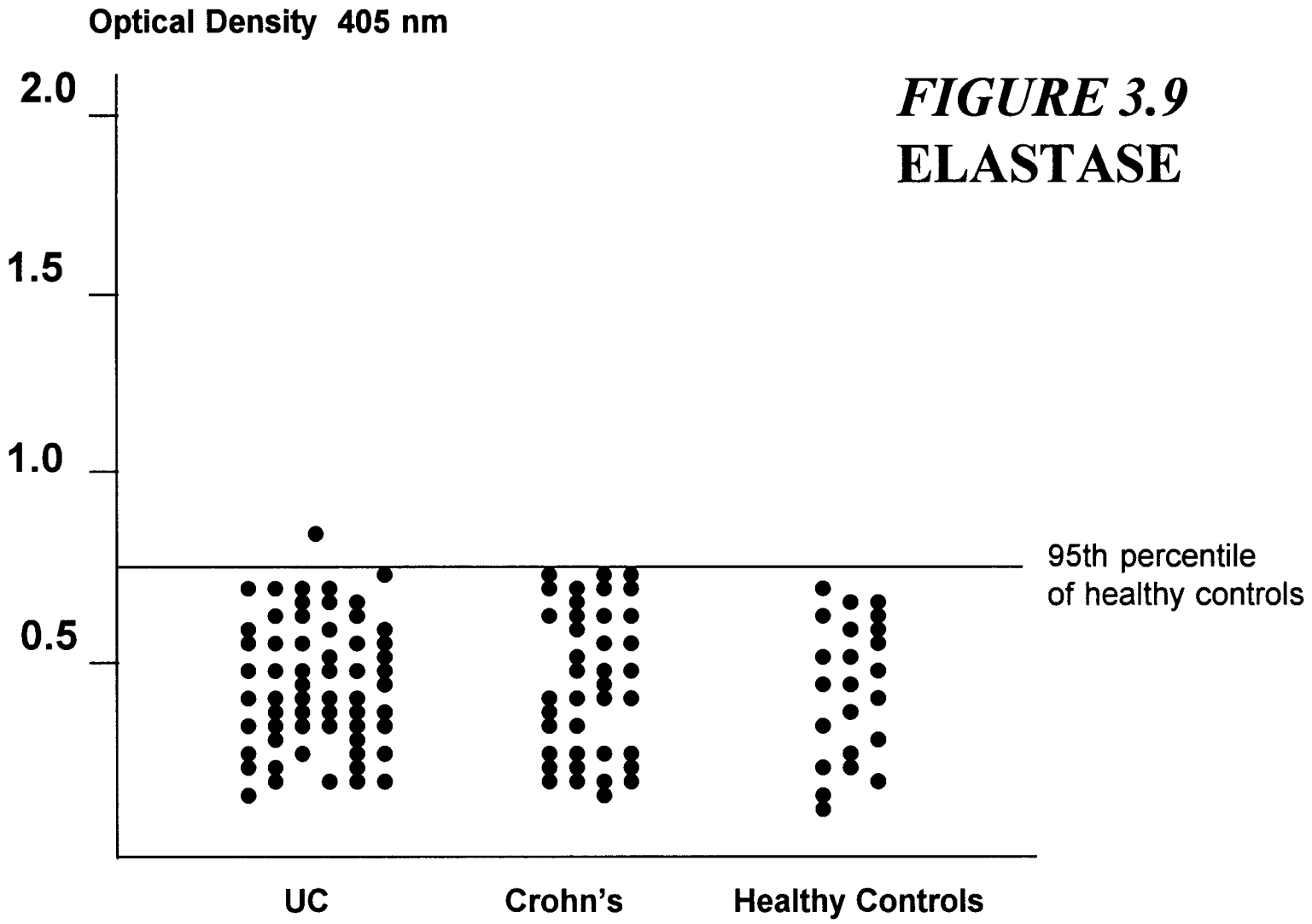



***FIGURE 3.9***
**ELASTASE**

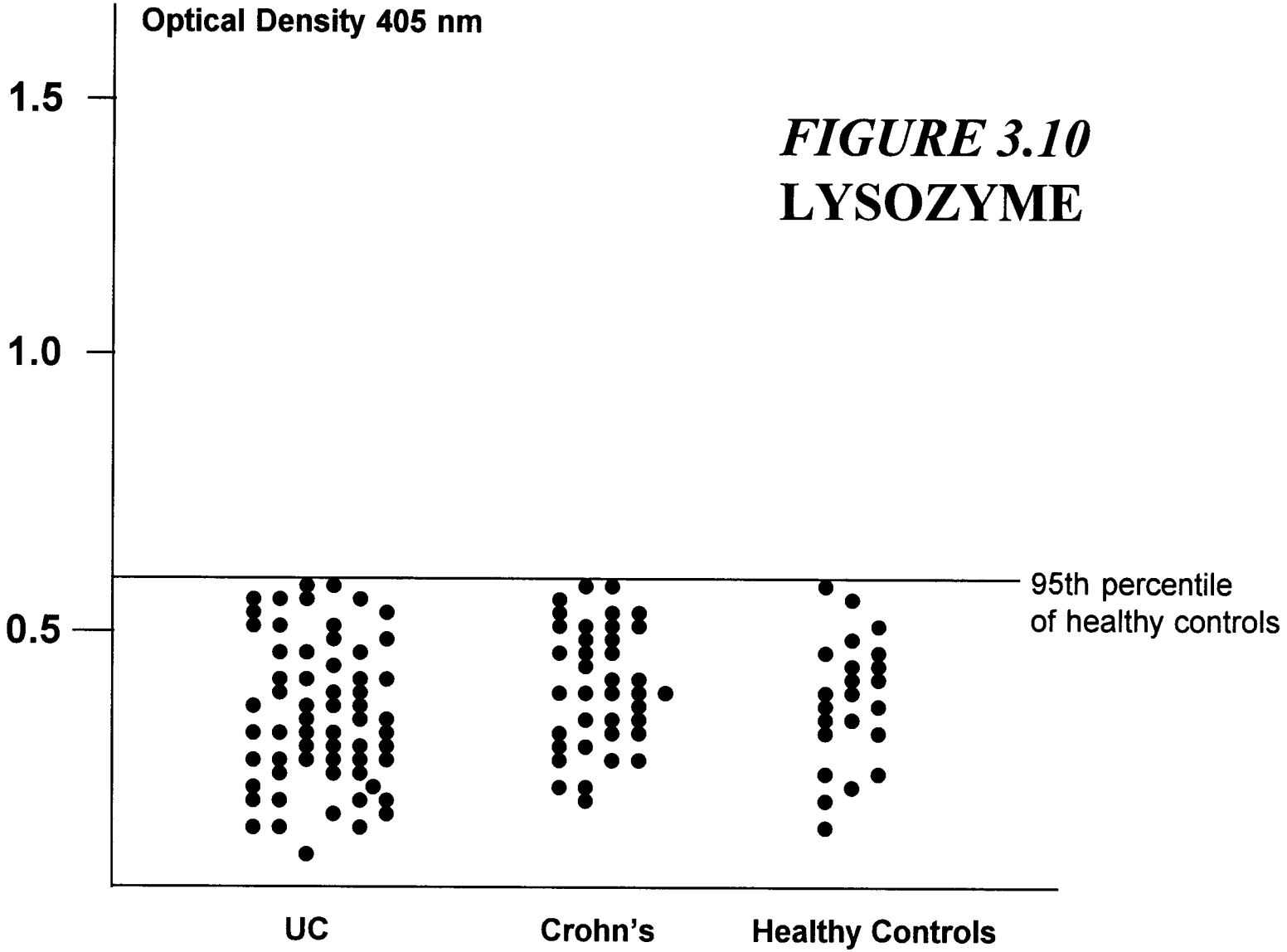



***FIGURE 3.10***
**LYSOZYME**

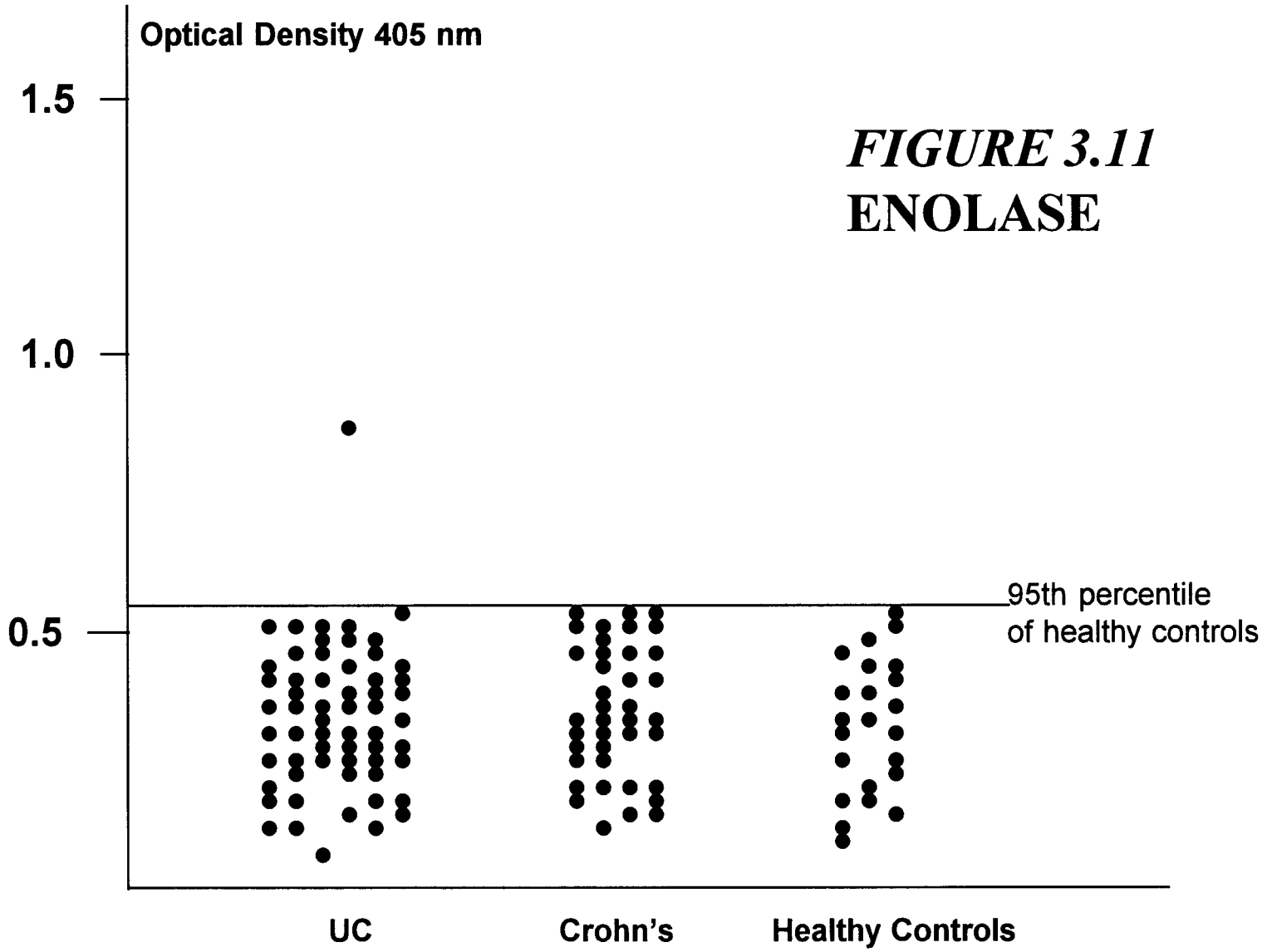



*FIGURE 3.11*
**ENOLASE**

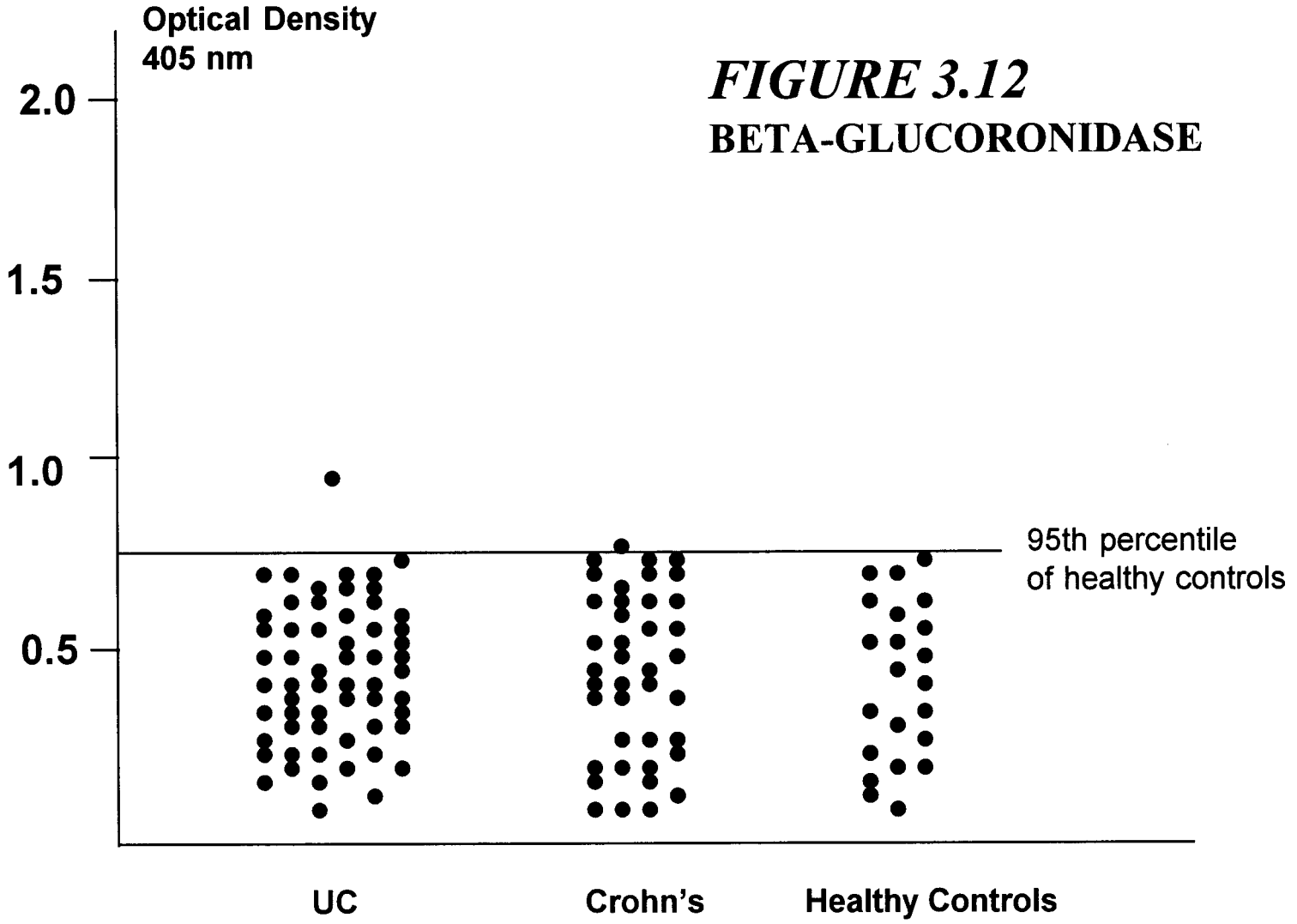



*FIGURE 3.12*

**BETA-GLUCORONIDASE**

Ulcerative colitis

Of the 71 patients with P-ANCA, 21 antigenic bands, ranging from 23-95 kDa, precipitated on the nitrocellulose strips. Only 18 antigenic bands (Figure 3.13) could be matched corresponding to known neutrophil antigens in UC. In 3 sera antigens between 60-65 kDa could not be matched on any known neutrophil enzyme.

Table 3.3 shows antigens detected by Western Blot and ELISA in UC.

| Antibody | Western Blot/ immunodetection | | ELISA | |
|---|---|---|---|---|
| anti-lactoferrin | 15/71 | 21.1% | 19/71 | 26.7% |
| anti-cathepsin G | 2/71 | 2.8% | 4/71 | 5.6% |
| anti-myeloperoxidase | 0/71 | 0.0% | 2/71 | 2.8% |
| anti-elastase | 0/71 | 0.0% | 1/71 | 1.4% |
| anti-enolase | 1/71 | 1.4% | 1/71 | 1.4% |
| anti-lysozyme | 0/71 | 0.0% | 0/71 | 0.0% |
| anti-ß-glucoronidase | 0/71 | 0.0% | 0/71 | 0.0% |
| Total | 18/71 | 25.3% | 27/71 | 38.0% |

Crohn's **disease**

In total, there were 10 out of 40 (25.0 %) patients with P-ANCA detected by indirect immunofluorescence. Of these 10 patients with P-ANCA, 5 antigenic bands were detected:- 3 77 kDa (lactoferrin), one 40kDa and the other 55 kDa. The 40kDa and 55kDa antigens could not be matched to any neutrophil a-granule enzymes.

The following matched antibodies which were detected are shown in the following table:-

Table 3.4 Western Blot and ELISA detection of ANCA antigens in Crohn's disease

| Antibody | Western Blot/ immunodetection | ELISA |
|---|---|---|
| anti-lactoferrin | 3/10 30% | 3/10 30% |
| anti-cathepsin G | 0/10 0% | 2/10 20% |
| anti-myeloperoxidase | 0/10 0% | 0/10 0% |
| anti-elastase | 0/10 0% | 0/10 0% |
| anti-enolase | 0/10 0% | 0/10 0% |
| anti-lysozyme | 0/10 0% | 0/10 0% |
| anti-ß-glucoronidase | 0/10 0% | 0/10 0% |
| Total | 3/10 10% | 5/10 50% |

In both UC and Crohn's bands were not detected in any of

the P-ANCA negative patients and only one band was detected per patient sera.

In essence, from this study the major antibody system which has emerged in UC and Crohn's disease is anti-lactoferrin antibody, and, to a lesser extent, anti-cathepsin G antibody (Figure **3.13)**.

## **3.6** Discussion

In this study, I have established, with the help of clinical and technical staff from the Depts of Physiology, Infection, Immunology and Surgery, the occurence of ANCA directed against lactoferrin and cathepsin G in inflammatory bowel disease.

Several different types of autoantibodies have been described in inflammatory bowel disease [Nielsen et al, **1983**; Snook et al, **1989**; Saxon et al, **1990**; Duerr et al, **1991**; Broberger et al, **1959**; Perlmann et al, **1967**; Das et al, **1984**; Skogh et al, **1982**; Skogh et al, **1986**; Chapman et al, **1986**; Snook et al, **1991]**. Although none of the autoantibodies have been proved to be of pathogenic significance, it has been shown in both ulcerative colitis and Crohn's, that IgG and complement can be present on the apical surface of enterocytes, in vivo [Green et al, **1975**; Hallstensen et al, **1990]**, possibly indicating a pathogenic role for **anti-epithelial/antibrush** border antibodies.

In this connection, and considering the high frequency of anti-lactoferrin antibodies found in the present study, it

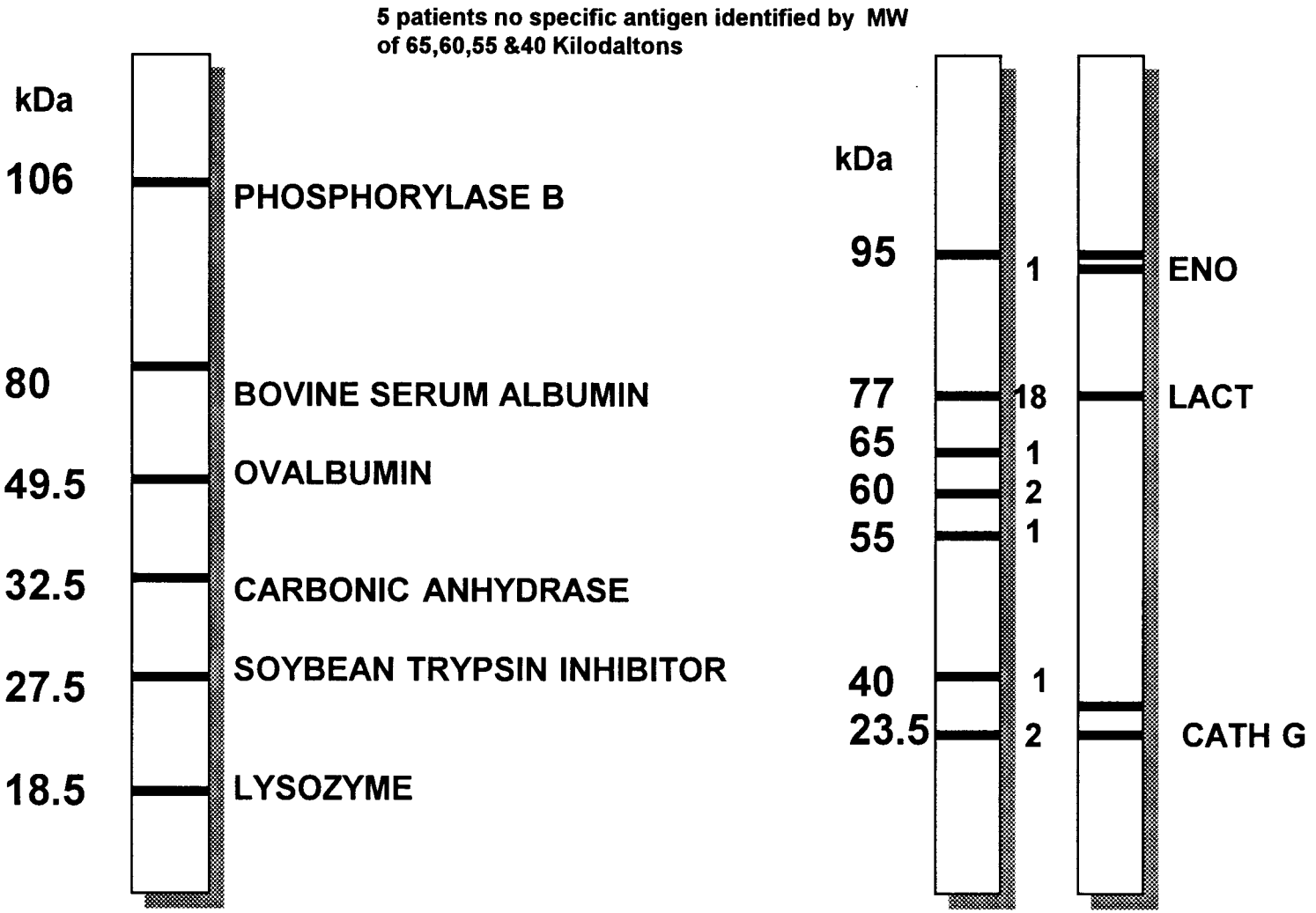



Figure 3.13 Summary of results by molecular weight using immunodetection on nitrocellulose strips

is interesting to note that lactoferrin has been reported to bind the intestinal brush border via a specific receptor [Davidsson et al, **1988]**.

It is possible that many of the anti-microbial antibodies described in inflammatory bowel disease and other inflammatory disease states, may, in fact, be reflections of immunisation against microbial heat shock proteins. These have been implicated in the pathogenesis of several autoimmune disease states [Winfield et al, **1991],** and may induce anti-lactoferrin antibodies [Perlmann et al, **1967].**

## Lactoferrin

Lactoferrin is an iron binding protein that occurs abundantly, not only in the specific granules of granulocytes [Furmanski et al, **1990],** but also in tears, milk and secretions at mucosal surfaces [Wang et al, 1984; Magnuson et al, **1990**; Kuizenga et al, **1987].** Raised levels of circulating lactoferrin are seen during active inflammatory bowel disease [Adeyami et al, **1990].** It exerts antibacterial effects by depriving bacteria of iron required for growth, and it has anti-inflammatory properties - for example, by preventing complement activation through inhibition of classical C3 convertase [Kievits et al, **1985].** Lactoferrin can also prevent the formation of hydroxyl ions by iron binding [Kuizenga et al, **1987].** Lactoferrin may thus be of great importance as a non-specfic anti-phlogistic defence factor at the primary

immunological barriers. It has been shown experimentally that binding of lactoferrin to anti-lactoferrin antibodies increases the amount and duration of hydroxyl radical formation by granulocytes [Britgan et al, 1989].

Hypothetically, antilactoferrin autoantibodies could, by counteracting the anti-inflammatory effects of lactoferrin, aggravate and prolong inflammation induced by several different mechanisms, and the antibodies, may, therefore have pathogenic significance, even though their occurence does not correlate with disease activity. Anti-lactoferrin antibodies may also have pathogenic effects by activation of primed granulocytes infiltrating the gut mucosa or adhering to vessel walls, in analogy with the other types of ANCA [Skogh et al, 1991; Charles et al, 1991; Falk et al, 1990b].

In addition, mucosal **lactoferrin/anti-lactoferrin** complexes may stimulate intestinal goblet cells to excessive mucus sedcretion, as do other immune complexes [Walker et al, 1977].

On the other hand, the occurence of anti-lactoferrin antibodies could be an epiphenomenon, but not one which is based solely on colonic inflammation, since ANCA do not occur in other diarrhoea1 illnesses, where there is colonic inflammation [Duerr et al, 1991]. The fact that the individual types of ANCA were found only in a minority (< 30%) of sera tested is an argument in favour of this. Alternatively, consumption of circulating antibodies cannot

be ruled out in the seronegative instances.

The origin of anti-lactoferrin autoantibodies is unknown. Although bovine and human lactoferrin have molecular and antigenic similarities [Magnuson et al, 1990], immunisation of experimental animals with bovine lactoferrin seldom seems to result in cross immunisation against human lactoferrin [Wang et al, 1984].

The lack of correlation between levels of anti-human lactoferrin and anti-bovine lactoferrin [Peen et al, 1993] contradicts the hypothesis that cross immunisation against dietary bovine lactoferrin results in the appearance of anti-human lactoferrin autoantibodies.

A point worth considering is the antigenic mimicry between the 65 kDa mycobacterial heat shock protein (HSP-65) and human lactoferrin, and the fact that immunisation against HSP-65 results in the production of anti-lactoferrin antibodies [Winfield et al, 1991]. Mycobacterial infection and immunisation against mycobacterial antigens/HSP-65 have been implicated in several autoimmune disease states, both experimental and clinical [Esaguy et al, 1991; Barnes et al, 1990; Lydyard et al, 1991; Shoenfield et al, 1988; Hampson et al, 1988]. It is also interesting to note that there have been reports of anti-HSP-65 antibodies in Crohn’s disease [Walker et al, 1977].

**ANCA** directed against Cathepsin G, myeloperoxidase, enolase and elastase.

Cathepsin G is a chymotrypsin like protease found in the primary granules of neutrophils. Antibodies to Cathepsin G have been reported in both ulcerative colitis and Crohn's disease [Halbwachs et al, **1992]** however the frequencies reported vary a great deal. In our study Cathepsin G was detected in only **4/101** patients **(4%)** with UC and **2/40 (5%)** with Crohn's disease.

Antibodies to other antigens were detected only in ulcerative colitis sera **(1/101** had anti-MPO, **1/101** had anti-elastase, **1/101** had anti-enolase)**.** This multispecificity of ANCA in inflammatory bowel disease, which has been shown by Western blotting suggests further immunological differences within the same disease and perhaps, the time has now come to grade these diseases not just clinically, but also immunologically. Only then shall we be able to classify comprehensively the symptom complex which has been termed chronic inflammatory bowel disease.

## 3.7 Summary

The antigens on the neutrophil which correspond to ANCA in inflammatory bowel disease have yet to be fully identified. In systemic vasculitis these antigens have been found to be enzymes in the a-granulesof the neutrophils.

The aims of this study were to determine the specificity of ANCA using **1)** purified neutrophil antigens (enzymes) 2) using sonicated neutrophil protein.

Methods: **1)** Immunoplates were coated with commercially obtained purified neutrophil enzymes: lactoferrin, cathepsin G, myeloperoxidase, elastase, enolase, lysozyme and ß glucoronidase. Test and control sera were added followed by alkaline phosphatase conjugated anti-human IgG and Substrate. The plates were read at 492 nm and results plotted. Values above the 95th percentile of normal controls were regarded as positive. 2) Sonicated neutrophil protein was applied to a SDS-PAGE column. The separated proteins were then tansferred to nitrocellulose membrane using Western Blotting. The membrane was cut into strips and diluted sera added, followed by HRP-conjugated anti-human IgG. AEC (brick red) was used as the Substrate. The antigens were determined using molecular weight corresponding to the markers on the membrane.

**i)Table** 3.5. **Using commercially purified neutrophil antigens the following results were obtained.**

**ii)Table 3.6. Using neutrophil sonicate the following results were obtained.**

Table 3.5 Antigen determination using ELISA in IBD

| Antibody | ELISA | ELISA |
|---|---|---|
| anti-lactoferrin | 19/71 26.7% | 3/10 30% |
| anti-cathepsin G | 4/71 5.6% | 2/10 20% |
| anti-myeloperoxidase | 2/71 2.8% | 0/10 0% |
| anti-elastase | 1/71 1.4% | 0/10 0% |
| anti-enolase | 1/71 1.4% | 0/10 0% |
| anti-lysozyme | 0/71 0.0% | 0/10 0% |
| anti-ß-glucoronidase | 0/71 0.0% | 0/10 0% |
| Total | 27/71 38.0% | 5/10 50% |

Table 3.6 Antigen determination using neutrophil sonicate in IBD

| Antibody | Western Blot & immunodetection | |
|---|---|---|
| anti-lactoferrin | 15/71 21.1% | 3/10 30% |
| anti-cathepsin G | 2/71 2.8% | 0/10 0% |
| anti-myeloperoxidase | 0/71 0.0% | 0/10 0% |
| anti-elastase | 0/71 0.0% | 0/10 0% |
| anti-enolase | 1/71 1.4% | 0/10 0% |
| anti-lysozyme | 0/71 0.0% | 0/10 0% |
| anti-ß-glucoronidase | 0/71 0.0% | 0/10 0% |
| Total | 21/71 25.3% | 3/10 10% |

Elisa was a more sensitive method for the detection of neutrophil antigens in IBD ANCA positive sera. IBD ANCA positive sera recognize different antigens which appear to be distinct from the commonly found antigens in systemic vasculitis namely proteinase 3 and myeloperoxidase.

Further studies using purified neutrophil enzymes may help to elucidate the unidentified antigens in ANCA positive sera.

# Chapter 4

# Target antigen identification of ANCA using confocal scanning laser microscopy

## 4.1 Introduction

The autoantigen(s)of ANCA in inflammatory bowel disease have yet to be fully identified. There have been two reports to date stating that lactoferrin [Peen et al, 1993] and Cathepsin G [Halbwachs et al, 1992] are the main target antigens, but these have been conflicting. However, both agree in the discussion parts of their papers that several other, as yet, unidentified antigens exist.

It is now 4 years since ANCA were first described in IBD [Saxon et al, 1990] and yet, the target antigens have not been found. This prompted me to look back to see whether I could identify the exact anatomical and morphological site of ANCA binding on the neutrophil.

I initially set out considering electron microscopy, but, was advised that this could not identify the fluorescence markers specifically. The light microscope did not provide enough resolution and therefore, I used confocal scanning laser microscopy.

### *4.11 Principles of confocal scanning laser microscopy*

An ordinary light microscope presents the operator with a 2-D image, consisting of an in-focus and an out-of-focus region of the specimen. In order to obtain greater resolution (3-D) of the specimen, several photographs have to be taken at different focus settings and at different levels of section.

This process is time consuming and has several technical

difficulties. A promising, relatively new method for 3-D studies is confocal microscopy [Carlsson et al, **1987]**.

The basic principle is that the specimen is illuminated one point at a time, and the detector only registers light emanating from the focussed illuminated point (Figure **4.1)**. Simultaneous viewing of a large number of points in this way enables the operator to view a section in real time, through the eyepieces of the microscope. A computer is used in conjunction, to process digitized images in such a way that out of focus information is filtered out [Agard et al, 1983; Erhardt et al, **1985]**.

The point illumination and point detection scheme results in imaging properties that are different from those of an ordinary light microscope. One such difference is that the resolution is improved and another is that a very pronounced depth discrimination is obtained. This means that different depth layers can be studied much more clearly, since no out of focus information is superimposed on the image.

Thus, this technique of recording the entire structure of the fixed neutrophil after application of ANCA with an immunofluorescence tag, refocusing the microscope between successful images, would allow a 3-D image of high resolution to be created.

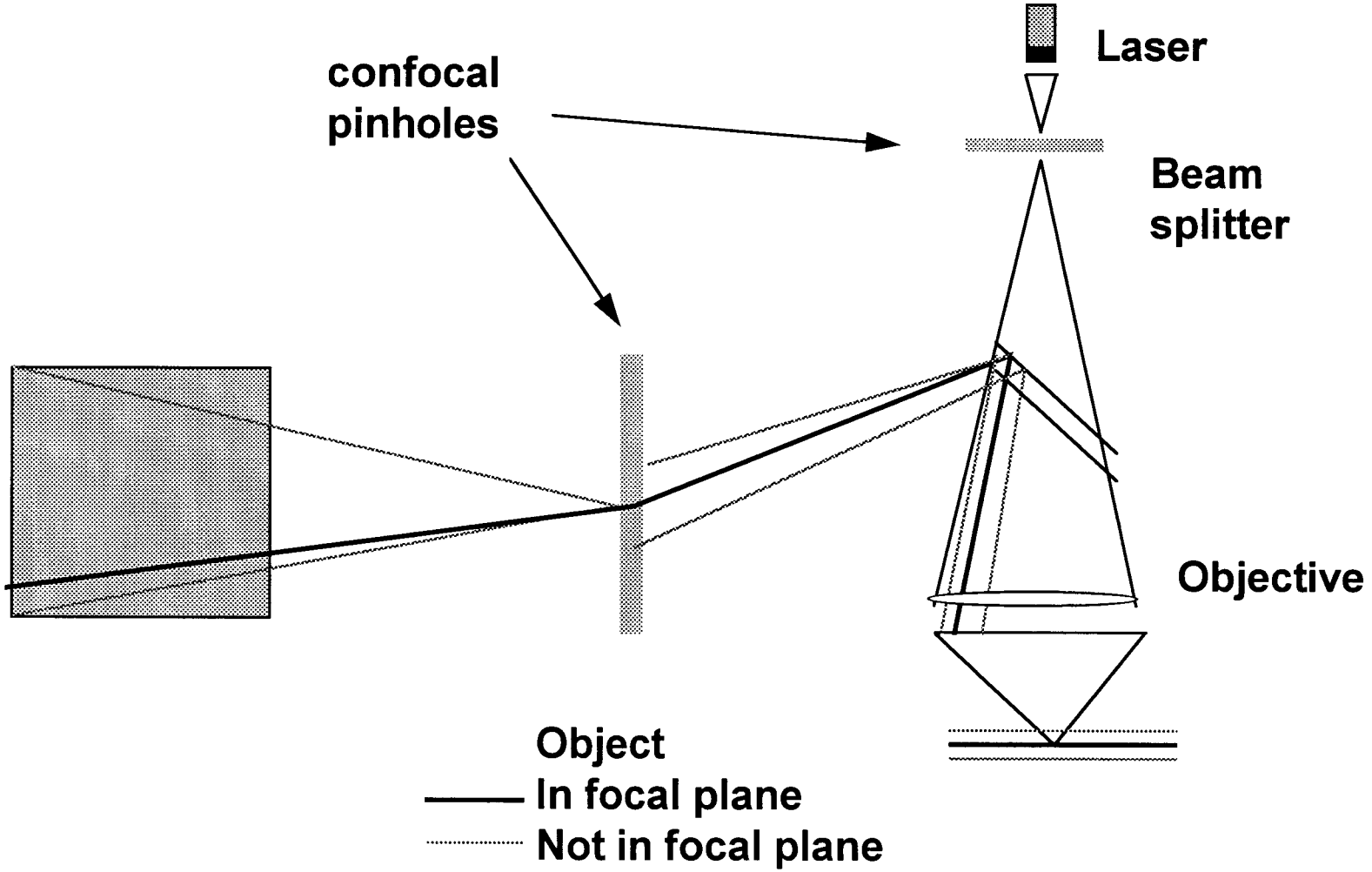





**Figure 4.1 Schematic of the confocal microscope principle; the pinhole is scanned across the objective focal plane to build the image**

## 4.2 Aims

The aims of this study were to determine the target site of ANCA ie. the exact morphological site of antigen-antibody binding which gives rise to the P-ANCA immunofluorescence patterns in IBD using confocal scanning laser microscopy.

## 4.3 Patients and methods

Sera from 125 patients (101 with UC and 24 healthy controls) were tested for the presence of ANCA, using the method of indirect immunofluorescence, as described in chapter 2. The disease activity of the patients with ulcerative colitis was assessed in a modified form, as described in chapter 2 [Kodner et al, 1990]. However, on this occasion the neutrophil nuclei were further stained with ethidium bromide. Ethidium bromide is a nucleus specific dye.

Table 4.1 Distribution of UC patient sera.

| Disease | Number |
|---|---|
| Mild UC | 15 |
| Moderate UC | 13 |
| Severe UC | 15 |
| Subtotal Colectomy for UC | 19 |
| Restorative proctocolectomy for UC | 39 |
| Healthy controls | 24 |
| Total | 125 |

# Method of indirect immunofluorescence:-

*HEALTHY NEUTROPHILS ISOLATED & FIXED WITH ETHANOL OR FORMALDEHYDE ONTO GLASS SLIDES*

*SLIDES INCUBATED WITH TEST OR CONTROL SERA **(1** IN **20)** FOR 30 MINUTES*

*WASHING*

*INCUBATION WITH FITC CONJUGATED ANTI-HUMAN IgG FOR **20** MINUTES*

*WASHING*

*INCUBATION WITH ETHIDIUM BROMIDE **(1** IN **10)***

*WASHING*

*SLIDES FIXED WITH DABCO*

*READ ON LEITZ ORTHOPLAN IMMUNOFLUORESCENCE MICROSCOPE BY MYSELF AND RON STOKES (IMMUNOLOGIST)*

**The slides with the brightest immunofluorescence patterns of P-ANCA and C-ANCA were read on the confocal laser microscope with the help of Gerald Johnson, Department of Immunology, University of Birmingham. The principle has been described in Figure 4.1.**

## 4.4 Results

The **P-ANCA** and **C-ANCA** patterns were indistinguishable on slides in which the neutrophils were fixed with formaldehdye, thus confirming previous studies which showed that these patterns are a result of the way in which neutrophils are fixed [Falk et al, 1988].

The **P-ANCA** and **C-ANCA** patterns were distinguishable by ethanol fixation of neutrophils, and overall clarity was better. **ANCA** was not detected in any of the healthy controls.

Table 4.2 **ANCA** distribution in patients with ulcerative colitis.

| Disease | Number | P-ANCA | C-ANCA |
|---|---|---|---|
| Mild UC | 15 | 9 | 0 |
| Moderate UC | 13 | 10 | 1 |
| Severe UC | 15 | 11 | 2 |
| Subtotal colectomy | 19 | 12 | 1 |
| Restorative proctocolectomy | 39 | 29 | 1 |
| Total | 101 | 71 | 5 |

### *4.41 3-D imagng of the neutrophil in different vertical planes using confocal imaging*

Figure 4.2 shows the image obtained for the **P-ANCA** pattern on ethanol fixed neutrophils, after staining the nucleus with ethidium bromide, using conventional light microscopy. One can see that it would be very difficult to interprete where exactly the immunofluorescence is emanating ie. from within the nucleus

Figure 4.2 P-ANCA patern after staining neutrophil nucleus with ethidium bromide

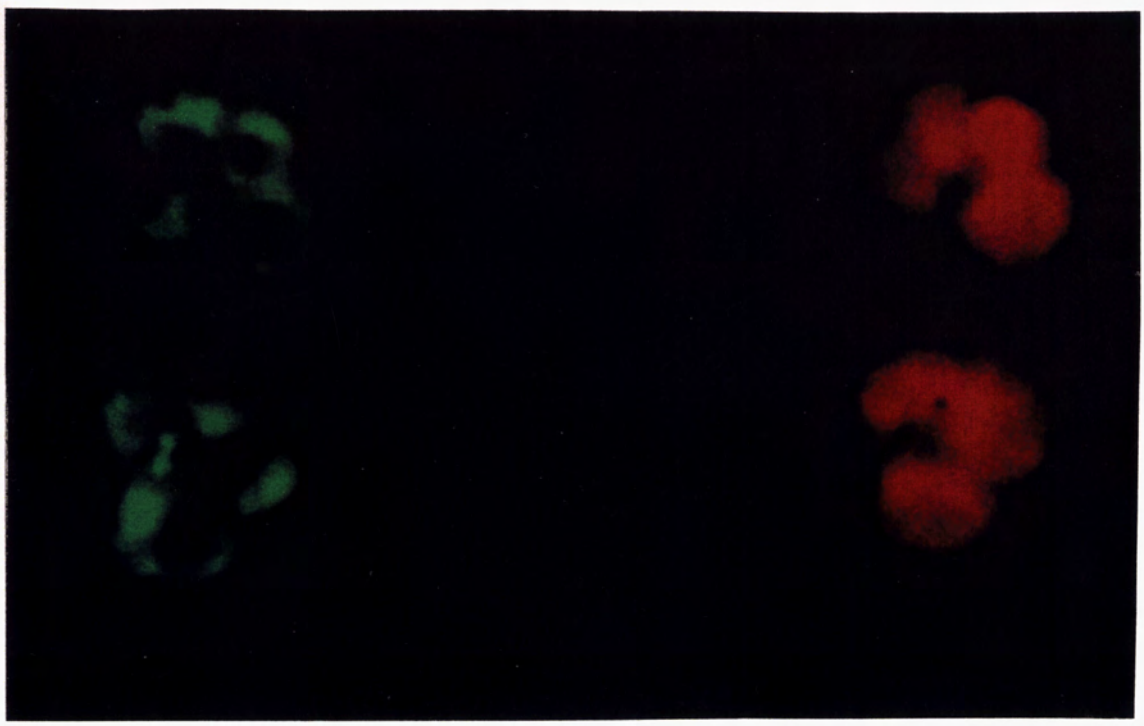


Figure 4.3 Separation of digital images of the red nuclear and green immunofluorescent stains.

or just outside.

Figure 4.3 shows separation of the digital images obtained on the confocal microscope of the nuclear stain from the immunofluorescence stain.

The computer attached to the confocal microscope was used to assess the amount of overlap between the red and the yellow. This, after examination of all the P-ANCA sera, revealed that >95% of the immunofluorescence was in fact intra-nuclear.

Figure 4.4 shows the images obtained when the confocal microscope was focussed from the level of the glass slide vertically upwards at cuts of approximately 0.1 microns. This showed that the maximum area of immunofluorescence emanated from the centre area of the neutrophil nucleus.

Having stored the information on the hard disk of the computer one could then assess the maximum area of immunofluorescence by looking at the neutrophil in cross-section ie., by looking at the spread out neutrophil on the slide from the side :- Figures 4.5 and 4.6. Using this technique again, I was able to determine that the maximal area of immunofluorescence emanated from the nucleus.

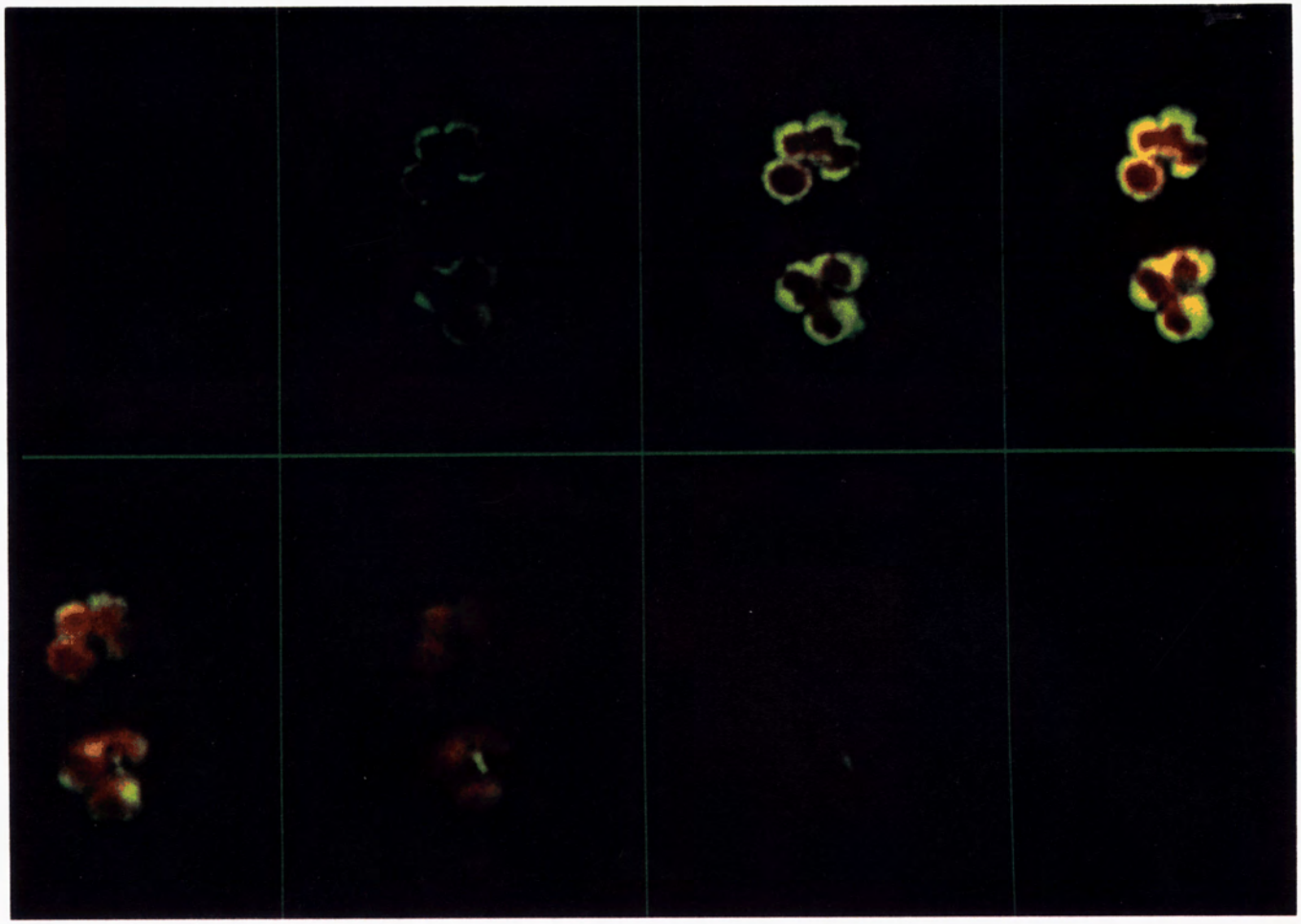


Figure 4.4 3D imaging of the fixed neutrophil in different vertical planes at 0.1 micron cuts

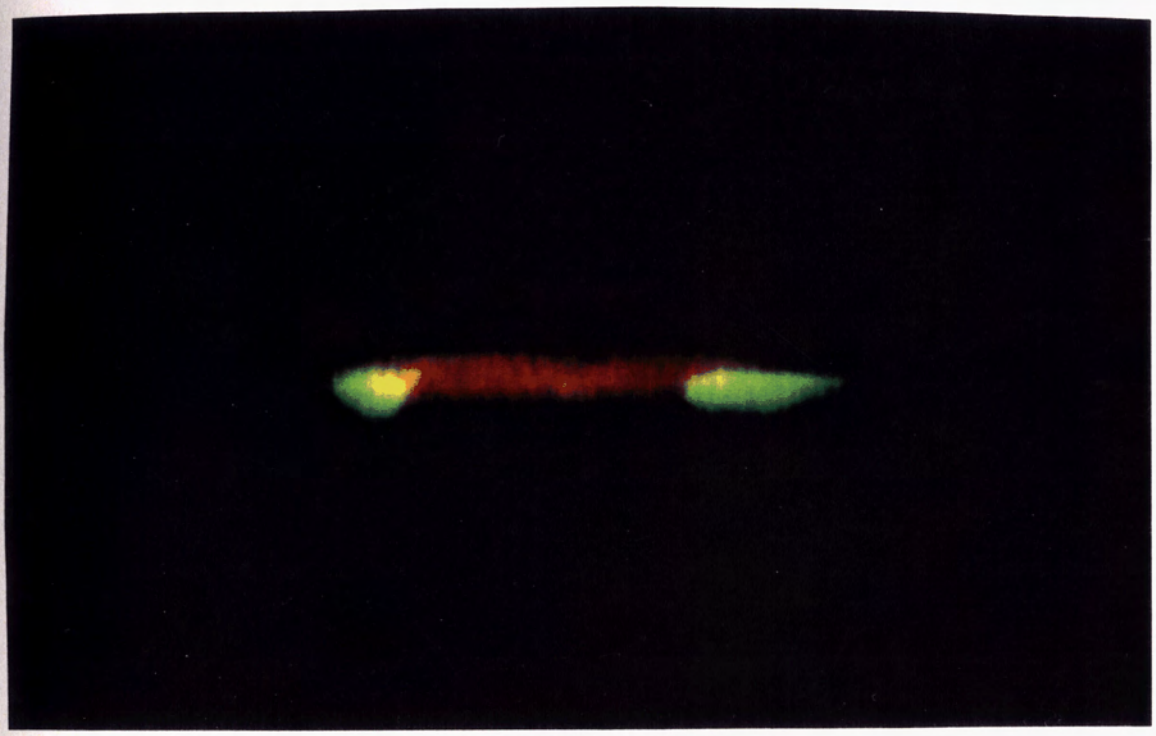


Figure 4.5 Neutrophil in vertical cross-section

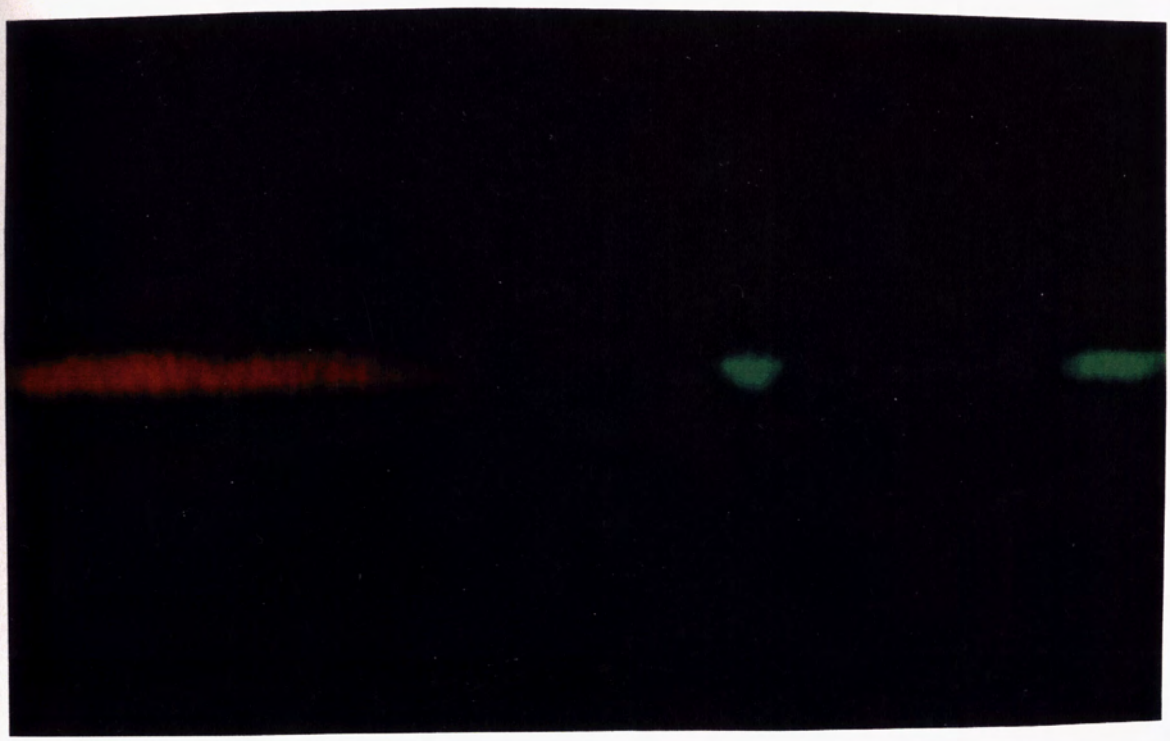


Figure 4.6 Separation of digital images of the red nuclear and green immunofluorescent stains on vertical section

## 4.5 Discussion

The most common and original screening method for detection of ANCA is still the indirect immunofluorescence technique on ethanol fixed human neutrophils [Gross et al, **1990]**. Fixation of the cells by ethanol allows discrimination between the different fluorescence patterns : C-ANCA and P-ANCA. P-ANCA is, indeed, an artefact of alcohol fixation [Falk et al, **1988]**. If fixation is done in formaldehyde, which prevents the translocation of basic (positively charged) proteins to the negatively charged nucleus, it is impossible to discrimminate between C-ANCA and P-ANCA (both exhibit the same cytoplasmic staining pattern). However, formaldehyde fixation enables distinguishing between ANCA and granulocyte specific antinuclear antibodies (GS-ANA). GS-ANA, in contrast to ANCA, exhibit a nuclear fluorescence pattern on formaldehdye fixed neutrophils. Further exclusion of GS-ANA is possible by testing the sera on Hep2 cells {The Binding Site, Birmingham}.

My findings, in this study, that **>95%** of the antibody binding occurs within the nucleus has led to several possibilities:-

a).neutrophil fixation with ethanol draws the antigens into the nucleus whereas formaldehyde fixtion of the neutrophils does not.

b) .Are the antigens drawn to the nucleus other than by a charge mechanism following fixation with alcohol?

c).Are there antigens for ANCA actually within the nucleus ie.histones?

d).Is this just simply an epiphenomenon?

I believe that the most likely reason is that alcohol fixation results in the inward migration of basic proteins by a charge mechanism. [Falk et al, **1988].**

The brightness of immunofluorescence was not related to disease activity in UC on confocal microscopy, and this again raises two important points for discussion:

i). Are ANCA in **IBD** a different group of antibodies entirely since they do not correlate with disease activity as has been shown in patients with, for example, Wegener's granulomatosis [Van der Woude et al, **1985].**

ii).Following total colectomy the titres of ANCA should fall, as the target organ has been removed by colectomy, however, this is not the case. In systemic vasculitis titres of ANCA fall with remission of the disease [Van der Woude et al, **1985;** Rasmussen et al, **1988].** If one is to postulate that ANCA are involved in the persistence of disease activity then the titres should fall with time. However, if ANCA are present, as a result of the loss of immunoregulation at some stage, then one can explore the possibilty of correcting this immunoregulatory defect in a select group of patients who have undergone restorative proctocolectomy, to assess new emerging immunoregulatory agents, such as cytokines or their inhibitors.

ANCA seem to be unique to **IBD** when compared to systemic vasculitis in that:

i) they do not correlate with disease activity

ii)titres do not relate to remission of disease

iii)they have different antigenic determinants

## 4.6 Summary

P-ANCA have been described to occur in UC, but the antigen(s) on the neutrophil have yet to be determined in this disease. The aims of this study were to localise the binding of ANCA onto alcohol fixed neutrophils, using confocal scanning laser microscopy.

A hundred and one sera from patients with UC were tested for ANCA, and of these 71 had P-ANCA. P-ANCA were further stained with ethidium bromide to identify the full extent of the neutrophil nucleus. Reading was then carried out on the confocal laser microscope.

This revealed that in P-ANCA sera the maximum immunofluorescence emanated from within the nucleus (90 %) in all cases which were studied. The immunofluorescence was located around the circumference within the nucleus, and not from the perinuclear area (outside the nucleus), as seen on conventional light microscopy.

These findings seem to suggest that the ANCA antigens are either located within the nucleus, or that the process of alcohol fixation of the neutrophils possibly draws these antigens by a charge mechanism into the nucleus, without causing too much architectural disturbance of the neutrophil.

# Chapter 5

## Anti-endothelial cell antibody in inflammatory bowel disease

## 5.1 Introduction

Anti-endothelial cell antibodies (AECA), were originally implicated as a pathogenic factor in the vascular injury induced in patients with systemic lupus erythematosus [Cines et al, **1984]**. Since then they have been reported to occur in Kawasaki syndrome [Leung et al, **1986**; Leung et al, **1987]**, haemolytic uraemic syndrome [Leung et al, **1988]**, and rheumatoid vasculitis [Heurkens et al, **1989**; Quadros et al, **1990]**. Recently, several studies have reported that sera from **30-60** % of patients, with polyarteritis and Wegener's granulomatosis, contain antibodies to endothelial cells [Ferraro et al, **1990**; Frampton et al, **1990**; Savage et al, **1991]**.

The role of AECA in the vasculitic disorders and mechanisms by which vascular injury may be brought, about are well documented [Cines et al, **1984**; Leung et al, **1986**; Leung et al, **1987**; Leung et al, **1988]**. Although vasculitis has been documented in inflammatory bowel disease, very little is known of the mechanisms involved. [Murch et al, **1992**; Wakefield et al, **1991]**.

Anti-neutrophil cytoplasmic antibodies (ANCA) have been described in many vasculitic disorders and also more recently in inflammatory bowel disease [Saxon et al, **1990**; Rump et al, **1990**; Cambridge et al, **1992**; Siebold et al, **1992]**. In systemic vasculitis ANCA have been postulated to cause vascular injury mediated by respiratory bursts, and degranulation of cytokine primed neutrophils [Falk et al,

1990B]. ANCA have also been shown to not only activate monocytes, but also to play a role in their growth regulation [Ewert et al, 1991]. In chapter 2, I showed that removal of the primary target site (the colon) by total colectomy has no apparent effect on the prevalence of ANCA, suggesting further that it is not just the colon which is targeted immunologically in this disease [Patel et al, 1994].

AECA and ANCA are thought to be two important antibody systems in the systemic vasculitides. AECA have been recently described in IBD [Romas et al, 1992, Stevens et al, 1993] however, relatively little is known of this antibody in this group of diseases.

## 5.2 Aims

The aims of this study were to investigate the role of AECA in inflammatory bowel disease and especially, to assess the effect of total colectomy on the prevalence of the antibody.

NB. This study was started in 1991 before studies of the existance of AECA in IBD were reported [Romas et al, 1992; Stevens et al, 1993].

## 5.3 Patients and methods

Sera from the following group of patients were collected and stored at *-80°C* : ulcerative colitis, n = 41; ulcerative

colitis who had undergone total restorative proctocolectomy, n=30; Crohn's disease, n=30 and 34 healthy controls. The diagnosis in all cases was established by prior clinical and histological features obtained on biopsies, or from gross specimens taken at the time of colectomy. None of the patients had any previous evidence of other vasculitic disorder. The disease activity of patients with ulcerative colitis was assessed using a modified score [Kodner et al, 1990] and in Crohn's disease using the Crohn's disease activity index (CDAI) [Harvey et al, 1980]

### *5.31* Endothelial ***cells***

These were obtained from fresh human umbilical veins by collagenase digestion. Cells between passes two and four were used for the experiments. The cells were coated in flasks precoated with 1% gelatin in M199 + 20 % human serum, 0.02 mmol/l glutamine, 15 mg/ml endothelial cell growth factor, 50 U/ml of heparin, 83 IU/ml penicillin, and 83 µg/ml streptomycin. For the experiments endothelial cells were detached with collegenase (1 mg/ml), seeded in microtitre plates at a concentration of **2** x $10^4$/well in endothelial culture medium containing 20 % FCS in place of human serum and cultured until confluent. The cells were confirmed to be endothelial cells by immunofluorescence staining with antibody to Factor VIII (RAG Dako).

### *5.32 Antibodies to endothelial cells*

These were detected by an ELISA [Hashemi et al **1987]** with minor modifications. Endothelial cells were fixed with **1%** paraformaldehyde for **10** minutes. After blocking for **1** hour in **0.1** M phosphate buffered saline (PBS)/ **2%** bovine serum albumin (BSA) (also used as the diluent for subsequent steps) at **37** ◦ C, sera from patients and controls were added at a dilution of **1/20** and incubated for one hour. After washing, horseradish Peroxidase conjugated sheep anti-human IgG(Fc) was added at a dilution of **1/10,000** followed by o-phenylenediamine in 0.05 M citrate buffer **(2.2** X $10^{-3}$ **M** final concentration) containing **6** X **10** $^{-3}$ % $H_2O_2$**.** The reaction was stopped with **20%** $H_2SO_4$ after **15** minutes, and the optical density (OD) of the wells read at **492** nm.

### *5.33 Inhibition ELISA to test for the specificity of the AECA assay*

Serum taken from a patient with ulcerative colitis, which was positive for AECA, was mixed at a dilution of **1/80** with an endothelial cell sonicate in a concentration range **0.07-2.5** μg/ml, and the AECA assay was performed as described above. The percentage inhibition was derived from the formula:

$$\frac{\text{OD without inhibitor - OD with inhibitor}}{\text{OD without inhibitor}} \times 100$$

## 5.34 *Antineutrophil cytoplasmic antibodies*

ANCA titres were measured by indirect immunofluorescence, as previously described [Lee et al, 1990]. Briefly, neutrophils were isolated on Percolll gradients and the cytocentrifuge preparation was susequently fixed with 95 % ethanol onto slides. The slides were then incubated for 30 mins with test sera diluted 1 in 20 in PBS. After washing incubation was carried out with FITC conjugated anti-human IgG for another 30 mins. After washing the slides were mounted with DABCO (fixing reagent) and examined under the Leitz fluorescence microscope at 50 X magnification. Depending on the brightness of immunofluorescence staining, the reactions were graded into negative (–), weak (+), positive (++), and strong (+++).

### *Anti–MPO antibodies*

Anti-MPO antibodies were detected by an ELISA, as previously described [Lee et al, 1990]. Briefly, Nunc ELISA plates were coated with MPO at a concentration of 10 micrograms/ml in 0.05 M bicarbonate buffer pH 9.6 for 16 hours at 37° C. Sequential incubations of the following were performed for 1 hour at 37° C. 0.1 M PBS/0.05 % Tween 20 (v/v) was used as diluting buffer, and the wells were washed between each incubation with 0.15 M NaCl/0.05%Tween 20 (v/v). (1) serum diluted 1/20 (2) sheep antihuman IgG (Fc) (Binding Site, Birmingham) at a dilution of 1/800

(3) p-nitrophenyl phosphate (1mg/ml)in 10 % diethanolamine pH 9.8. The OD of the wells was read at 405 nm.

### *5.35 Adsorption studies to remove ANCA*

This was done in order to determine whether ANCA and AECA were two separate antibody systems. Human group O +ve neutrophils were isolated and fixed in ethanol onto plates as previously described [Savage et al, **1991].** Samples of serum (100 $\mu$l) were incubated for 60 min with 5 X $10^7$ fixed neutrophils. Supernatants were then harvested and adsorbed against a second batch of cells. Before use, protein concentrations of the samples were adjusted to those of the unadsorbed samples. The samples were then tested for the presence of AECA, as described above.

### 5.36 Statistics

Differences between the various disease groups of patients and healthy controls were analysed by the Wilcoxon rank sum test. The significance of the prevalence of AECA with ANCA was determined using the Mann-Whitney test.

## 5.4 Results

### *5.41 IgG anti-endothelial cell antibodies*

The inter-assay and intra-assay coefficients of variation for the AECA assay was 9.1 % and 4.2 % respectively. IgG

AECA levels in the control sera were not normally distributed, so the 95th percentile of 34 healthy control sera was used to define the upper limit of normal. The distribution of AECA in the various patient groups is shown in Figure 5.1. In ulcerative colitis and Crohn's disease AECA levels did not correlate with disease severity.

In the test sera, AECA were found in 15 out of 41 patients with ulcerative colitis, 12 out of 30 patients who had undergone restorative proctocolectomy and 6 out of 30 patients with Crohn's disease. Overall, 33 patients out of 101 with inflammatory bowel disease had detectable AECA (Table 5.1). AECA was not detected in any of the control sera taken from healthy patients.

Table 5.1. Number of patients with AECA and ANCA in the disease study groups.

| Disease | n | AECA | ANCA |
|---|---|---|---|
| Ulcerative colitis | 41 | 15 | 31 |
| Restorative proctocolectomy | 30 | 12 | 21 |
| Crohn's disease | 30 | 6 | 13 |
| Total | 101 | 33 | 65 |

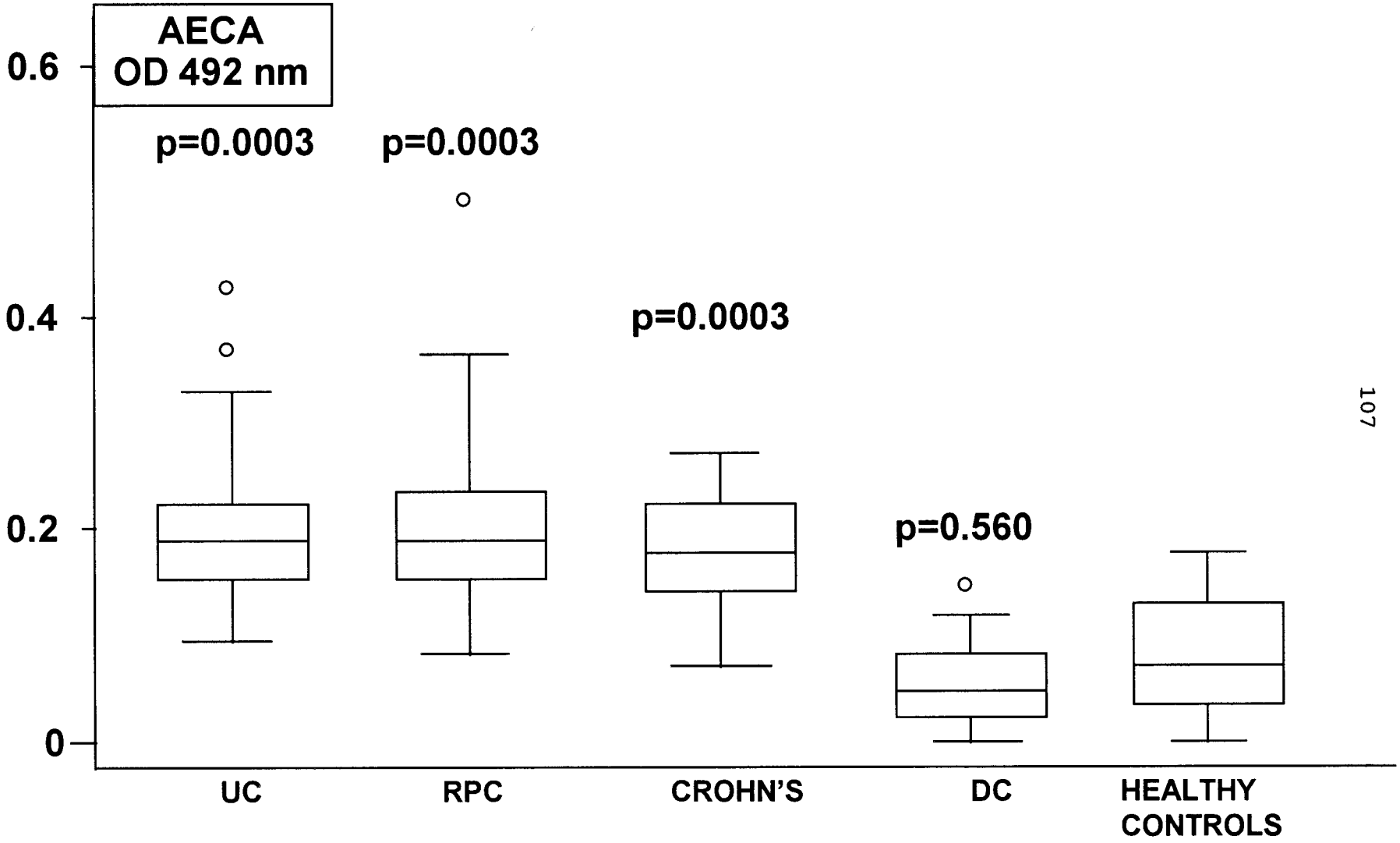





**Figure 5.1** Distribution of Anti-endothelial cell antibody in the various patient groups (RPC=Restorative proctocolectomy; DC=Disease controls)

### *5.42 Inhibition of anti-endothelial cell antibodies with an endothelial cell sonicate.*

The specificity of the AECA assay was assessed by inhibiting the binding of AECA from a patient's serum with an endothelial cell sonicate. Figure 5.2 shows maximum inhibition of 94% of AECA binding by 2.5 μgrams/ml of endothelial cell sonicate.

### *5.43 AECA and ANCA*

Table 5.1 shows the results for the 101 patients with inflammatory bowel disease, tested for ANCA using indirect immunofluorescence in relation to AECA. Table 5.2 shows the various gradations of brightness as seen by indirect immunofluorescence microscopy of positive ANCA sera.

Table 5.2. ANCA titres measured by indirect immunofluorescence.

| Disease | n | - | + | ++ | +++ |
|---|---|---|---|---|---|
| Ulcerative colitis | 41 | 10 | 13 | 10 | 8 |
| Restorative proctocolectomy | 30 | 9 | 13 | 5 | 3 |
| Crohn's disease | 30 | 17 | 7 | 4 | 2 |
| Total | 101 | 36 | 33 | 19 | 13 |

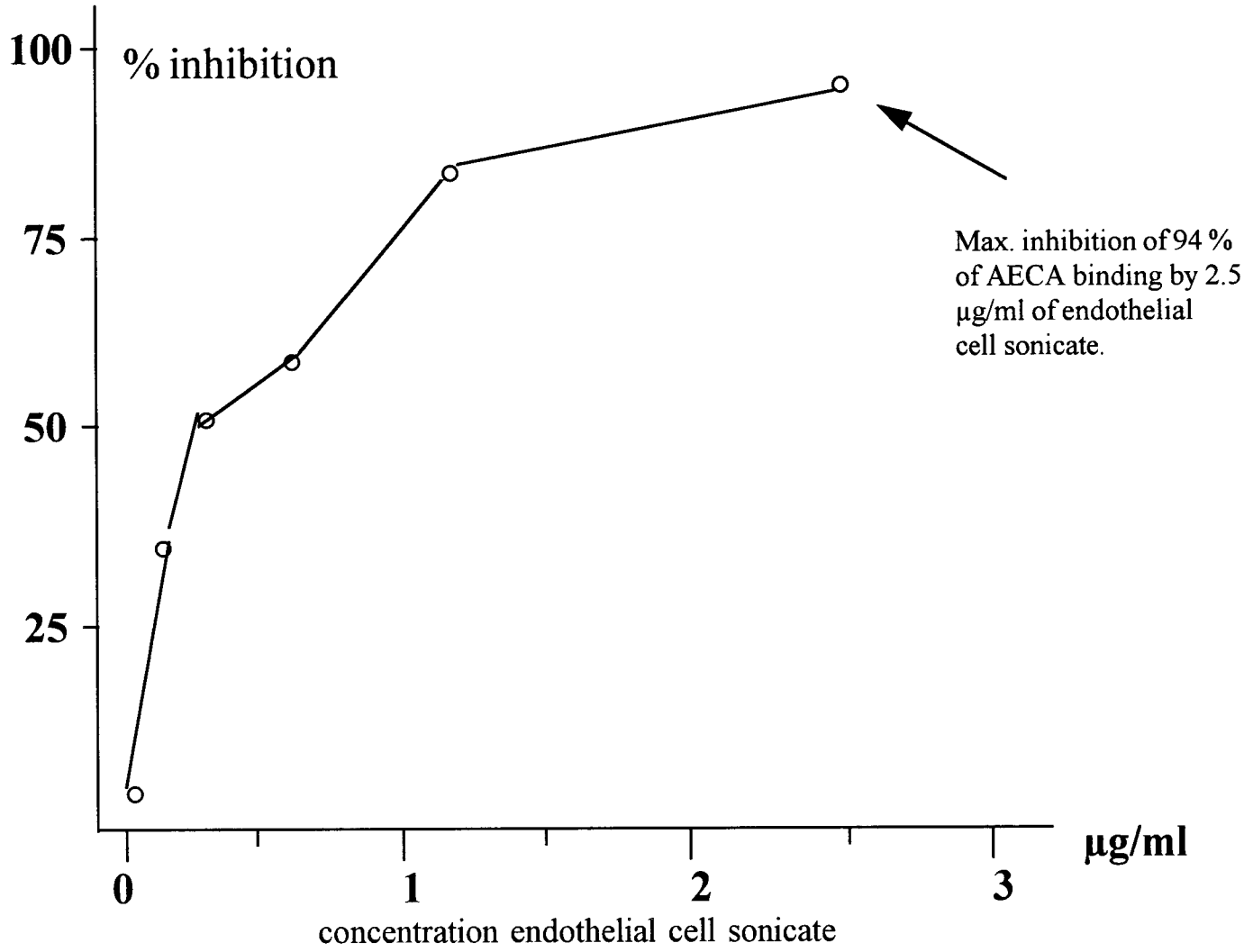



Figure 5.2 Inhibition of binding of anti-endothelial cell antibody to endothelial cells by an endothelial cell sonicate.

AECA titres were higher in sera with a positive ANCA (median OD = **0.20;** range = **0.094 - 0.469),** than in sera with a negative ANCA (median = 0.**13;** range **0.020** - 0.25)**;** and the difference was significant, (P = **0.0001)** using the Mann-Whitney test. Twenty seven out of the **33** patients who had AECA were also ANCA positive, and by contrast only **6** out of **36** patients who were ANCA negative had a positive AECA.

Anti-MPO ***antibodies***

It is interesting to note that none of the IBD patients had anti-MPO antibodies, where as, in systemic vasculitis this represents a major antibody system.

## *5.44 Adsorption studies*

The sixty-five ANCA positive sera were incubated with ethanol fixed normal neutrophils. After adsorption with neutrophils, ANCA were no longer detectable using an indirect immunofluorescence assay. Adsorption of ANCA from **33** AECA positive sera slightly reduced AECA levels, from median **0.19** range **0.14 - 0.29** to median **0.17** range **0.14 - 0.24.** There was no significant loss of AECA activity after adsorption of serum with neutrophils ( **p=0.09** ).

## *5.5* Discussion

AECA may represent one group of autoantibodies which cause vascular injury in systemic vasculitis. However, although the general consensus, at present is that they have a pathogenic potential. The mechanisms by which vascular injury and inflammation are brought about is poorly understood, and several hypotheses have been put forward.

This study shows that AECA exists in UC, a disease, which has not previously been reported to exhibit classical vasculitic features. The hypothesis that anti-MPO antibodies bind to endothelium bound MPO, and that this may be a possible mechanism for endothelium damage in systemic vasculitis [Varagunam et al, **1991]**, cannot be applied to **IBD** since this study together with other studies did not demonstrate anti-MPO antibodies in the sera of patients with **IBD.**

The pathogenic potential of AECA has been studied most extensively in Kawasaki disease [Leung et al, **1986;** Leung et al, **1987].** Kawasaki disease is a necrotizing arteritis that affects children predominantly and occurs worldwide. Clinical manifestations include fever, polymorphous erythematous rash, diffuse mucosal inflammation, non-supparative lymphadenopathy, and in some patients, cardiac failure due to coronary arteritis.

Leung and associates have studied autoimmune events in Kawasaki disease that appear to be involved in the pathogenesis of vasculitis. They have observed that

patients with Kawasaki disease have circulating antibodies which react with cytokine inducible non-major histocompatibility complex (MHC) molecules on endothelial cells, resulting in endothelial cell lysis in vitro. Specifically, they detected IgG and IgM AECA that cause complement mediated lysis of human umbilical and saphenous endothelial cells, that have been pretreated with gamma interferon, interleukin-1, or tumour necrosis factor. From these observations, a pathogenic mechanism for vasculitis in Kawasaki disease would entail two events:

a. Production of AECA (possibly related to the polyclonal B-cell activation that occurs in patients with Kawasaki disease), and increased cytokine production (possibly related to the increased activity of CD4 T lympbocytes and monocytes that also occurs in Kawasaki disease).

b.The AECA would bind to up-regulated endothelial antigens, and cause endothelial death and vascular inflammation.

The above immunological mechanisms may be operating in IBD, however, studies have shown that AECA cross-reacts with other cells. It has been shown that AECA, in patients with rheumatoid vasculitis and some collagen-vascular disorders, specifically cross-reacts with fibroblasts, and non-specifically to peripheral blood mononuclear cells, and thus, do not appear to be specific for endothelial cells

[Heurkens et al, **1989**; Quadros et al, **1990**]. This cross reactivity could be a possible mechanism by which abnormal mononuclear expression of cytokines and fibroblast proliferation is brought about.

Little is known of the significance of AECA in IBD. This study has shown that although IBD is a completely different group of inflammatory diseases, there appear to be immunological similarities to the systemic vasculitides. This phenomenon is perhaps not just coincidence since ANCA has also been found to occur in both groups of diseases. Histologically, there is unequivocal evidence of vasculitis in Crohn's [Wakefield et al, **1991**] but perhaps this is not ***so*** well demonstrated in UC [Murch et al, **1992**. Granulomas are not a universal finding in Crohn's disease and a spectrum of vascular inflammation occurs in Crohn's disease ranging from a lymphocyte-predominant pattern to granulomatous vasculitis with giant cell formation [Wakefield et al, **1989**]. Patients with a non-granulomatous Crohn's disease, show a lymphocyte-predominant pattern of vasculitis and it is notable that this same spectrum of vascular inflammation is seen in Wegener's granulomatosis [Devaney et al, **1990**]. It is also interesting to note that both IBD and systemic vasculitis not only share two similar antibody systems namely ANCA and AECA but also respond to immunosuppressive measures, namely steroid therapy.

Another observation in this study was that, despite total colectomy for UC, AECA was still prevalent suggesting that

the immunological processes which initiate IBD are not halted by total colectomy. This observation also applies to the previous study on ANCA [Patel et al, 1994]. Whether AECA represents a cause or effect of the various vasculitic diseases still needs to be elucidated.

## *5.6* Summary

Anti-endothelial cell (AECA) and anti-neutrophil cytoplasmic antibodies (ANCA) are known to occur in systemic vasculitis. While ANCA has been described in inflammatory bowel disease (IBD) little is known of the role of AECA. Steroids remain the cornerstone in the treatment of both groups of diseases. The aims were to assess the prevalence and effect of total colectomy on AECA in IBD. Sera from **101** patients with IBD were tested for AECA by an ELISA using human umbilical vein endothelial cells, **41** with UC, **30** with UC who had undergone restorative proctocolectomy, and **30** with Crohn's disease. Sera from **34** healthy individuals were used as controls.

Overall **33/101** patients were AECA positive :- **15/41** with **UC, 12/30** who had undergone restorative proctocolectomy and **6/30** with Crohn's. All **5** patients who had pouchitis were AECA positive. 27 out of **33** patients who had AECA were also found to have ANCA. Neutrophil adsorption studies of ANCA were performed to differentiate between ANCA and AECA.

These results show AECA occurs as a distinct antibody system in IBD, and that immunological similarities of IBD and systemic vasculitis are not just coincidence. Despite total colectomy, AECA persists, suggesting continuing immunological processes. Colonic metaplasia in the ileal pouch or pouchitis may be possible reasons for this. AECA like ANCA represents a major antibody system in IBD which needs further investigation.

# Chapter 6

## Anti-epithelial cell, anti-tropomyosin antibodies, AECA and ANCA

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## 6.1 Introduction

Circulating autoantibodies to colonic epithelial cells have been shown, by various techniques, in a proportion of patients with ulcerative colitis. The best characterised of these is the 'classical' anticolon antibody (Anti-EPI) with affinity for colonic mucopolysaccharide [Broberger et al, **1959**; Broberger et al, **1963**; Wright et al, **1966**; Chapman et al, **1986**; Snook et al, **1991]**. From the literature however, it is unclear whether cytotoxic antibody, which has been shown to be capable of mediating colonocyte specific cytotoxicity [Shorter et al, **1970**; Das et al, **1984**; Auer et al, **1988]**, is the same antibody as Anti-EPI. The relation between these two characterised antibodies, if different, is unclear and their importance in the pathogenesis of ulcerative colitis is uncertain.

A possible target antigen for this anti-colon antibody has been described to be a 40kDa epithelial membrane protein [Das et al, **1992]**. This protein is expressed not only by the colon but also by gall bladder and skin, possibly providing an explanation for the biliary and cutaneous manifestations of ulcerative colitis [Das et al, **1990**; Das et al **1992]**. Recently, this protein has been identified to be an isoform of tropomyosin [Das et al, **1993]**.

ANCA have been implicated in the pathogenesis of systemic vasculitis. However in IBD their pathogenic role is unclear. Are ANCA directed solely against neutrophils or are they cross-reactive with other cells?

## 6.2 Aims

The aims of this study were to assess:-

1.The prevalence rate of Anti-EPI and anti-tropomyosin antibody in inflammatory bowel disease.

2.To assess whether there was any cross-reactivity of Anti-EPI antibody or anti-tropomyosin antibodies with ANCA by adsorption studies.

3.To assess any correlation with disease activity or treatment of any of these antibodies.

## 6.3 Patients and methods

Sera from the following groups of patients were collected and stored at -80°C until studied :

i)ulcerative colitis, n = 41; median age **37** range 18- 65 years.

ii)patients with ulcerative colitis who had undergone total restorative proctocolectomy, n = **30;** median age 40 range 19 **- 70** years. 6 of these patients had pouchitis at the time of sampling.

iii)Crohn's disease, n = 30, median age **30,** range 18-60 years. Eleven patients had disease confined to the colon and rectum of whom four had had a total proctocolectomy. Six had disease confined to the ileum and thirteen had disease involving both colon and ileum.

The diagnosis, in all cases was established by clinical criteria, and by histology on biopsies or from gross

specimens taken at the time of colectomy. None of the patients had any clinical or histological features of a systemic vasculitis.

## 6.31 Controls

Sera from 34 healthy individuals an **10** patients wit colonic inflammation due to salmonella (n=2), Campylobacter (n=5) and ischaemic colitis (n=3) were used as controls.

## 6.32 Disease activity

The disease activity in patients with Crohn's disease was recorded using the Crohn's disease activity index (CDAI) [Harvey et al, **19801,** and in patients with ulcerative colitis the Sutherland score was used [Sutherland et al, **1987].**

## 6.33 Treatment

Treatment with steroids +/- aminosalicylates was recorded in all cases.

## 6.34 ANCA

ANCA was measure( by indirect immunofluorescence, as previously described in chapter **2.** The immunofluorescence staining pattern was either graded as positive or negative in this study.

### *6.35* Antibodies to epithelial cells (ANTI-EPI)

Epithelial cells of the colon cancer line HT29 were cultured in tissue culture flasks with RPMI, **10%** foetal calf serum, **2.0** mmol/l glutamine, **83** IU/ml of penicillin and **83** $\mu$g/ml streptomycin. The cells were then transfered to **96** well immunoplates at a concentration of **2** x $10^4$ / well, cultured until confluent and fixed with **1%** formaldehyde for **10** minutes.

The antibodies to epithelial cells were detected using the method as described below:-

After blocking for **1** hour in **0.1** M phosphate buffered saline(PBS)/ **2%** bovine serum albumin (BSA) (also used as the diluent for subsequent steps) at **37°** C, sera from patients and controls were added at a dilution of **1/20** and incubated for one hour. After washing with PBS-Tween, horseradish Peroxidase conjugated sheep antihuman IgG(Fc) [British Biotech) was added at a dilution of **1/10,000** followed by o-phenylenediamine in *0.05* M citrate buffer **(2.2** x $10^{-3}$ **M** final concentration) containing 6 X **10** $^{.3}$ % $H_2O_2$**.** The reaction was stopped with **20%** $H_2SO_4$ after **15** minutes and the optical density (OD) of the wells read at **492** nm.

### *6.36* Antibodies to tropomyosin

These were detected as previously described [Das et al, 1993]. Tropomyosin **(T-2400,** Sigma) was coated onto a 96

well Nunc immunoplate at 0.3 μg per well in 100 μl of carbonate buffer, pH 9.6, overnight at 4°C. Thereafter, wells were washed with saline-Tween. Plates were blocked with 5% BSA and 1% sheep serum for 1 hour at 37°C. 100 μl of test or control sera diluted 200 fold were added and incubated for one hour at 37°C and washed. The conjugate sheep alkaline phosphatase antihuman IgG (The Binding Site , Birmingham) was added (1/1600) to each well and incubated for one hour at 37°C. After washing the subtrate, P-nitrophenyl phosphate in 1 mM magnesium chloride, and 50mM sodium carbonate, pH 9.8 (Sigma) was added. The plates were read at 405nm.

## **6.37** Adsorption studies

Human group O +ve neutrophils were isolated and fixed in ethanol onto plates, as previously described [Savage et al, 1991]. Samples of serum (100 μl) were incubated for 60 min with 5 X $10^7$ fixed neutrophils. Supernatants were harvested and adsorbed against a second batch of cells. Before use for the antibody ELISA, protein concentrations of both the pre- and post-adsorption samples were measured by spectrophotometry at 260nm and 280nm and adjusted to those of the unadsorbed samples. The samples were tested for the presence of ANCA by indirect immunofluorescence, as described in Chapter 2 to ensure, complete adsorption.

### 6.38 Statistics

Results are given as the median and range of observations. Differences, between the groups of subjects studied were analysed by the Kruskal-Wallis analysis of variance and differences between pairs of observations by the Wilcoxon rank sum test. The Bonferroni correction was used for multiple comparisons. Correlations were sought using Spearman's rank correlation test.

## 6.4 Results

### 6.41 Disease controls

Autoantibodies were not detected in any of the disease controls.

### 6.42 ANCA

ANCA results are summarized in Table 6.1. Thirty one out of 41 patients (76%) with UC had a positive ANCA as did 21 of 30 patients (70%) with UC who had had a restorative proctocolectomy and 13 out of 30 patients (43%) with Crohn's colitis. The pattern of ANCA staining was perinuclear in all sera. ANCA were not detected in any of the healthy or disease controls.

### 6.43 Anti-epithelial cell antibodies (Anti-EPI)

IgG anti-EPI antibody levels also were not normally

distributed, and the 95th percentile of 34 healthy control sera have been used to define the upper limit of normal. The inter-assay and intra-assay coefficients of variation for the anti-EPI assay was 9.1 % and 4.2 % respectively. Anti-EPI antibody was detected in 20 out of 101 (20%) patients (Table 6.1). The distribution of anti-EPI antibodies was significantly different between the groups studied ($p = 0.029$), although comparisons between any of the patient groups and healthy controls did not show any significant differences (Figure 6.1). Anti-EPI antibodies were not detected in any of the disease controls.

### 6.44 **Antibodies** to tropomyosin

The inter-assay and intra-assay coefficients of variation for the anti-tropomyosin assay were 9.8 % and 10.2 % respectively.

IgG anti-tropomyosin antibody levels were not normally distributed (median OD 0.081, range 0.001-0.774), so the 95th percentile of 34 healthy control sera was used to define the upper limit of normal. Twelve out of 41 patients (29%) with UC had anti-tropomyosin antibodies, and these antibodies were found in three of 30 patients (10%) with UC who had had a restorative proctocolectomy, and eight out of 30 patients (27%) with Crohn's colitis (Table 6.1). The distribution of anti-tropomyosin antibodies was significantly different between the UC (n=41) and Crohn's disease (n=30) groups compared to controls, but not between

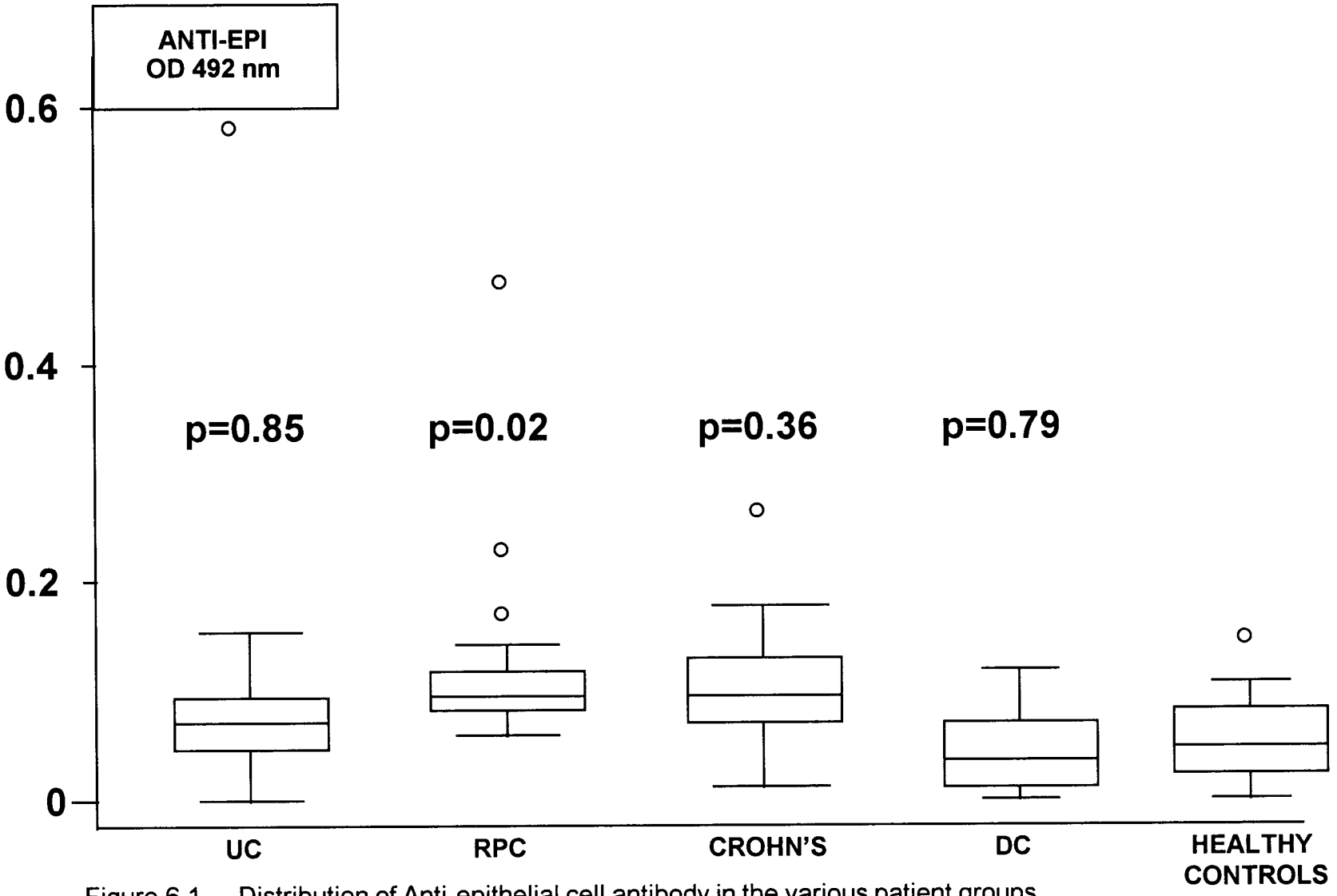



Figure 6.1 Distribution of Anti-epithelial cell antibody in the various patient groups (RPC=Restorative proctocolectomy; DC=Disease controls)

controls and UC patients who had undergone restorative proctocolectomy (Figure 6.2).

Table 6.1 Number of patients with ANCA, AECA, anti-EPI and anti-tropomyosin antibodies in the disease study groups.

| | n | ANCA | AECA | ANTI-EPI | ANTI-TROPOMYOSIN |
|---|---|---|---|---|---|
| UC | 41 | 31 | 15 | 6 | 12 |
| RPC | 30 | 21 | 12 | 6 | 3 |
| Crohn's | 30 | 13 | 06 | 8 | 8 |
| Total | 101 | 65 | 33 | 20 | 23 |

## 6.45 Anti-EPI antibodies, anti-tropomyosin antibodies and **ANCA**

Anti-EPI antibodies were significantly higher in patients with a positive ANCA (median 0.08 range 0.02-0.57) as compared to patients with a negative ANCA (median *0.06* range 0-0.23) ($p = 0.003$).

Anti-tropomyosin titres were not significantly higher in sera with a positive ANCA (median OD = 0.18; range = 0.07 - 0.47) than in sera with a negative ANCA (median = 0.16; range 0.02 - 0.25) ($p = 0.11$).

Correlation of the four autoantibodies studied with each other is shown in the correlation matrix (Table 6.2).

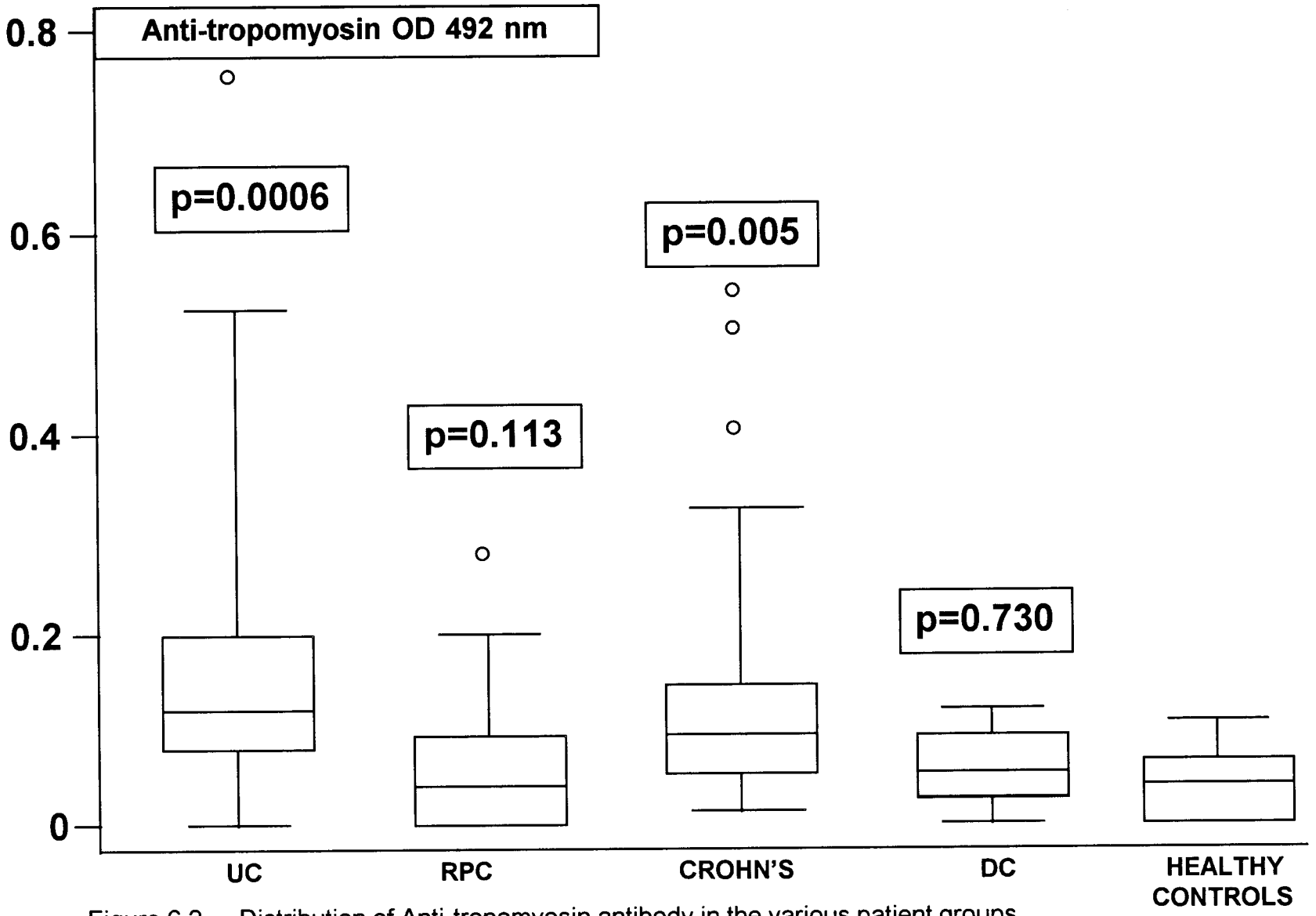



Figure 6.2 Distribution of Anti-tropomyosin antibody in the various patient groups (RPC=Restorative proctocolectomy; DC=Disease controls)

Table 6.2 Correlation matrix of the autoantibodies.

| | ANCA | AECA | ANTI-EPI |
|---|---|---|---|
| AECA | 0.390*<br>P<0.05 | - | - |
| ANTI-EPI | 0.410*<br>P<0.05 | 0.1245<br>0.5<P<0.2 | - |
| ANTI-TROPOMYOSIN | 0.087<br>P>0.05 | 0.108<br>P>0.05 | -0.165<br>P>0.20 |

## 6.46 Adsorption studies

The sixty-five ANCA positive sera were incubated with ethanol fixed normal neutrophils. After adsorption with neutrophils, ANCA were no longer detectable using an indirect immunofluorescence assay. Adsorption of ANCA from **20** anti-EPI positive sera significantly reduced anti-EPI antibodies, from median 0.08 range **0.02 - 0.76** to median **0.03** range **0.00-0.18**, p = **0.001**.

Adsorption of ANCA, from **23** anti-tropomyosin positive sera, did not significantly reduce anti-tropomyosin antibodies, from median **0.17** range **0.02 - 0.47** to median **0.15** range 0.01-0.30, p = **0.012**.

## 6.47 Disease activity scores and presence of autoantibodies

The sera were obtained from a wide spectrum of disease activity in both ulcerative colitis and Crohn's disease. However no significant correlation was found between any of the autoantibodies detected with disease activity (Table **6.3).**

Table **6.3.** Correlation of autoantibodies with disease activity in UC and Crohn's disease (r-value with corresponding P value).

| | ANCA | AECA | ANTI-EPI | ANTI-TROPOMYOSIN | CRP | α-1 GLYCO PROTEIN |
|---|---|---|---|---|---|---|
| UC N=41 | 0.206 P>0.20 | 0.149 P>0.20 | -0.010 p>0.50 | 0.071 p>.50 | 0.41* p<0.05 | 0.26 p>0.20 |
| CROHN'S N=30 | 0.059 P>0.50 | -0.245 P>0.10 | -0.056 P>0.50 | 0.139 p>0.20 | 0.47* p<0.05 | 0.34* p<0.05 |

## 6.48 Treatment

No significant correlation was found between autoantibody titres and treatment with steroids +/- aminosalicylates (Table **6.4).**

**Table 6.4. Correlation of the autoantibodies and treatment with steroids +/-aminosalicylates in UC and Crohn's disease (r-value with corresponding P value).**

| Disease | ANCA | AECA | ANTI-EPI | ANTI-TROPO MYOSIN |
|---|---|---|---|---|
| UC<br>N=41 | O.042<br>P>0.50 | -0.154<br>P>0.20 | 0.104<br>p>0.20 | -0.032<br>p>0.50 |
| CROHN'S<br>N=30 | 0.019<br>P>0.50 | 0.121<br>P>0.20 | -0.165<br>P>0.20 | 0.019<br>p>0.50 |

## 6.49 Crohn's Disease

**Table 6.5 shows the four patients with Crohn's disease who had undergone total proctocolectomy for severe colitis in whom the following autoantibodies were found:**

| | ANCA | AECA | ANTI-EPI | ANTI-TROPOMYOSIN |
|---|---|---|---|---|
| Crohn's<br>n=4 | 2 | 1 | 1 | 0 |

## *6.5* Discussion

The presence of antibodies to colonic epithelium has been reported widely by many investigators since the late **1950's** [Broberger et al, **1959**; Broberger et al, **1963**; Wright et al, **1966**; Shorter et al, **1970**; Chapman et al, **1986**; Auer et al, **1988]**, but, more recently a new antibody has been described to be an isoform of tropomyosin, which forms part of the cytoskeletal protein of cells [Das et al, **1993]**.

Das et al have shown that sera from patients with UC but not Crohn's disease contain antibodies to tropomyosin, isolated from the normal colon of patients undergoing colectomy for cancer. The tropomyosins are actin binding structural proteins found in most cell types. A possible explanation for the presence of antibodies, directed against several cell types in UC would be that these antibodies were directed against different isoforms of tropomyosin.

Data on whether anti-EPI antibodies have any correlation with disease activity in ulcerative colitis, or any capability for cell cytotoxicity in vitro, have been conflicting [Shorter et al, **1970**; Das et al, **1990**; Snook et al, **1991**; Auer et al, **1988]**. To date, there has been no data published on correlation of autoantibodies in inflammatory bowel disease with each other, especially with ANCA. In addition, the prevalence of anti-tropomyosin antibodies to disease activity has not yet been demonstrated.

The major observations in this study were, to confirm the prevalence rate of **20%** for anti-EPI antibodies in inflammatory bowel disease, with that of both recent and older other studies [Wright et al, **1966;** Snook et al, **1991].** However, the prevalence of **29%** of antitropomyosin antibodies in ulcerative colitis in this series is considerably less than the **90%** recently reported by Das and colleagues. Conversely, the prevalence rate for this antibody in Crohn's disease is greater than that reported by Das and colleagues. Since there is only one other report of the existence of antitropomyosin antibodies, it is difficult to comment what the difference in prevalence rate **is** due to. Strong contenders for this difference could be methodological or different selection groups.

There was a significant association between anti-EPI antibodies and ANCA. Anti-EPI antibody levels were significantly reduced following adsorption of sera by neutrophils, suggesting that these antibodies recognised antigens also present on neutrophils. This finding is promising, since it would enable a more rational explanation for the presence of ANCA in UC, where a gross vasculitis is seldom demonstrated.

Finally, there was no significant correlation between the levels of anti-EPI antibodies with, either AECA or, anti-tropomyosin antibodies, therefore, they are quite distinct antibody systems.

None of the autoantibodies discussed and investigated, including ANCA, AECA, ANTI-EPI and anti-tropomyosin, correlate with disease activity. They all persist despite total colectomy thus they cannot just be related to colonic inflammation. Indeed, patients with colonic inflammation due to non-IBD causes such as infective diarrhoeas, do not have any evidence of ANCA, AECA or ANTI-EPI antibodies. Thus, it can be inferred that, simply removing the colon ie the target organ does very little to the actual immunological disturbance which gave rise to ulcerative colitis.

Increased prevalence of ANCA and AECA have also been reported in pouchitis following restorative proctocolectomy. Perhaps the pouchitis is simply a pseudo-ulcerative phenomenon, occurring in ileal mucosa which has undergone a degree of colonic metaplastic change, and villous atrophy, reported to occur in ileoanal pouches with time [De Silva et al, **1991**; Pemberton, **1993]**. This hypothesis is further supported by the fact that pouchitis responds to treatment using aminosalicylates and steroids although metronidazole is undoubtedly, the first line agent. Reports have suggested that metronidazole **is** probably exerting an immunosuppressive, rather than an antimicrobial role in pouchitis [O'Connellet al, **1986**; Kmiot et al, **1993]**. However stasis in the ileoanal pouch and increased proliferation of bacteria, and their subsequent sensitivity to metronidazole, is another argument.

Overall, ANCA was found more frequently than AECA or anti-EPI or antitropomyosin antibodies in IBD. There was a higher prevalence of ANCA and AECA in patients with ulcerative colitis, as compared with Crohn's disease, and yet it is Crohn's disease where vasculitic features have been more commonly described. Anti-EPI antibodies were slightly more common in Crohn's disease but this was not statistically significant.

My observations show that there is a subgroup of patients with ulcerative colitis and Crohns disease who have ANCA, AECA, anti-EPI and antitropomyosin antibodies. It **is** as yet, unclear whether these antibodies are of pathogenic significance in inflammatory bowel disease but this study has made it clear that total colectomy has no influence on the prevalence of these autoantibodies.

## 6.6 Summary

To date, there have been few studies looking simultaneously at the prevalence, association with each other, relation to disease activity and treatment for the autoantibodies:- ANCA, AECA, ANTI-EPI and the recently described antibody to tropomyosin.

The prevalence rates of these autoantibodies were determined in patients with ulcerative colitis (UC), n=41; UC following restorative proctocolectomy (RPC), n=30 and Crohn's disease (CD) n=30 using ELISA.

Table 6.1 Autoantibody prevalence in the study groups:-

| | n | ANCA | AECA | ANTI-EPI | ANTI-TROPOMYOSIN |
|---|---|---|---|---|---|
| UC | 41 | 31 | 15 | 6 | 12 |
| RPC | 30 | 21 | 12 | 6 | 3 |
| Crohn's | 30 | 13 | 6 | 8 | 8 |
| Total | 101 | 65 | 33 | 20 | 23 |

A strong association of ANCA with ANTI-EPI (P=0.003) antibodies was noted.

Significant cross-reactivity was noted between ANCA and ANTI-EPI antibodies **(P=0.001)** but not between ANCA and AECA **(P=0.09)** or between ANCA and anti-tropomyosin (P=0.12) by removal of ANCA from the sera (adsorption) using isolated neutrophils fixed onto microtitre plates.

The prevalence of these autoantibodies showed no correlation with either disease activity or with treatment.

All **antibodies persisted despite total colectomy, indicating that these antibodies do not merely reflect colonic inflammation. The cross-reactivity of** ANCA **with ANTI-EPI antibodies may help to explain the high prevalence of** ANCA **in ulcerative colitis.**

# Chapter 7

## Circulating cell adhesion molecules in inflammatory bowel disease

## 7.1 Introduction

Leucocytes are prominent in the inflammatory processes which occur in chronic inflammatory bowel disease. These leucocytes arrive at the site of inflammation from the blood stream, by diapedesis through the endothelium. Recently, it has been shown that adhesion of leucocytes and their transmigration across the endothelium, is dependent on cell adhesion molecules (CAMS), which include intercellular adhesion molecule 1 (ICAM-1)[Simmonset al,. 1988], E-Selectin [Polte et al, 1989] and vascular cell adhesion molecule 1 (VCAM-1) [Bevilacqua et al, 1989]. ICAM-1 and VCAM-1, belong to the immunoglobulin supergene family and bind respectively to the ß2 integrins (LFA1 and **MAC1)** [Marlin et al, 1987; Diamond et al, 1991] on lymphocytes, granulocytes, monocytes and to the B1 integrin (VLA4) [Hemler et al, 1987] on lymphocyes, eosinophils, basophils and monocytes.

ICAM-1 is found on many cell types, whilst VCAM1 is found mainly on endothelial cells, follicular dendritic cells, neural cells and glomerular parietal epithelium [Garner et al, 1993].

E-Selectin is found only on activated endothelium and binds to granulocytes, monocytes and some memory T-cells (CLA+) [Shimizu et al, 1991; Picker et al, 1991]. Raised levels of soluble forms of these intercellular cell adhesion molecules :- SICAM-1, SE-Selectin and sVCAM-1 have been

found in the plasma of a variety of disease states, including chronic inflammatory liver disease [Adams et al, 1992a], diabetes [Ballantyne et al, 1991], some carcinomas [Adams et al, 1992b], allograft rejection [Gearing et al, 1992; Harning et al, 1991] and systemic vasculitides [Hauschild et al, 1992].

## 7.2 Aims

The aims of this study were to determine levels of the soluble **CAMs**, in patients with chronic inflammatory bowel disease, in relation to prevalence, treatment and disease activity.

## 7.3 Patients and methods

Plasma from 115 patients with chronic inflammatory bowel disease, and 24 healthy controls were collected and stored at ***-80°*** **C**. The diagnosis of inflammatory bowel disease in the study group had been made by prior clinical, radiological, endoscopic and histopathological methods.

### 7.31 Ulcerative colitis

Sera were obtained from 81 patients with ulcerative colitis, median age 39, range 18-70 years of whom 39 were males. These patients were placed in two groups depending on whether they had **UC** (n=49) or whether, they had **UC** and had undergone restorative proctocolectomy (n=32).

From the RPC group, 8 patients had clinical pouchitis at the time of investigation. In addition, two patients had undergone prior liver transplantation for sclerosing cholangitis.

## 7.32 Crohn's Disease

There were 34 patients with Crohn's disease, median age 30, range 18-60 of whom 18 were males. 10 patients had disease confined to the colon and rectum, 10 patients had disease confined to the small bowel alone, and 14 had disease affecting the large and small bowel. 4 of the above Crohn's patients had an ileostomy after undergoing proctocolectomy. Active IBD was defined by the presence of symtoms such as diarrhoea, rectal bleeding, constipation, abdominal pain, fever and weight loss; together with endoscopic and histological evidence of mucosal inflammation.

Inactive disease was defined by the absence of symptoms and endoscopic normal mucosal appearances. Treatment with non-steroidal anti-inflammatory drugs, steroids or both was undertaken in all the active disease study groups. Assessment of the disease activity, by measurement of haemoglobin, platelet count, albumin and C-reactive protein, was recorded in all patients.

The control group consisted of plasma taken from 24 healthy individuals whose median age was 34 years, range 21-41 years.

## **7.33** SICAM-1 and sE-Selectin ELISA

The levels of SICAM-1 and sE-selectin were measured by commercially available two site ELISA (British Biotechnology Products (BBP)). Sequential incubations were performed in microtitre plates as follows, the plates were washed three times between each incubation, with wash buffer: (a) anti-human ICAM-1 or E-Selectin monoclonal antibody; (b) plasma samples diluted 1 in 10 for SICAM-1 assay and 1 in 5 for sE-selectin assay, standard concentrations of SICAM-1 (3.25-52 ng/ml) or of sE-selectin (1.875-30 ng/ml) (respectively) and biotinylated anti-human ICAM-1 or E-selectin (respectively) monoclonal antibody, incubated for 2 hours at room temperature; (c) **streptavidin-horseradish** Peroxidase (HRP) anti-human IgG conjugate, incubated for 1 hour at room temperature and (d) tetramethylbenzidine Substrate. The optical density was read after 10 minutes at 450 nm. The SICAM-1 and **sE-**selectin concentrations in the test plasma, were determined from the standard curve.

## **7.34** sVCAM-1 ELISA

The levels of sVCAM-1 were measured by a two site ELISA. Microtitre plates were coated with mouse anti-human VCAM-1 monoclonal antibody (The Binding Site, Birmingham), at 2.5 μg/ml in 0.05M bicarbonate buffer pH 9.6, for 16 hours at 4°C. The plates were blocked with 1% bovine serum albumin

(BSA) in 0.1M phosphate buffered saline (PBS)/0.05% Tween 20 pH 7.4. Sequential incubations of the following were made for 1 hour at 37°C, and the plates were washed between incubations with PBS/Tween: (a) Plasma samples diluted 1 in 10 in 0.1M PBS/0.05%Tween 20 pH 7.4; (b) goat polyclonal anti-human VCAM-1 (British Biotech) diluted 1 in 2000 in 0.1M PBS/0.05% Tween 20 pH 7.4, which had been incubated with 2% normal mouse serum (NMS) for 1 hour at 37°C; (c) HRP conjugated donkey anti-goat IgG (Fc) (The Binding Site) diluted 1 in 20000 in 0.1M PBS/0.05% Tween 20 pH 7.4, which had been incubated with 2% NMS for 1 hour at 37°C prior to use; (d) o-phenylenediamine Substrate in 0.05M citrate phosphate buffer pH 5. The reaction was stopped with 20% sulphuric acid after 15 minutes and the OD read at 492 nm.

## 7.35 C-reactive protein and *a-1* glycoprotein

Serum levels of C-reactive protein (CRP) and of *a-1* glycoprotein were measured by latex immunoturbidometric assay.

## 7.36 Statistics

The data were not normally distributed and therefore, are expressed as medians with interquartile ranges. Differences in adhesion molecule distributions between the patient groups were analysed by the Kruskal-Wallis one way analysis of variance. The significance of the differences between

patients and controls were determined by the Wilcoxon rank sum test, and P values adjusted by the Bonferroni method for multiple comparisons. The Spearman rank correlation coefficient test was used to determine associations. The 5% level was used to indicate statistical significance.

## 7.4 Results

### 7.41 Plasma ICAM-1

The median(ng/ml) and range of SICAM-1 was 250(140-670) for UC, 290(140-510) for RPC and 280(90-630) for CD. These values were significantly different from those obtained from the 24 healthy controls 125(10-250). Subgroup analysis in relation to disease activity revealed significant differences between active and inactive disease states in UC (P=0.031), RPC (pouchitis regarded as active disease in this group) (P=0.0004), and CD (P=0.045)(Figure 7.1 and Table 7.1).

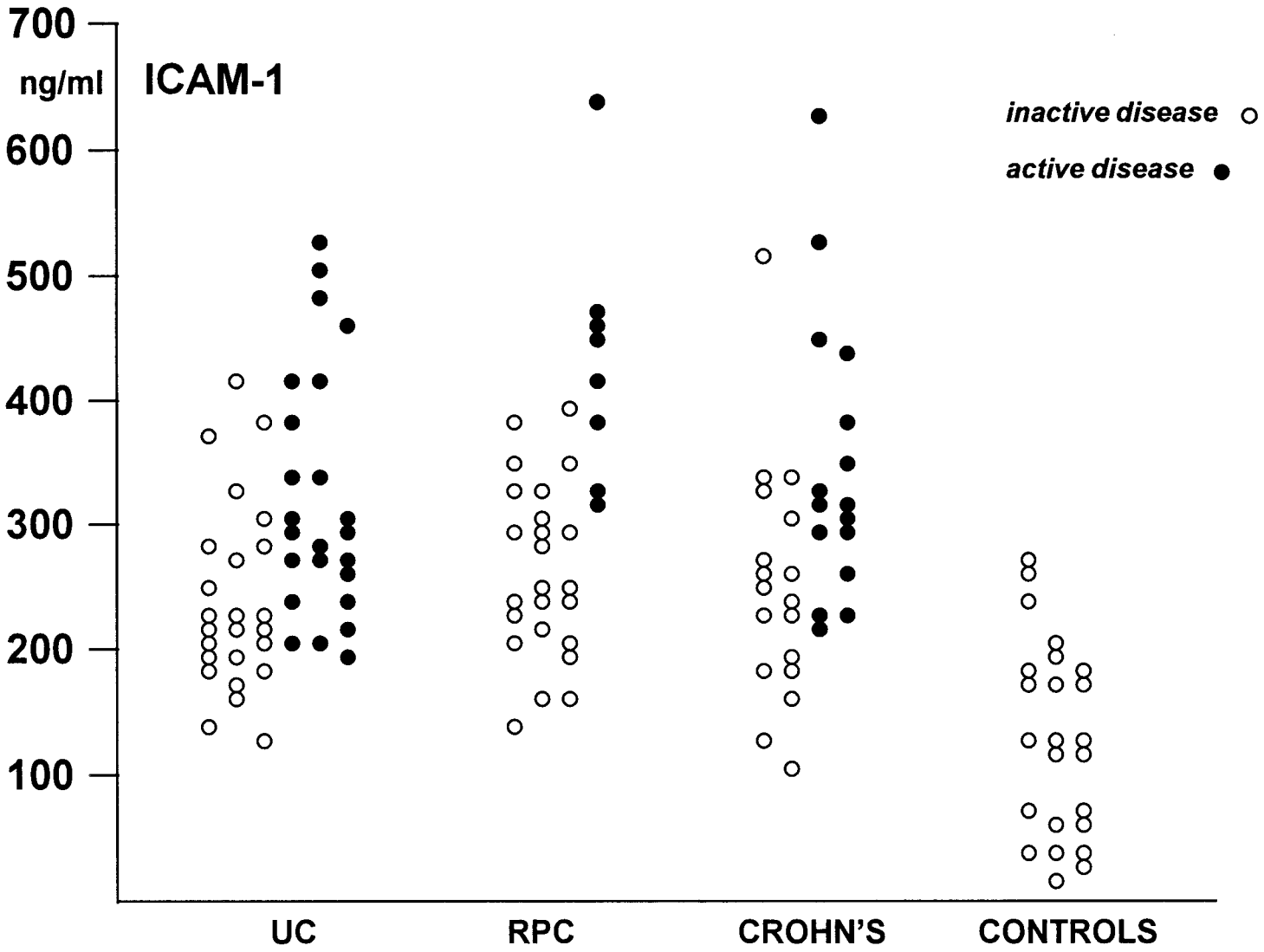



Figure 7.1 Distribution of soluble ICAM-1 in the various disease groups

**Table 7.1. Medians with ranges of soluble CAMs in relation to disease activity.**

| | sICAM-1 (ng/ml) | sE-SELECTIN (ng/ml) | sVCAM-1 (OD VALUE) |
|---|---|---|---|
| UC | | | |
| active | 270(90-510) | 55(40-140) | 1.44(0.74-1.77) |
| inactive | 225(140-420)* | 45(20-80)* | 1.10(0.68-1.84) |
| RPC | | | |
| pouchitis | 415(310-670) | 90(45-145) | 1.65(0.98-2.02) |
| non-inflamed | 260(140-380)* | 45(20-90)* | 1.22(0.74-1.88) |
| CD | | | |
| active | 305(200-630) | 78(30-115) | 1.03(0.66-1.83) |
| inactive | 245(90-520)* | 48(25-90)* | 1.07(0.86-1.86) |

Values are medians with ranges. Mann Whitney *P<0.05

**There was no correlation found between levels of SICAM-1 and treatment with steroids and/or non-steroidal anti-inflammmatory drugs using analysis of variance (F=1.16, P=0.33). Significant correlation was found between the levels of a-1 glycoprotein, but not CRP with ICAM-1 (Table 7.2).**

Table 7.2 Spearman correlation matrix for soluble CAMs, C-reactive protein and al-glycoprotein.

| | sICAM | sE-SELECTIN | sVCAM-1 | CRP |
|---|---|---|---|---|
| sE-SELECTIN | 0.464 # | - | - | - |
| sV-CAM1 | 0.043 * | 0.098 * | - | - |
| CRP | 0.126 * | 0.203 † | 0.026 * | - |
| α1-GLYCOPROT | 0.223 † | 0.133 * | 0.072 * | 0.246† |

# P < 0.001, † P < 0.05, * P > 0.10

## 7.42 Plasma E-Selectin

The median(ng/ml) and range of distribution of SE-Selectin was 55(20-140) for UC, 53(20-135) for RPC and 60(25-115) for CD. These values were significantly greater than those obtained from the controls 25(5-105). Subgroup analysis in relation to disease activity revealed significant differences between active and inactive disease states in UC (P=0.0025),RPC (P=0.0012)and CD (P=0.0199)(Figure 7.2 and Table 7.1). There was no correlation found between levels of SE-Selectin and treatment with steroids or non-steroidal anti-inflammatory drugs using analysis of variance (F=0.62, P=0.61). Significant correlation was found between the levels of CRP but not a-1 glycoprotein with SE-Selectin (Table 7.2). The highest values of sICAM1 (630 ng/ml) and SE-Selectin (180 ng/ml) were seen in a

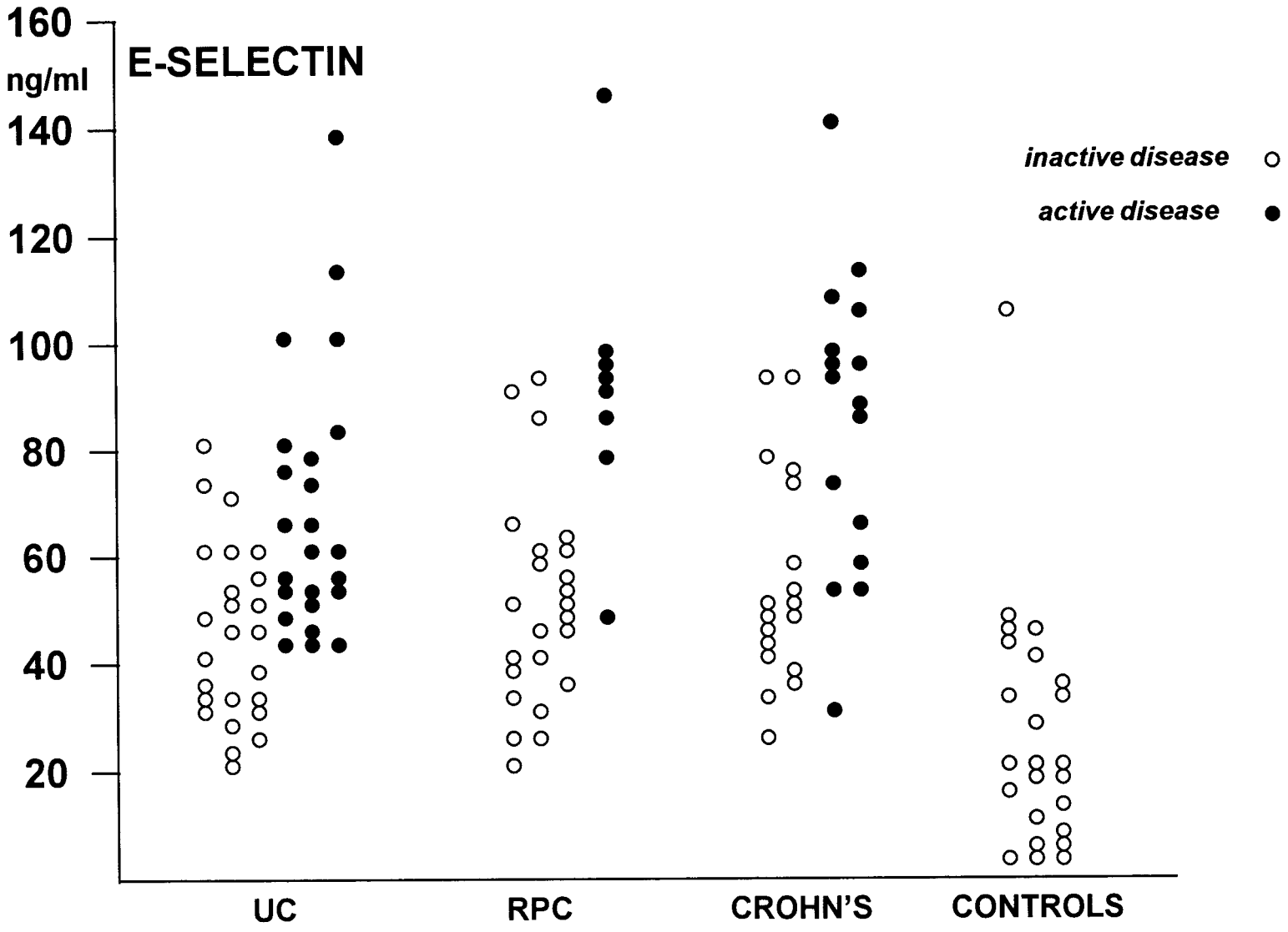



Figure 7.2 Distribution of soluble E-Selectin in the various disease groups

patient who had undergone restorative proctocolectomy during an acute episode of pouchitis.

### 7.43 Plasma VCAM-1

The median optical density (OD) and range of distribution of sVCAM-1 was 1.354 (0.676-1.845) for UC, 1.279 (0.737-2.023) for RPC and 1.073 (0.663-1.859) for Crohn's disease. These values were not significantly greater than those obtained from the controls 1.073 (0.663-1.859). Subgroup analysis in relation to disease activity revealed no significant differences between active and inactive disease states in UC (P=0.100),RPC (P=0.296) and Crohn's disease (P=0.569)(Figure 7.3 and Table 7.1).

No significant correlation was found between the levels of both C-reactive protein and a-1 glycoprotein with sV-CAM1 (Table 7.2).

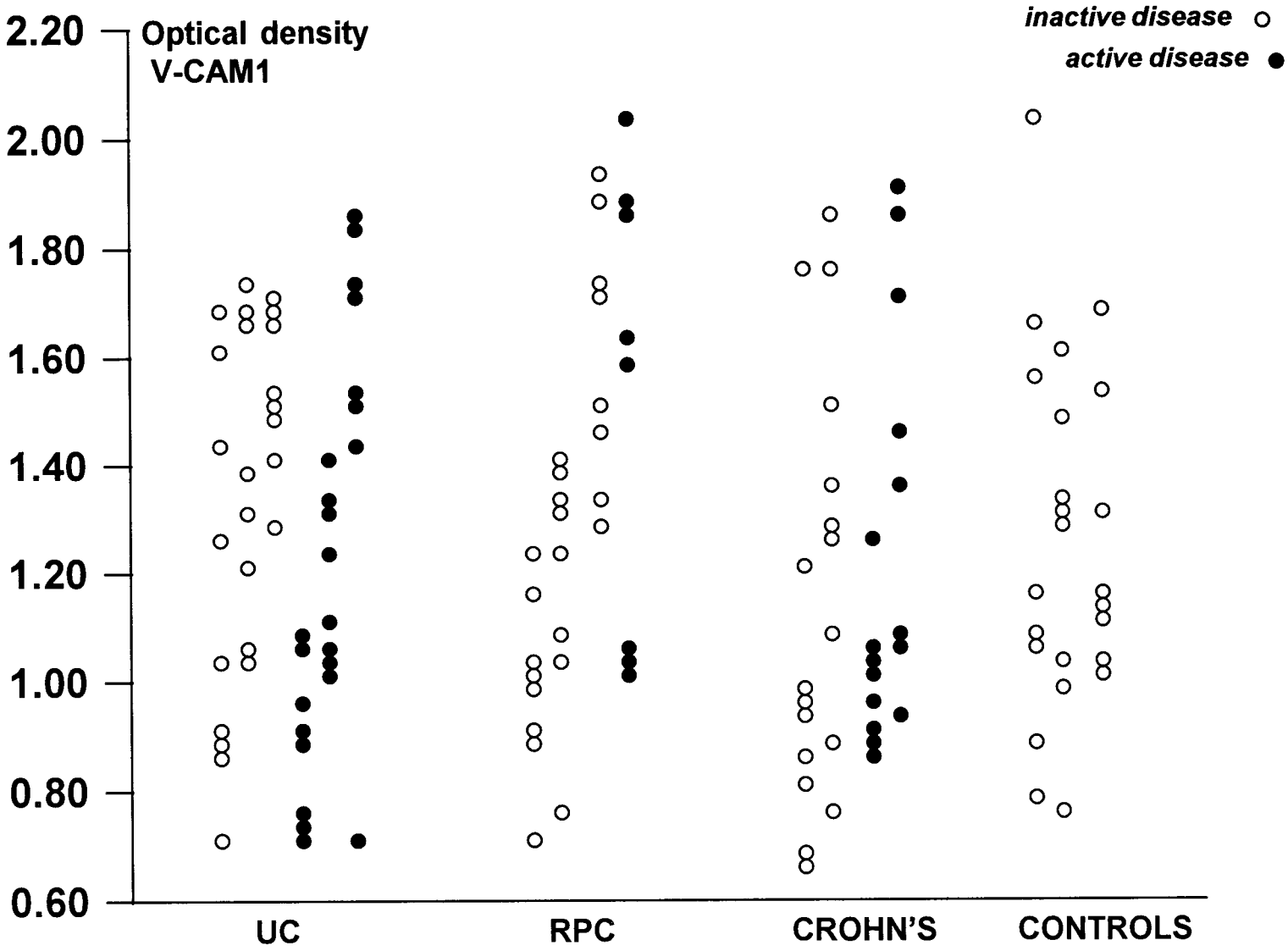



Figure 7.3 Distribution of soluble VCAM-1 in the various disease groups

## *7.5* Discussion

Leucocyte migration is a tightly regulated process, involving CAMs expressed on endothelial cells as well as leucocytes. Circulating adhesion molecules appear to arise from proteolytic cleavage of the extracellular domain of adhesion molecules [Adu et al, **1994**; Rothelin et al, **1991**]. Increased cell surface expression of these adhesion molecules is regulated by cytokines such as IL-1, TNF and yinterferon [Kishimoto et al, **1989**; Keelan et al, **1992**]. It seems likely that the expression of these CAMs would be increased on endothelium in the vicinity of tissue inflammation and this would in turn lead to an increase in plasma levels of soluble CAMS. It is also possible that soluble CAMs compete for inflammatory cell binding and thus, may act in a negative feedback manner limiting, rather than promoting cell adhesion [Barrett et al, **1993**]. This study demonstrates that circulating soluble adhesion molecules sICAM1 and SE-Selectin are detectable in greater amounts in the plasma of patients with chronic inflammatory bowel disease, as compared with healthy controls. Since ICAM-1 is found on many cell types as well as on endothelium, the raised circulating levels could reflect lymphocyte activation and non endothelial tissue injury, as well as endothelial injury.

Another interesting possibility comes from the discovery that ICAM-1 is the major cell surface receptor for **90%** of human rhinovruses. The gene for this receptor maps to

chromosome **19** which also contains the gene for other picornavirus receptors. Increased expression of **SICAM-1** has been shown to inhibit rhinovirus infection of cells from the upper respiratory tract [Marlin et al, **1990].** It seems conceivable that **ICAM-1** may play an antiviral role in the vicinity of bowel mucosa.

Vasculitis has previously been described in IBD [Murch et al, **1992;** Wakefield et al, **1991]** however, the mechanisms involved are poorly understood. E-selectin is found only on activated endothelium and elevated levels provide further proof that there is endothelial activation in chronic IBD. Recent evidence using immunoelectron microscopy has shown ultrastructural evidence that E-Selectin is a secretory product of endothelial cells in IBD, released via exocytosis [Ohtani et al, **1992].**

Binding of soluble CAMS to their target cells may also deliver an activating stimulus. Recombinant sE-Selectin has been shown to be a powerful neutrophil chemoattractant, and to activate the integrin CR3 [Lo et al, **1991].**

This study has shown increased levels of **SICAM-1** and sE-Selectin in both active inflammatory bowel disease and pouchitis. Recently increased pro-inflammatory cytokine expession has also been reported [Gionchetti et al, **1993].** It is well known that in the ileoanal pouch colonic metaplasia and villous atrophy occur. Similar expression of adhesion molecules and cytokine expression in pouchitis and active UC suggest perhaps that pouchitis represents a

reactivation of the immunological mechanisms which brought about UC.

In this study levels of sVCAM1 did not differ significantly in chronic IBD from healthy controls. Immunohistochemical localization studies on frozen tissue sections have also reported similar findings [Dean et al, 1993]. This may possibly be due to the restricted expression of this molecule on certain cell types which, perhaps are not actively involved in the inflammatory processes in chronic IBD.

This study suggests that SICAM-1 and SE-Selectin levels are increased during active IBD and pouchitis, and that the measurement of circulating levels of these molecules may be used as sensitive markers of ongoing inflammation. Inhibition of these leucocyte-endothelial cell interactions may bring about decreased leucocyte transmigration to the site of inflammation, and hypothetically may provide a new target for the control of IBD.

## 7.6 Summary

Levels of SICAM-1, SE-Selectin and sVCAM-1 might reflect endothelial activation and injury, and could be useful markers of disease activity in IBD.

The aims of this study were to determine levels of the soluble forms of ICAM-1, E-Selectin and VCAM-1, in relation to prevalence, treatment and disease activity in IBD.

Plasma was obtained from the following patients:- UC (n=49); UC who had undergone RPC [n=32 (8 of whom had a clinical pouchitis)], Crohn's disease (n=34) and 24 healthy controls.

Plasma SICAM-1 levels [median with ranges (ng/ml)] were significantly higher in patients with active UC [270 (90-510)], pouchitis [415 (310-670)], and active Crohn's [305 (200-630)] compared to inactive UC [225 (140-425)(P=0.031)], non-inflamed ileoanal pouch [260 (140-380)(P=0.0004)] and inactive Crohn's [245 (90-520)(P=0.045)] respectively and controls. SE-Selectin levels were also significantly higher in patients with active UC [55 (40-140)], pouchitis [90 (45-145)], and active Crohn's [78 (30-115)] compared to inactive UC [45 (20-80)(P=0.003)], non-inflamed ileoanal pouch [45(20-90)(P=0.001)] and inactive Crohn’s [48 (25-90)(P=0.020)] respectively and controls.

Increased levels of SICAM-1 and SE-Selectin occur during active IBD and pouchitis, which may be used as sensitive markers of ongoing inflammation.

# CHAPTER 8

## Pro-inflammatory cytokines in inflammatory bowel disease

## 8.1 Introduction

The activation of the immune system in IBD affects all cell systems associated with the mucosal immune system. Cytokines are important in orchestrating the interactions of cells involved in immunological and inflammatory reactions. Cytokines are multifunctional peptides, and the functions of the pro-inflammatory cytokines in relation to IBD are given in Chapter1.

In this chapter, studies of the local mucosal levels and plasma levels of IL-8, TNFa, IL-1ß and IL-6 are individually shown, following a brief introduction to provide clarity.

## 8.2 Aims

The aims of this study were to investigate the role of the pro-inflammatory cytokines:- IL-8, TNFa, IL-1ß and IL-6 in inflammatory bowel disease.

Plasma levels of these cytokines were determined at the same time as trying to establish the local production in the mucosa at different anatomical sites some of which were/were not involved by chronic inflammation by means of tissue biopsies.

## 8.3 Patients and methods

### 8.31 Plasma samples

Plasma samples were taken in heparinised tubes, spun down and stored in endotoxin free tubes.

Table 8.1 Number of patients in disease categories:-

| Disease | n | Age (yrs) median (range) | amino-salicylates | systemic steroids | predsol enemas |
|---|---|---|---|---|---|
| Active UC | 15 | 41(20-61) | 15 | 15 | 9 |
| Inactive UC | 15 | 50(24-72) | 0 | 0 | 0 |
| Crohn's colitis | 9 | 36(21-55) | 7 | 9 | 6 |
| Healthy controls | 15 | 49(21-65) | 0 | 0 | 0 |
| Total | 54 | - | 22 | 24 | 15 |

### 8.32 Disease activity and treatment

The disease activity was assessed in UC and Crohn's, using a simple score as previously described [Kodner et al, **1990]** and the Crohn's disease activity index [Best et al, **1976]** respectively. **4** out of **9** patients with Crohn's colitis also had evidence of ileal Crohn's disease.

Treatment with aminosalicylates +/- steroids was recorded in all patients including details of local steroid therapy using enemas.

## 8.33 Mucosal biopsies

Control healthy mucosal biopsies were obtained from patients undergoing colectomy for non-IBD disorders such as colon cancer and slow transit bowel disorders with a histologically normal colon and terminal ileum.

The mucosal biopsies were taken simultaneously with plasma samples in the operating theatre, immediately after colectomy together with an ileal biopsy if this was available or at the time of colonoscopy. Biopsies were taken from:-

1. rectosigmoid colon between 20 and 60cm
2. ileum (when available).

The biopsies were placed immediately in cool Hanks buffered saline (Hanks) supplemented with glutamine, penicillin, streptomycin (GPS) and 10% foetal calf serum (FCS). The biopsies were minced using 23 gauge needles and a microscope as shown in Figure 8.1, and washed. (Each washing step consisted of spinning the cells in a centrifuge at 1600 **RPM** for 5 minutes followed by the removal of the supernatant and the addition of fresh culture medium. 0.75 mM EDTA in Hanks/GPS/FCS was used to lyse the epithelial cells, followed by washing. 1 mM dithiothrietol in Hanks/GPS/FCS was used to remove surface mucous and washed.

Next, the tissue matrix was subjected to enzymatic dispersion at 37°C with collagenase (Sigma) at conc 0.5mg/ml

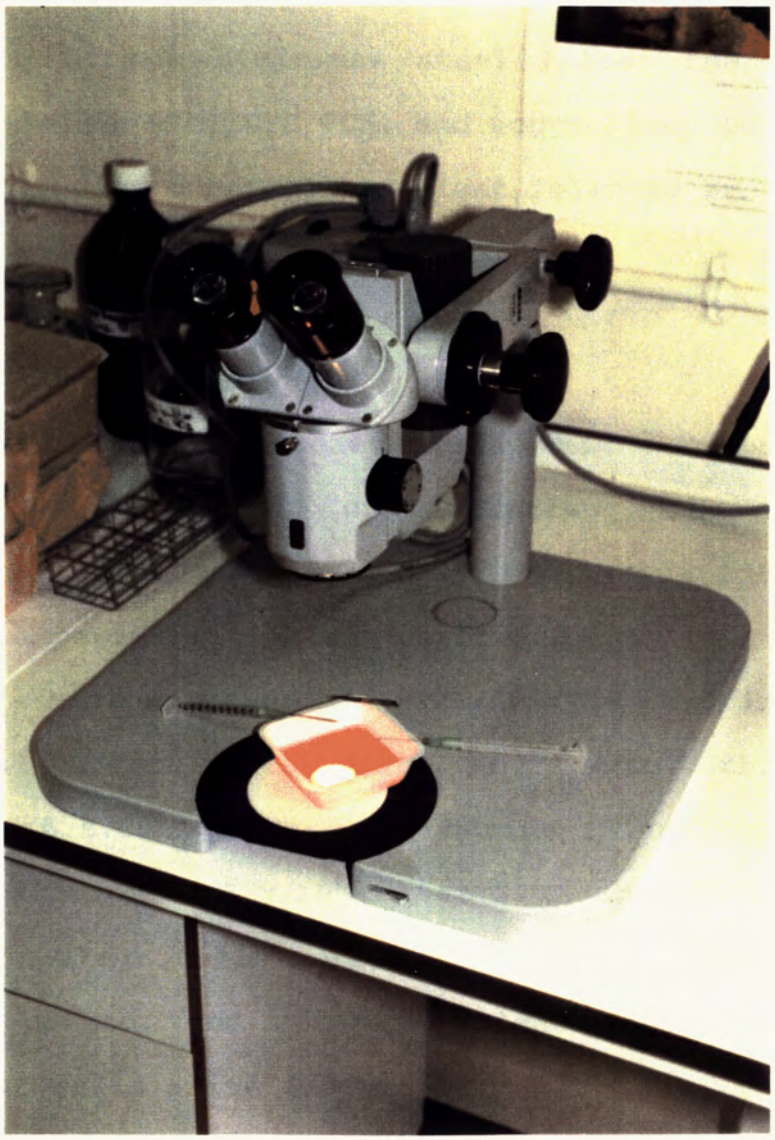


Figure 8.1 Microscope used for mincing mucosal biopsies

and hyaluronidase (Sigma), at conc 1 mg/ml in RPMI/GPS/FCS, for 4 hours, with pipetting every 20-30 minutes. After filtering with a stainless steel filter the cells were resuspended in RPMI/GPS/FCS, and mononuclear cells further separated using a Percollgradient followed by counting in an improved Neubauer chamber.

***Identity and viability***

Identity of the mononuclear cells was assessed by flow cytometry (Department of Immunology, University of Birmingham) and viability using Trypan blue **1:1,** if >95%, further experimentation was performed.

The cells were suspended in RPMI/GPS/FCS, and incubated in Nunc cell culture plates for 48 hours with nothing or with 1 μg/ml of E.Coli lipopolysaccharide (Sigma)(control), at a concentration of $2 \times 10^5$/ml, at 37°C, in a 5% CO, chamber. The mononuclear cells were quantitated at the end of the culture period, and cytokine release corrected for the number of cells alive and viable.

Maximal secretion of the cytokine by E.Coli LPS (Sigma) was performed, to elucidate the optimal concentration of LPS required to produce maximal cytokine production by the isolated mononuclear cells. Biopsies were taken from 5 healthy patients, and cytokine secretion assessed using LPS at concentrations of 0.001, 0.01, 0.1, 1.0, and 10 micrograms/ml (Appendix 2). Potency of the LPS used in these studies was confirmed by experiments on mononuclear

cells isolated from healthy mucosa (Appendix 2).

E Coli LPS (1.0 μg/ml) was also used to assess cytokine production in both healthy and inflamed colon biopsies.

Both the plasma and the supernatants were stored at -80 °C in endotoxin free (Falcon) tubes. The mononuclear cells were quantitated at the end of the culture period and cytokine release corrected for the number of cells alive and viable.

Levels of serum C-reactive protein (CRP) and al-acid glycoprotein were also measured by latex immunoturbidometric assay.

## **8.34** Cytokine Elisa

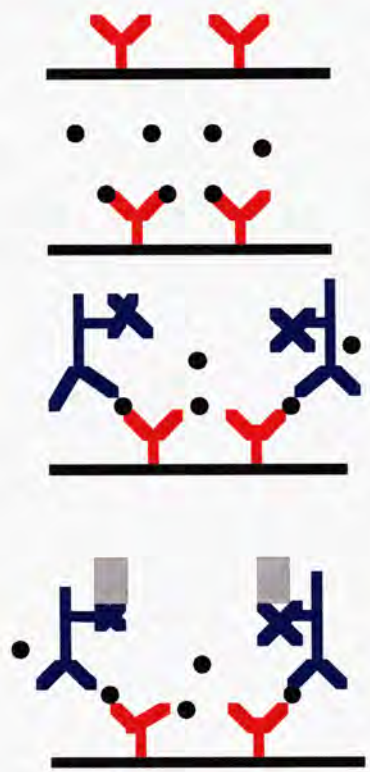
Monoclonal antibody for TNFa (British Biotechnology, UK), IL-1ß (Janssen, Belgium), IL-6 (Monosan, Belgium) and IL-8 (British Biotechnology) were individually coated overnight, in coating buffer (pH 9.6)(Appendix *2),* onto Nunc 96 well plates, at a concentration of 10 μg/ml, except for IL-8 which was coated at 2.5 μg/ml. After washing with PBS-Tween, which was used for all steps of washing, the plates were blocked for one hour with 1% BSA PBS-Tween at 37°C. After washing, 100 μl of standard or test sample (supernatant or plasma) was incubated for one hour at 37°C. After washing, alkaline phosphatase conjugated polyclonal antibody (British Biotechnology) to the specific cytokine was incubated for one hour at 37 °C. The Substrate,

orthophenylene diamine, was then added after further washing, and 20% $H_2SO_4$ was added, after 15 mins, to stop the reaction. The optical density of the plate was then read at 492 nm, these steps are summarized in Figure 8.2.

Results were plotted against a standard curve (Figure 8.3) for each cytokine. High values of test samples were determined by serial dilutions. These assay systems had previously been validated by the Department of Infection, University of Birmingham and were regularly performed in the Department of Renal Medicine, Queen Elizabeth Hospital. The same methods described above were used to measure local mucosal production of TNFa, IL-1ß AND IL-6 in the supernatants. I had also previously carried out cytokine ELISAs on plasma of septic patients [Patel et al, 1994b; Helle et al, 1990].

## 8.35 Statistical analysis

Results were not normally distributed, and therefore have been expressed as medians with ranges. The Mann-Whitney U test has been employed to show differences between the various groups and Wilcoxon rank sum test for comparing paired data. Analysis of variance was carried out using the general linear model.



1. Precoated plate with monoclonal antibody

2. Sample (supernatant or plasma)

3. Polyclonal antibody - conjugate

4. Substrate

5. Optical density measured at 492 nm and the amount of cytokine present determined from a standard curve.

Figure 8.2 Summary of steps for the cytokline ELISA for IL-1, IL-6, IL-8 and TNF.

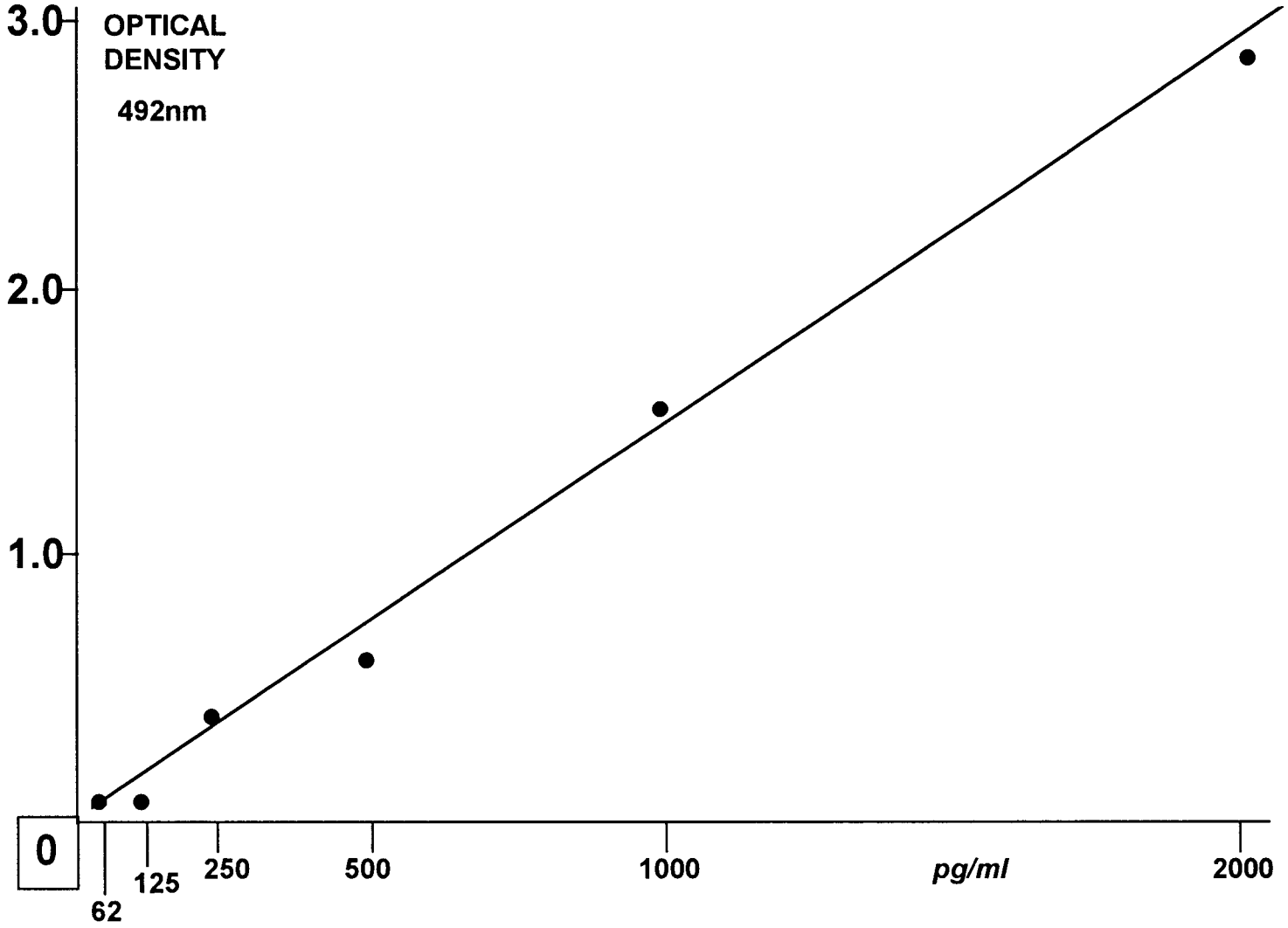



Figure 8.3 Standard curve used for estimation of cytokine concentration.

# 8.4 Interleukin 8

## 8.41 Introduction

Neutrophil accumulation in the inflamed intestinal mucosa is a prominent feature in ulcerative colitis. The granules of neutrophil granulocytes contain a number of enzymes, for example myeloperoxidase and lactoferrin, which are important in the combat against bacteria [Klebanoff, 1975]. These granule enzymes, some of which are proteolytic, can be released upon stimulation, together with cytotoxic oxygen metabolites [Weissmann et al, 1980]. Therefore, activated neutrophils may contribute to tissue damage at sites of inflammation. The factor(s) responsible for the mucosal recruitment of neutrophils in ulcerative colitis are not certain. Human neutrophil activating peptide (NAPl)/interleukin-8 is a single non-glycosylated peptide chain, its main activities include activation and chemoattraction of neutrophils [Peveri et al, 1988; Thelen et al 1988; Lindley et al, 1988]. It was originally isolated from endotoxin treated monocytes but can also be produced by a variety of other cells upon stimulation with a number of cytokines including IL-1 and TNFa [Striter et al, 1988; Striter et al, 1989, Watson et al, 1988].

## Aims, patients and methods

As described earlier.

## 8.42 Results (IL-8)

There was no significant difference in the values of plasma IL-8 in the study groups compared with controls (Figure 8.4) .

However, there were significant differences in IL-8 production by lamina propria mononuclear cells, in vitro, from the colonic and ileal biopsies in the disease study groups when compared with healthy controls as shown in Figure 8.5 and 8.6 respectively.

Significantly higher values of IL-8 were recorded, from rectosigmoid biopsies, in patients with active UC and Crohn's colitis, then from those with inactive UC and healthy controls ($P < 0.001$) {Figure 8.5).

Out of the 15 patients with active UC, 9 who were receiving steroid enemas, had significantly lower IL-8 levels recorded median (range; pg/ml) 480 (280-910), than those who didn't 640 (500-1300).

The distribution of IL-8 in the ileal biopsies revealed no significant differences between active and inactive UC with healthy controls but significantly higher values of IL-8 were demonstrated between Crohn's ileum and healthy controls (Figure 8.6).

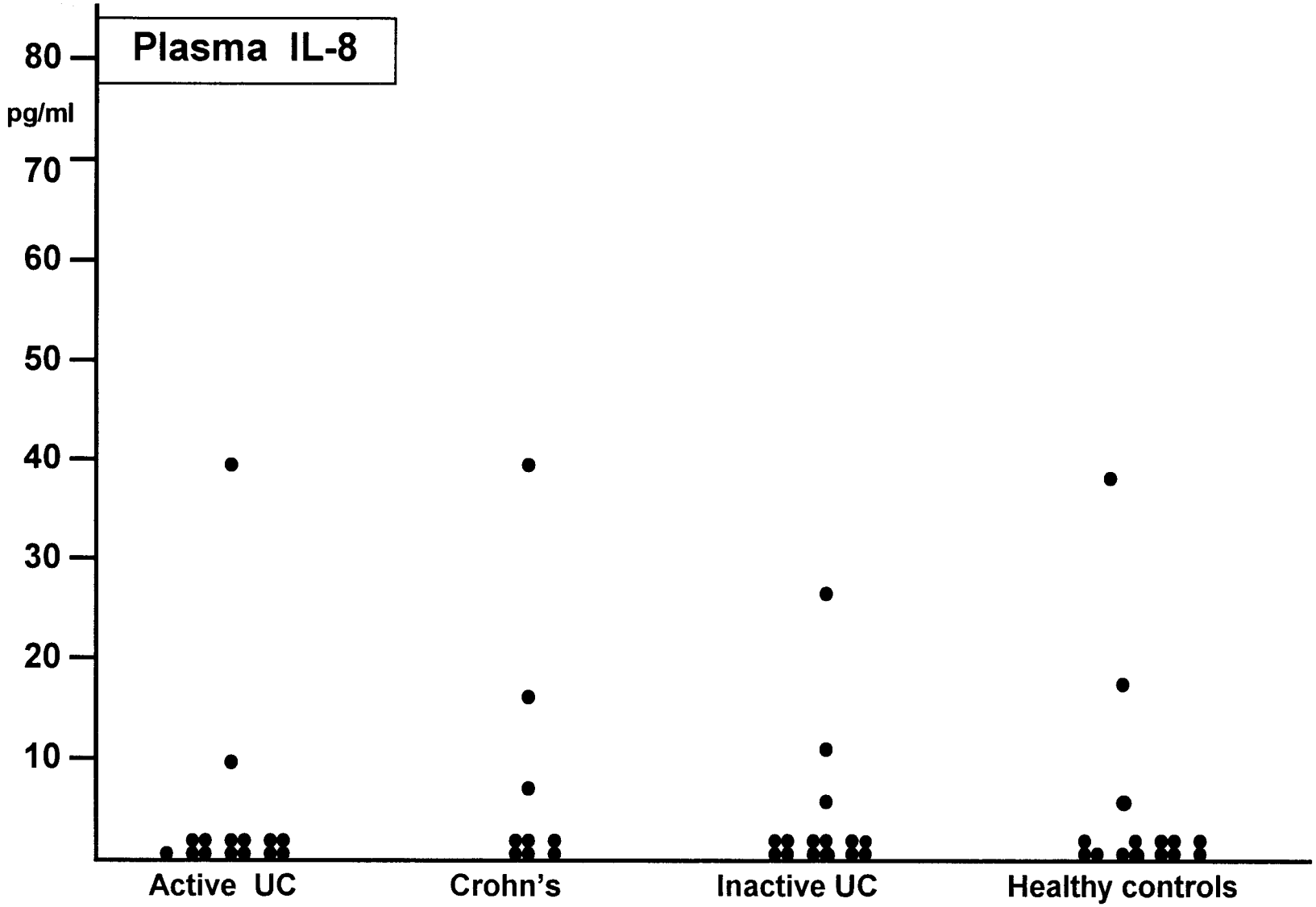



Figure 8.4 Plasma levels of IL-8

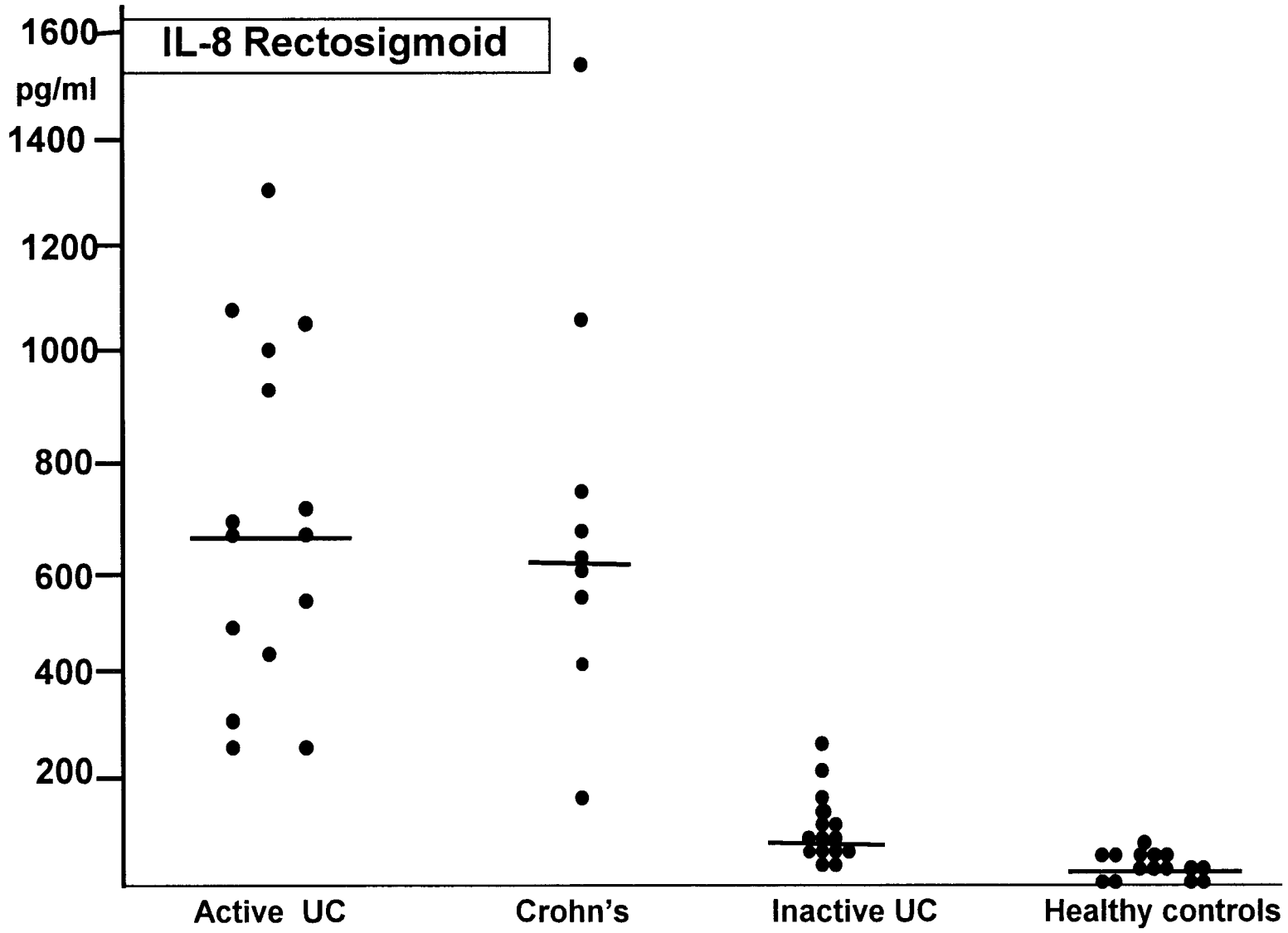



Figure 8.5 IL-8 production from mononuclear cells from rectosigmoid colon

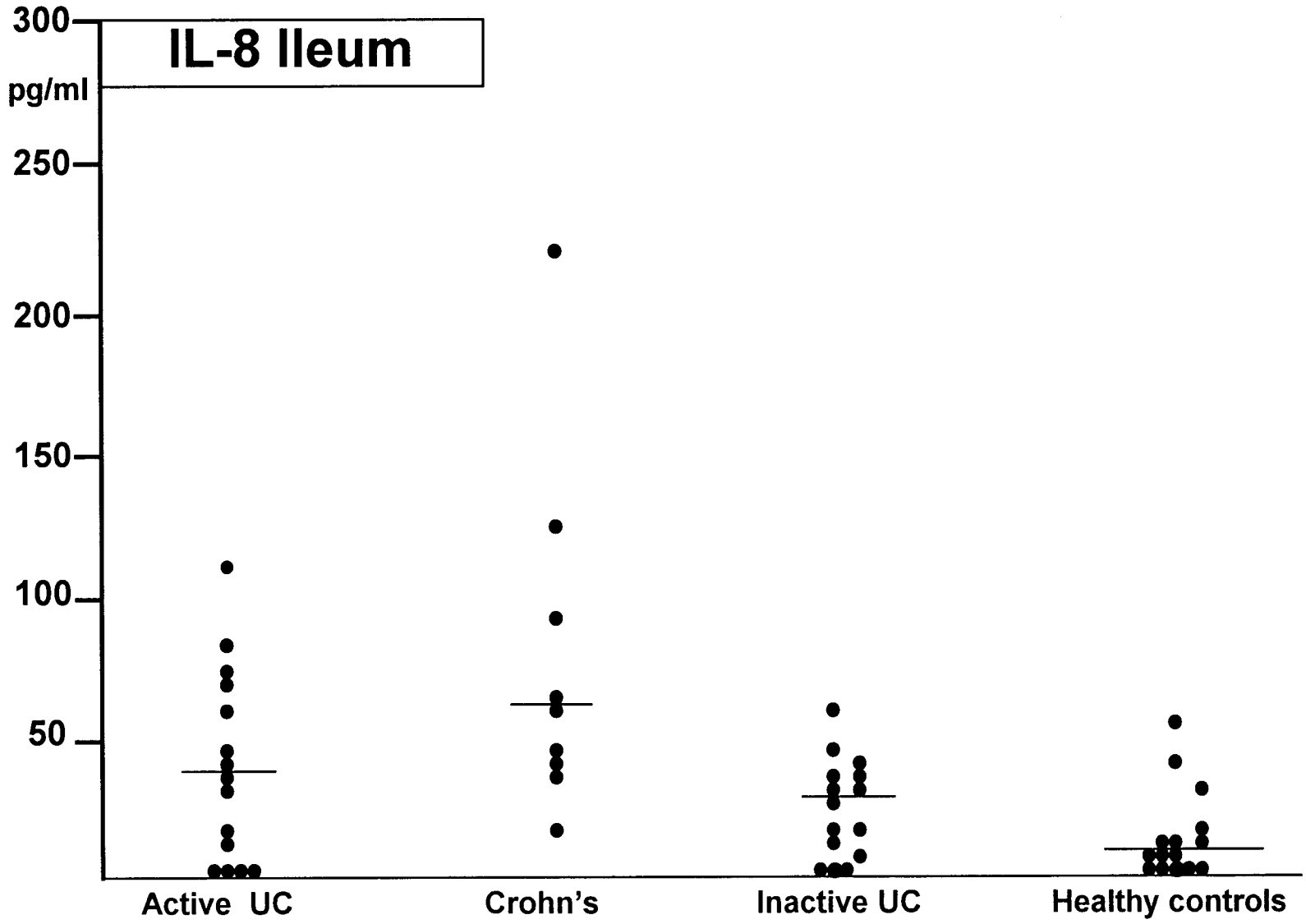



Figure 8.6 IL-8 production by mononuclear cells from ileum

Table 8.2 P-values comparing IL-8 levels in inflammatory bowel disease with healthy controls (P<0.05*).

| | PLASMA | ILEUM | RECTOSIGMOID |
|---|---|---|---|
| UC(ACTIVE)* | 0.220 | 0.113 | 0.043* |
| CROHN'S* | 0.066 | 0.045* | 0.014* |
| UC(INACTIVE)* | 0.332 | 0.340 | 0.120 |

Analysis of variance did not reveal any significant difference from the use of systemic steroids and IL-8 production from isolated mucosal mononuclear cells (F=0.79, P>0.10).

Stimulation of isolated rectosigmoid mononuclear cells by 1.0 μg/ml of E. Coli LPS (Sigma) revealed increased production of IL-8 from [median, range, pg/ml]:- 670 (250-1340) to 710 (200-1200) in inflamed UC colon; and increased production in healthy colon from 30(20-70) to 40(20-100). This difference was statistically significant in the two groups (P<0.05).

The intra-assay and inter-assay coefficients of variation for the IL-8 ELISA assay were 7.5% and 9.1% respectively. Preliminary studies showed that the amount of IL-8 produced by mononuclear cells isolated from inflamed rectosigmoid mucosa in IBD was usually in the range detected by the assay.

## 8.43 Discussion IL-8

These results show that the degree of disease activity was proportional to the levels of IL-8 measured from the colonic biopsies but not from the plasma or ileal biopsies in UC.

Recent studies have also reported that plasma IL-8 levels do not reflect the local disease activity in IBD [Raab et al, 1993]. Increased IL-8 production locally in the inflamed colonic mucosa in active UC and Crohn's colitis may provide an explanation for the early recruitment of neutrophils and their subsequent activation [Raab et al, 1993].

Bacterial LPS, immune complexes and several cytokines including IL-2, IL-3, TNFa , GM-CSF and IL-1 are strong inducers to IL-8 production in mononuclear phagocytes [Baggiolini et al, 1989; Striter et al, 1988; Striter et al, 1989; Watson et al, 1988]. It is highly likely that IL-8 is a principal mediator of inflammation in ulcerative colitis, although many other cytokines are now being implicated.

Possible steps leading to colonic inflammation can be illustrated thus:-

Autoimmune stimulus for UC

Macrophages and T-cell activation

Increased IL-8 production

Neutrophils

Tissue inflammation

The mechanisms recruiting and activating mononuclear cells and macrophages in the intestinal mucosa are probably multifactorial. The cellular source of IL-8 in inflamed mucosa has been shown to be from macrophages, T cells and also from neutrophils to a lesser extent. Intradermal IL-8 has been shown in animals to cause plasma exudation and massive neutrophil infiltration [Colditz et al, 1989]. It also causes these cells to degranulate and exhibit a respiratory burst.

IL-8 probably plays an important role in neutrophil diapedesis through vascular endothelium and the focal recruitment of neutrophils at inflamed sites [Peveri et al, 1988; Thelen et al, 1988; Lindley et al, 1988, Van Damme et aï, 1988, Baggiolini et al, 1989].

Adhesion molecules also play a significant role in the trasmigration of neutrophils across vascular endothelium as discussed in Chapter 7.

Considering the part IL-8 plays in the disease process it may be possible to control tissue damage and inflammation that occurs in UC and Crohn's by controlling levels of IL-8 locally.

2 possible methods are:-

i.Steroids (used currently)

ii.Anti-interleukin-8 therapy

## Steroids

In this study, treatment with systemic steroids had little

significance on cytokine production in vitro. However in those patients who received steroid enemas in addition had lesser degree of inflammation in the rectosigmoid region and significantly lower production of IL-8 by isolated MNCs.

But I noted that the inflammation was reduced only in the rectosigmoid region on opening the colectomy specimens and not along the whole length of the colon. Perhaps, in time, a method of local application of steroids, along the whole length of the colon might become feasible but this cannot be the answer in the long term as steroid therapy is fraught with disadvantages.

## Anti-IL-8 therapy

Recent technology has made possible the production of anti-IL-8 antibodies. The advantages of the use of anti-IL-8 is that it is specific and thus could effectively block the cascade process illustrated. Several disadvantages are apparent:-

i) UC and Crohn's are chronic diseases, and therefore multiple doses of anti-IL-8 would have to be administered.

ii) Anti-IL-8 is expensive to manufacture at present

iii) Generalised side effects eg, fever, rashes, metabolic disturbances and sweating are likely (these side effects I witnessed first hand, when administering anti-TNF monoclonal antibody to patients with severe sepsis).

iv) Long term safety and efficacy of anti-IL-8 have yet to be established.

# 8.5 TNFa

## 8.51 Introduction

Tumour necrosis factor a (TNFa)/cachectin is a cytokine produced mainly by activated macrophages and monocytes. In the last 4 years there has been immense interest in TNF, both as a mediator of tissue maturation and local immunity, and as a destructive agent capable of causing profound cachexia and tissue injury. The balance of beneficial and deleterious effects are determined by its level of production and its interaction with other mediators [Tracey et al, **1988**; Beutler et al, **1988**; Ziegler, **1988**; Tracey et al, **1989**].

TNFa is produced in response to inflammation and sepsis. In addition to its role in the elimination of pathogens, TNFa induces necrosis of certain types of tumour in vivo [Carswell et al, **1975**] and is cytotoxic for many transformed cell lines in vitro [Tracey et al, **1989**]. However, high levels of TNFa in vivo can be detrimental since they induce metabolic acidosis, wasting, and suppression of haematopoiesis. Abnormal expression of this pleiotropic molecule leads to many of the systemic manifestations of chronic disease. These include cachexia [Beutler et al, **1988**], suppression of haemopoeitic colony formation and increased red blood cell destruction leading to anaemia [Tracey et al, **1988**], and acts as an endogenous pyrogen [Dinarello et al, **1986**].

The local and systemic effects of the inflammatory bowel diseases together with the observation that the intestinal tract contains receptors for TNFa [Beutler et al, **1985]** suggests that TNF may be involved in many of these effects.

## Aims, patients and methods

As described earlier.

## 8.52 Results

The distribution of TNFa in the plasma, rectosigmoid colon and ileum is shown in Figures **8.7 - 8.9** respectively.

There was no significant difference noted in the values of plasma TNFa in the study groups compared with controls (Figure **8.7)** in UC but slightly higher levels of TNF were noted in patients with Crohn's.

However, there were significant differences in the TNFa production by lamina propria mononuclear cells from colon in patients with active UC and Crohn's colitis when compared with healthy controls ($P < 0.001$)(Figure **8.8).**

Out of the **15** patients with active UC, **9** who were receiving steroid enemas had significantly lower TNFa levels recorded median (range; pg/ml) **90** (0-440), than those six who didn't **190 (50-710).**

The distribution of TNFa in the ileal biopsies revealed no significant differences between active and inactive UC with healthy controls but significantly higher values of TNFa

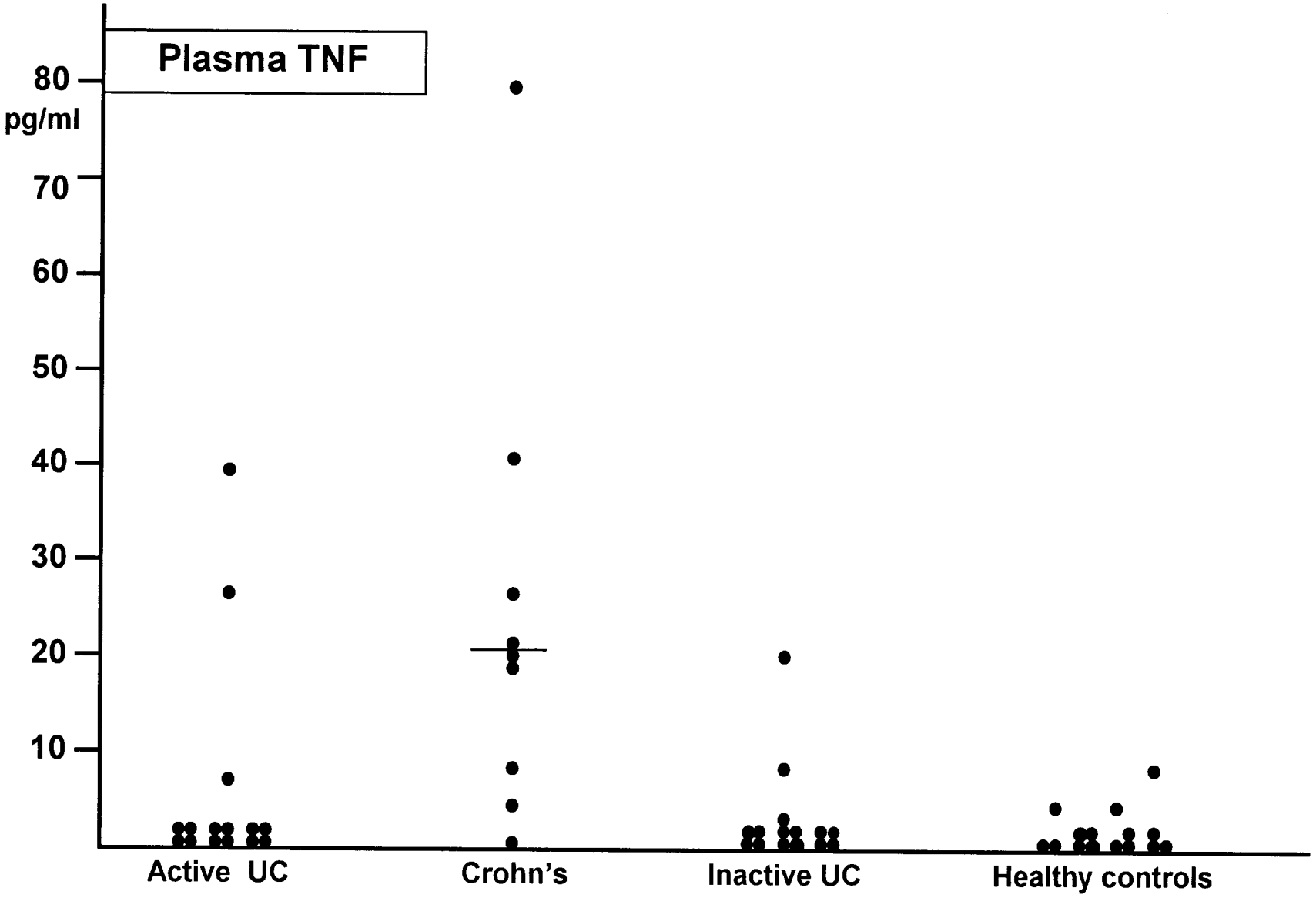



Figure 8.7 Plasma levels of TNF

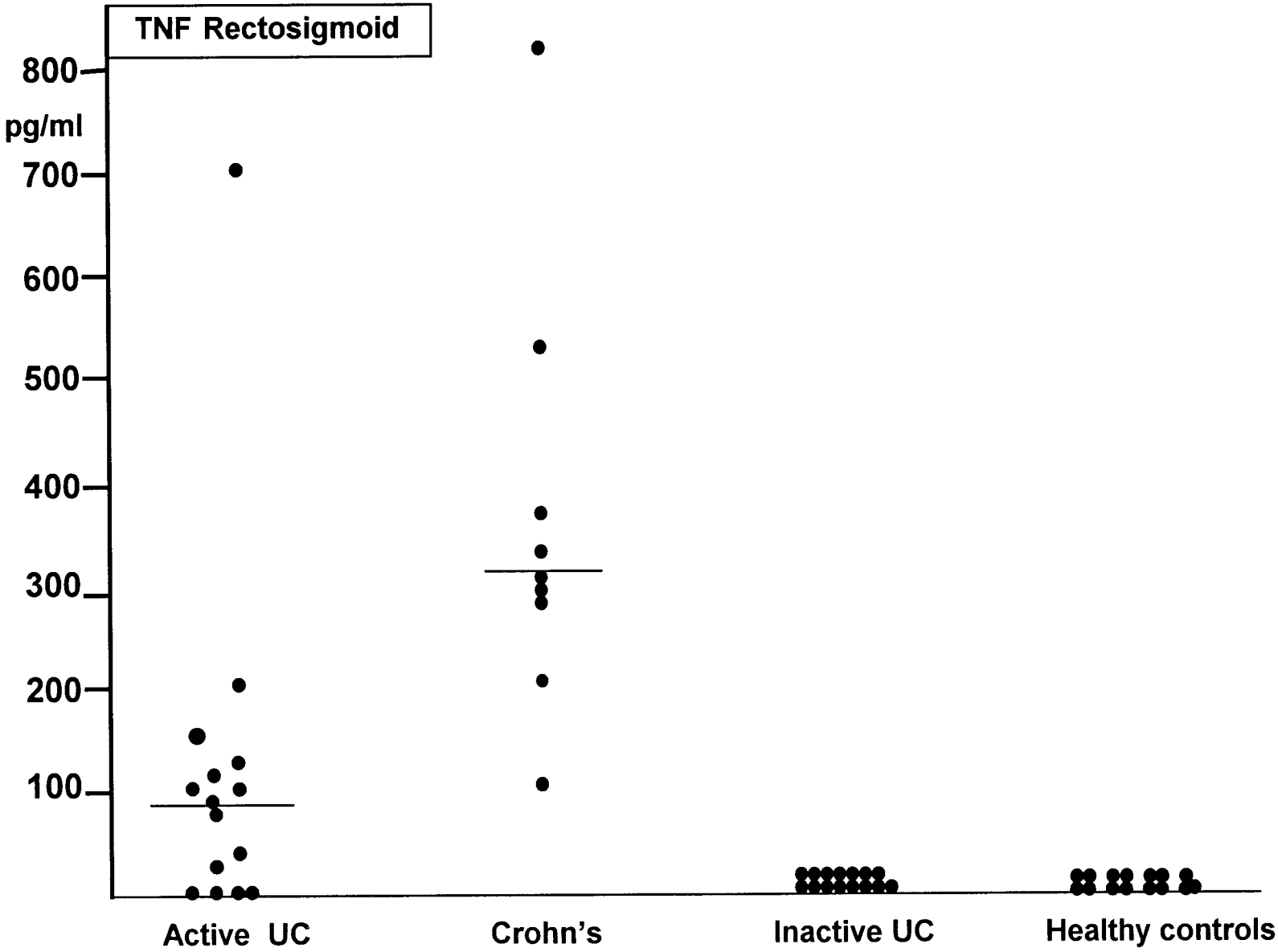



Figure 8.8 TNF production from mononuclear cells from rectosigmoid colon

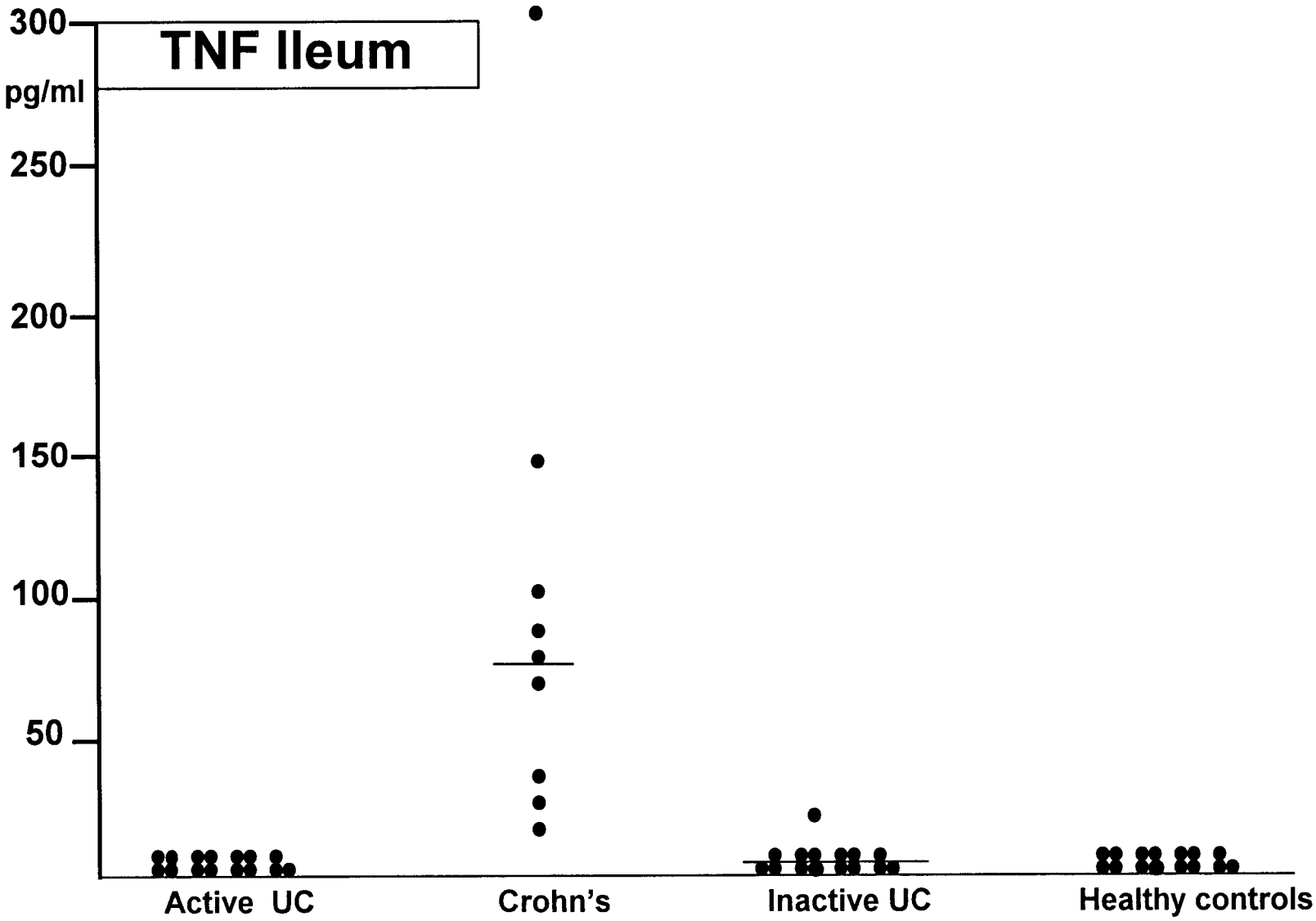



Figure 8.9 TNF production by mononuclear cells from ileum

were demonstrated between Crohn's ileum and healthy controls (Figure 8.9). Increased TNFa levels were demonstrated, from MNCs isolated from ileum, in patients with Crohn's disease, which was reported as histologically normal.

Table 8.3. P-values comparing TNFa levels in inflammatory bowel disease with healthy controls ($p<0.05$*).

| | SERUM | ILEUM | RECTOSIGMOID |
|---|---|---|---|
| UC(ACTIVE)* | 0.420 | 0.520 | 0.032* |
| CROHN'S* | 0.676 | 0.021* | 0.034* |
| UC(INACTIVE)* | 0.452 | 0.650 | 0.320 |

Analysis of variance did not reveal any significant difference from the use of systemic steroids and TNFa production from isolated mucosal mononuclear cells ($F=0.32$, $P>0.10$).

Stimulation of isolated rectosigmoid mononuclear cells by 1.0 μg/ml of E Coli LPS (Sigma) revealed increased production of TNFa from [median, range, pg/ml]:- 100 (0-710) to 120 (0-600) in inflamed UC colon; and increased production in healthy colon from 0(0-40) to 20(0-50). This difference was statistically significant in the two groups ($P<0.05$). The intra-assay and inter-assay coefficients of variation for the TNF ELISA assay were 6.7% and 11.2% respectively. Preliminary studies showed that the amount of

TNFa produced by mononuclear cells isolated from inflamed rectosigmoid mucosa in IBD was usually in the range detected by the assay.

## 8.53 Discussion [TNF]

TNFa production is increased in the mucosa of patients with active UC and Crohn's colitis compared with controls. Possible reasons for this are that this is directly proportional to the degree of colonic inflammation from where the biopsies were taken. On the other hand, if this was simply the case, why then was increased TNFa expressed in Crohn's ileum which was histologically normal?

As is well known, at present, there is no satisfactory animal model of IBD and particularly it is worth noting that TNFa has not been found to be increased in experimental models **(1-chloro-2,4-dinitrobenzene**{DNCB}) of colitis in rabbits, compared to controls [Mack et al, **1992].**

The mononuclear cells from mucosal biopsies taken from patients with Crohn's disease, secreted relatively more TNFa compared to active UC. This cannot simply be explained on the degree of colonic inflammation. TNFa production in Crohn's disease has been linked to the production of granulomas and this may have some bearing on the difference seen.

As with IL-8, these results looking at TNFa also did not

correlate with systemic steroid therapy. Again, this can be explained by the fact that the mucosal biopsies taken from areas of severe inflammation had mononuclear cells which are already activated and these cells are unresponsive or refractory to steroid therapy.

It is quite evident from the numerous reports in the literature that it is a multifunctional cytokine [Beutler et al, **1987]**. TNFa infused systemically into rats causes intestinal haemorrhage and necrosis [Sun et al, **1988]**. This is due to breakdown in the intestinal vascular network caused by TNF-induced pro-coagulant activity of the endothelial cells and increased neutrophil adherence and mediator release [Cerami et al, **1988]**. TNFa is also angiogenic and may help in the healing of diseased mucosa [Schroder et al, **1987]**. TNFa has also been shown to be important in the granulomatous response to mycobacteria in mice [Kindler et al, **1989]**.

**A** macrophage influx is a feature of both ulcerative colitis and Crohn's disease. However, it is still unknown why granulomas are seen only in Crohn's disease.

TNF also has cachectic activity [Oliff et al, **1987]** and may contribute to the weight loss frequently seen in children with IBD (as do other factors such as malnutrition).

Raised plasma CRP levels are also associated with IBD. Infusion of graded doses of TNFa into patients leads to a dose dependant increase in plasma CRP levels [Michie et al, **1988]**.

The finding of increased TNFa in colonic mucosa in active IBD supports the idea that TNFa is one possible mediator of enhanced IL-8 synthesis in inflamed mucosa.

Attempts by some investigators [Macdonald et al, 1990] to identify the mucosal cell type secreting the TNFa have been equivocal. It is likely that more than one particular cell population is responsible for TNFa release and also that numerous other mediators are also released simultaneously in the inflamed mucosa.

In recent years there has been much interest in TNF being a major mediator in the inflammatory processes related to sepsis, other inflammatory diseases, neoplastic diseases and autoimmune diseases. Indeed animal and human studies have reported clinical benefit in the use of anti-TNFa monoclonal therapy in severe sepsis [Tracey et al, 1987; Hinshaw et al, 1990]. Recently, I have participated in a large, prospective, multicentre, multinational trial on the efficacy of murine monoclonal antibody to tumour necrosis factor in severe sepsis. Outcome in the study was assessed as survival to 28 days. Results have not been finalised but the interim analysis is encouraging. A definite improvement in mortality by 9% has been found when given to patients with septic shock , compared to placebo. In chronic IBD the role of anti-TNFa still needs further study.

It is also unlikely that TNFa production is specific for IBD and that as more enteropathies are studied TNFa production may be a common feature.

# 8.6 Interleukin 1ß

## 8.61 Introduction

Interleukin 1 (IL-1) is a polypeptide produced, predominantly, by stimulated macrophages and monocytes. Two structurally distinct forms of IL-1, IL-a and IL-1ß have been identified. They are coded by two separate genes on chromosome 2 and messenger RNA for IL-1ß predominates over that coding for IL-a [Dinarello, 1988].

Although they share only 26% sequence homology, their biological activities are, for the most part, identical [Enders et al, 1987]. This could be explained by identical cell surface receptors for the two forms of IL-1 [Dower et al, 1986].

Interleukin 1 exerts an influence over a wide range of biological functions. It acts as an endogenous pyrogen and induces hepatocytes to synthesize acute phase proteins – for example serum amyloid A, C-reactive protein and fibrinogen [Dinarello, 1988; Enders et al, 1987; Dinarello, 1984].

It activates T-lymphocytes [Mizel, 1982], increases antibody synthesis by B-cells [Falkoff et al, 1983] and induces granulocyte release from the bone marrow [Kampschmidt, 1981]. It enhances fibroblast collagen production [Schmidt et al, 1982] and alters prostaglandin production.

Information, at present, on IL-1ß production in UC and

Crohn's disease is sparse.

## Aims, patients and methods

As described earlier.

## 8.62 Results

The distribution of IL-1ß in the plasma, rectosigmoid colon and ileum is shown in Figures 8.10, 8.11 and 8.12 respectively.

There was no significant difference in plasma IL-1ß values between the study groups, Figure 8.10.

Preliminary studies showed that the amount of IL-10 produced by mononuclear cells isolated from inflamed rectosigmoid mucosa in IBD was usually In the range detected by the assay. There was significantly more IL-1ß produced by mononuclear cells isolated from active ulcerative colitis and Crohn's disease mucosa compared to normal mucosa and inactive UC ($P<0.01$)(Figure 8.11).

Out of the 15 patients with active UC, 9 who were receiving steroid enemas had lower IL-8 levels recorded median (range; pg/ml) 190 (20-420), than those six who didn't, 220 (40-440).

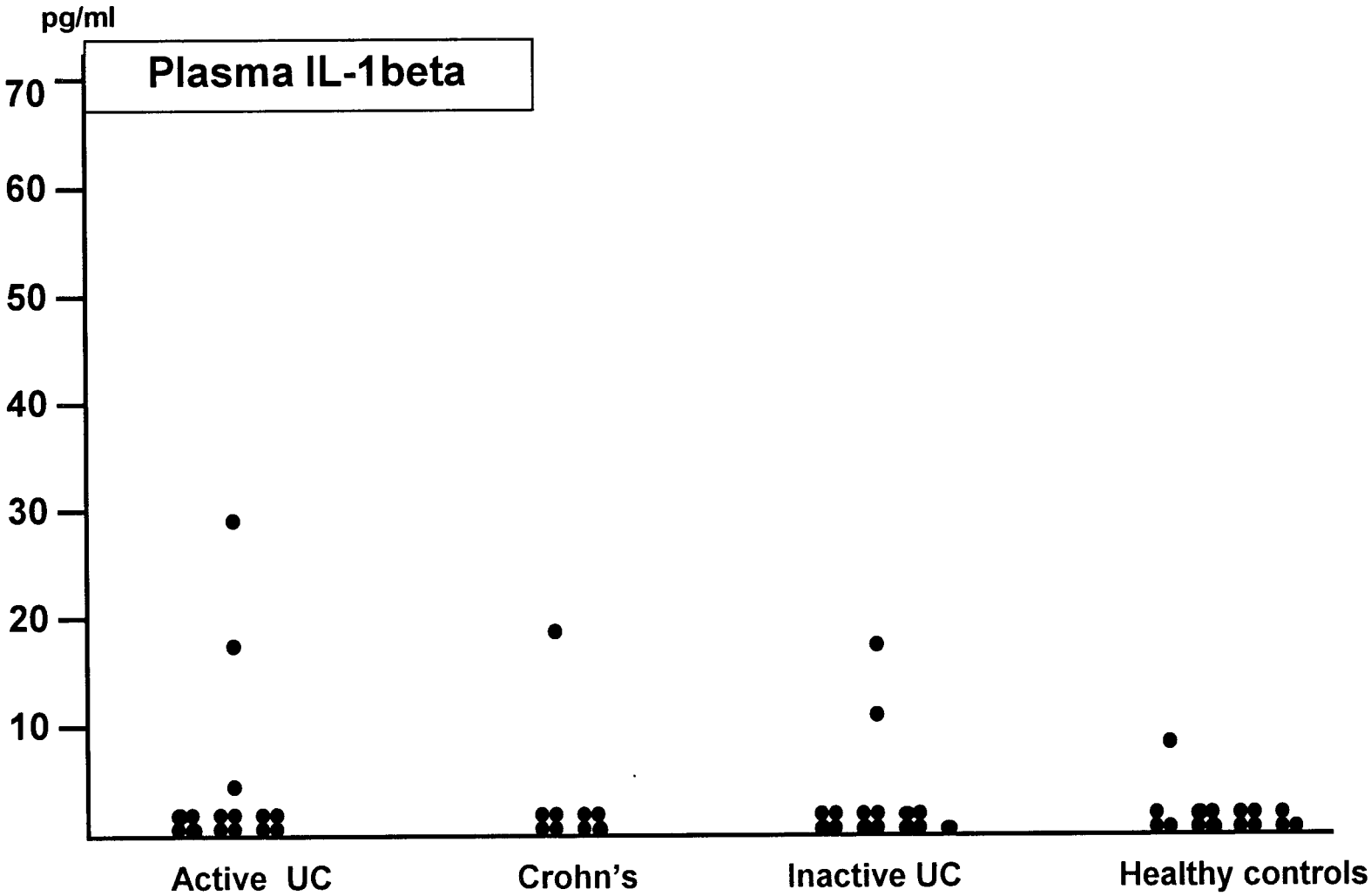



Figure 8.10 Plasma levels of IL-1beta

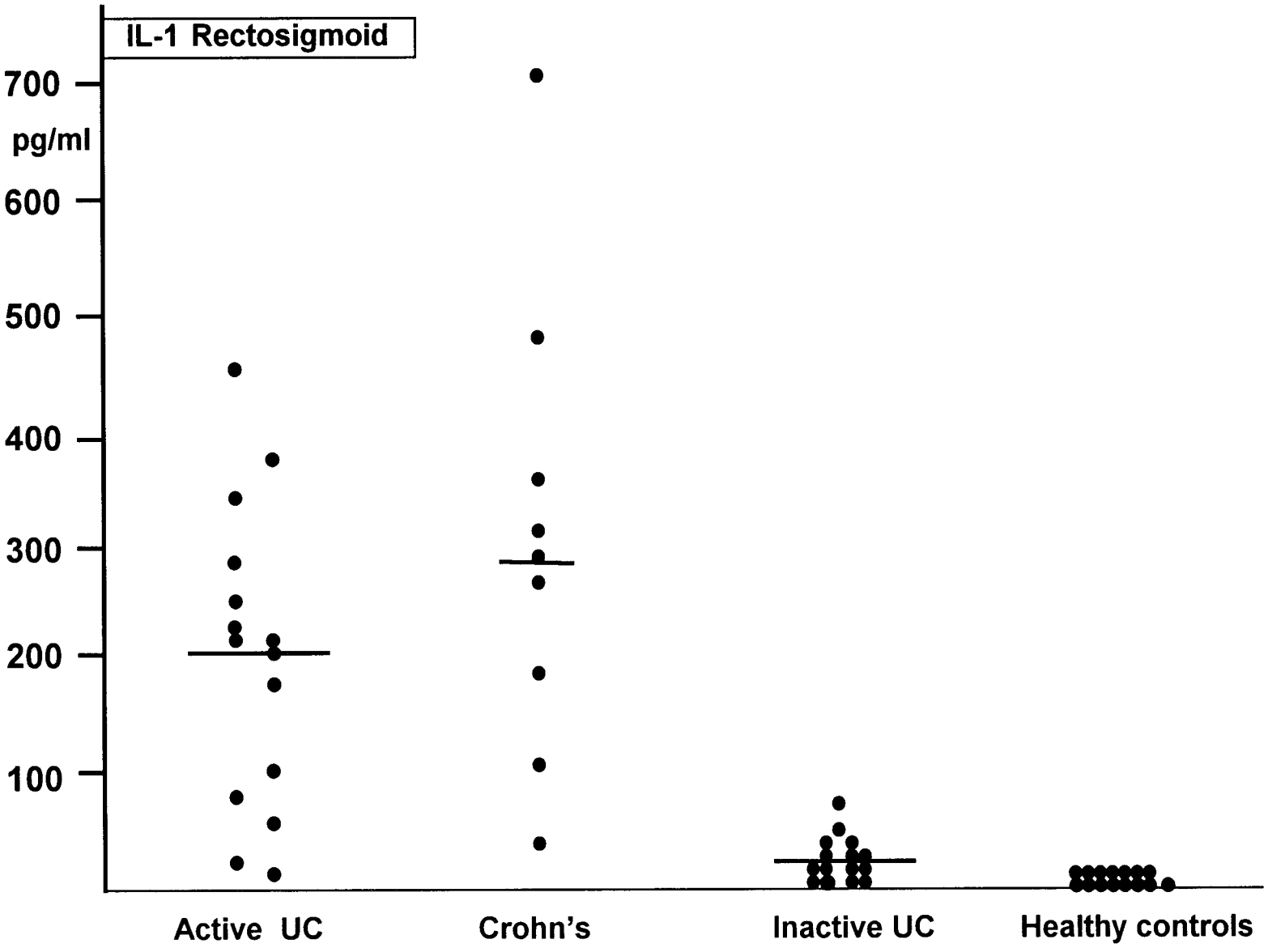



Figure 8.11 IL-1 production by mononuclear cells from rectosigmoid colon

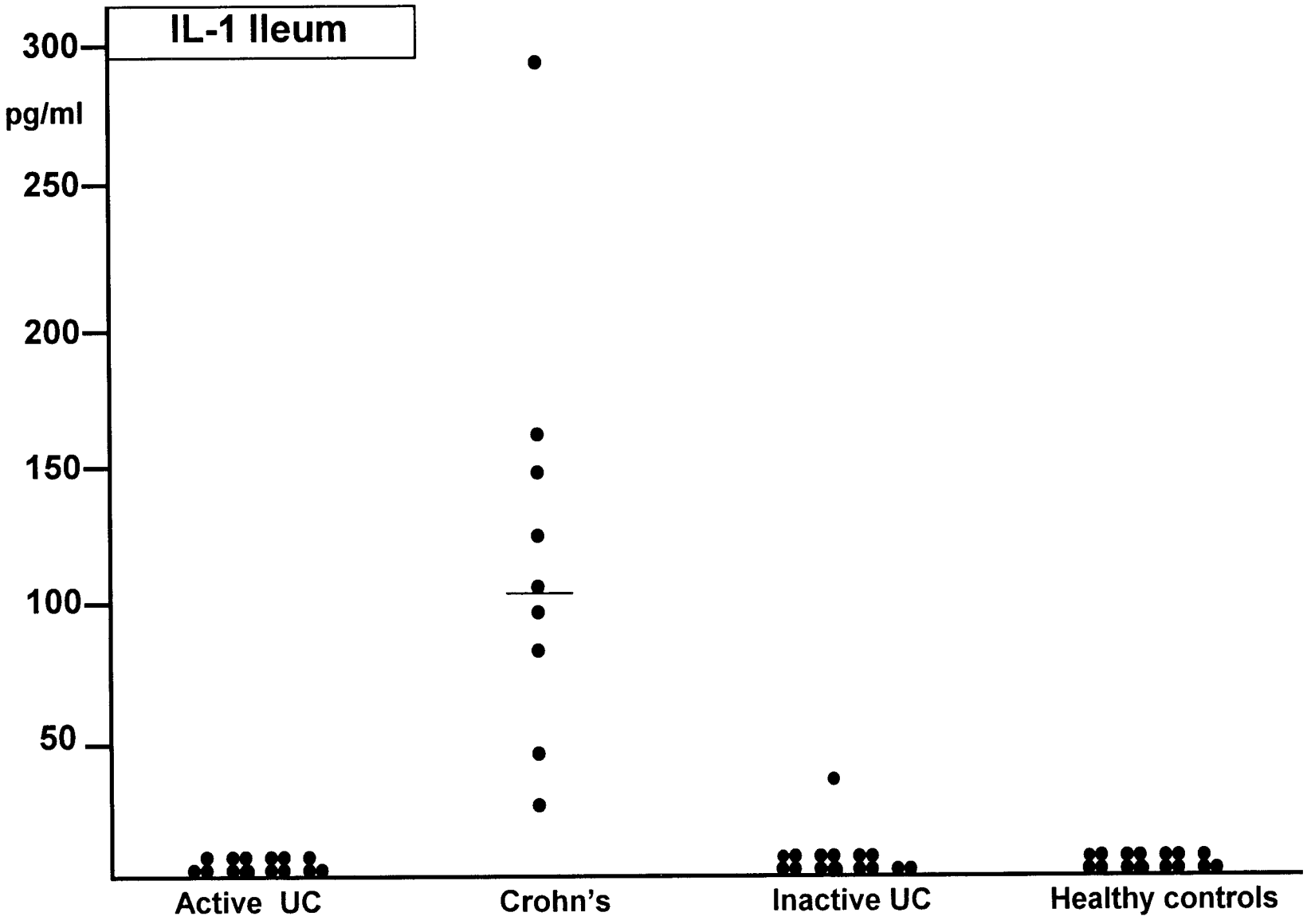



Figure 8.12 IL-1 production by mononuclear cells from ileum

Table 8.4 P-values comparing IL-10 levels in inflammatory bowel disease with healthy controls **(P<0.05*).**

| | PLASMA | ILEUM | RECTOSIGMOID |
|---|---|---|---|
| UC(ACTIVE)* | 0.220 | 0.113 | 0.043* |
| CROHN'S* | 0.066 | 0.045* | 0.014* |
| UC(INACTIVE)* | 0.332 | 0.340 | 0.120 |

Significantly higher values of IL-1ß were recorded from ileal biopsies in patients with Crohn's colitis than from any other group (Figure 8.12).

Analysis of variance did not reveal any significant difference from the use of systemic steroids and IL-10 production from isolated mucosal mononuclear cells (F=0.54 **P>0**.10)**.**

Stimulation of isolated rectosigmoid mononuclear cells by 1.0 μg/ml of E. Coli LPS (Sigma) revealed increased production of IL-10 from [median, range, pg/ml]:- 220 (20-450) to 240 (30-500) in inflamed UC colon; and increased production in healthy colon from 20(0-60) to 40(20-120). This difference was statistically significant In the two groups (P<0.05).

The intra-assay and inter-assay coefficients of variation for the IL-10 ELISA assay were 11.5% and 12.4% respectively.

## 8.63 Discussion

This study shows that mononuclear cells isolated from colonic mucosa of patients with active IBD cultured ***in*** vitro produced much greater amounts of IL-1ß than mononuclears isolated from normal colonic mucosa.

This increase appears to be on a per cell basis as there was no significant difference in the proportion of macrophages present in the mononuclear cell population from normal or inflamed colonic mucosa.

Enhanced production of IL-1ß by cells isolated from inflamed colons is likely to be caused by the presence of activated macrophages [Besedovsky et al, 1986].

Stimulation with LPS enhanced production of this cytokine, by mononuclear cells isolated from inflamed and normal colonic mucosa - this is in contrast to the findings by Mahida et al who found that LPS resulted in enhanced production of IL-1ß only in inflamed mucosa only [Mahida et al, 1989]. These particular findings have not been borne out by other investigators or by myself, in any case there does not appear to me, to be any significance from LPS stimulation because LPS has been shown to induce cytokine production in all mononuclear cells irrespective of tissue type. However, the study by Mahida et al, seems to suggest that inflammation is caused by an elite population of monocytes. The same reasoning can be applied to IL-8, TNFa and IL-1ß production by LPS.

Macrophages are the major producers of IL-1ß although other

cells have also been shown to produce it [Dinarello, 1988]. Mahida et al have shown that IL-10 production is markedly reduced by depleting the macrophages, by panning with a monoclonal antibody [Mahida et al, 1989].

Activation of mononuclear cells has previously been shown in IBD [Mee et al, 1980; Doe et al, 1982], in addition, increased monocyte turnover has been demonstrated [Meuret et al, 1978]. It seems likely that during an inflammatory response, further monocytes are recruited into the mucosa by the action of IL-10, which may then produce further release of IL-10 and the generation of oxygen radicals, thus triggering the development of an even greater inflammatory response.

The majority of the IBD patients studied were receiving corticosteroid treatment. Corticosteroids have been shown to inhibit production of pro-inflammatory cytokines in vitro [Kern et al, 1988]. In the current study, biopsies taken from inflamed sites had greater cytokine production compared to non-inflamed sites, I believe these results although, also, of an in vitro study more accurately show what is happening in vivo.

In addition to being an important mediator of inflammatory and immunological reactions [Dinarello, 1988; Maury, 1986] in IBD, IL-1ß is likely to be involved in the repair of damaged connective tissue by synthesizing and remodelling components of the matrix like collagen, fibronectin and proteoglycans [Schmidt et al, 1982; Postlethwaite et al,

**1983] and may contribute to fibrosis. It may also be involved in the induction *of* mucus secretion. Endothelial cells and fibroblasts also produce IL-8 in response to IL-1ß and** TNFa **[Striter et al, 1988; Striter et al, 1989; Schroeder et al, 1989], this process indirectly leads to neutrophil activation, diapedesis and degranulation.**

# 8.7 Interleukin-6

## 8.71 Introduction

Increased serum concentrations of acute phase proteins such as a1-acid glycoprotein, al anti-trypsin, CRP, al antichymotrypsin, ß2 microglobulin, serum amyloid A have been reported to be useful markers for the assessment of disease activity in chronic IBD [Weeke et al, 1971; Marner et al, 1975; Descos et al, 1979]. These acute phase proteins are non-specific markers of inflammation and are synthesized mainly in the liver **[Koj**, 1985].

Several cytokines such as IL-6 [Gauldie et al, 1987; Andus et al, 19871; IL-1 [Ramadori et al, 1985], TNF [Darlington et al, 1986], IFN [Zuraw et al, 1990], TGFß [Mackiewicz et al, 1990] have been shown to stimulate acute phase protein synthesis in hepatocytes of various species. However, in the human, IL-6 has been found to be the most important inducer of acute phase protein synthesis (Figure 8.13 – IL-6 and acute phase protein production) whereas, IL-1ß and TNFa play only minor roles [Castell et al, 1988, Castell et al, 1989].

IL-6 is synthesized by several cell types including macrophages, T-cells, monocytes, endothelial cells and fibroblasts. These cells can all be activated independantly by various different stimuli such as LPS, TNFa, IFN, and viruses.

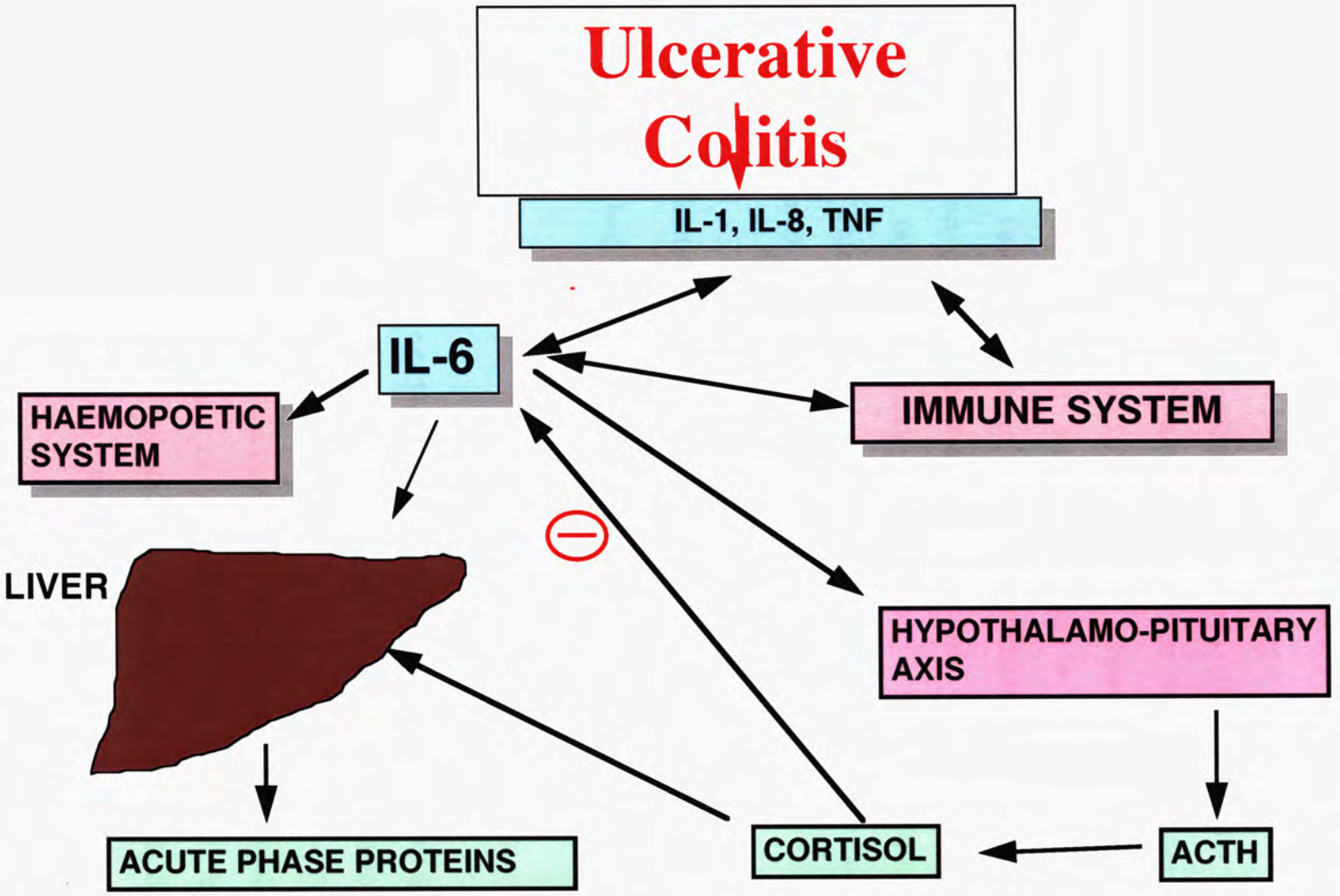



Figure 8.13 IL-6 and the acute phase reaction in UC

Increased serum IL-6 has been detected during various acute phase conditions such as major surgery [Nichimoto et al, **1989]**, renal graft rejection [Van Oers et al, **1988]** and acute pancreatitis [Leser et al, **1991]**. In acute pancreatitis IL-6 has been reported to be a more sensitive marker of disease severity than CRP [Leser et al, **1991]**.
It has been reported that in Crohn's disease there is increased levels of serum IL-6 detectable through continuos stimulation of IL-6 producing cells but this was not found in active UC sera [Gross et al, **1992]**. However there have been few studies looking at plasma levels simultaneously with mucosal levels in the literature.

## Aims, patients and methods

As described earlier.

## 8.72 Results

The distribution of IL-6 in the plasma, rectosigmoid colon and ileum is shown in Figures **8.14**, **8.15** and **8.16** respectively.
Significantly higher values of IL-6 were recorded from rectosigmoid biopsies in patients with active UC and Crohn's colitis from those with inactive UC and healthy controls ($P < 0.001$) {Figure **8.15)**. Out of the **15** patients with active UC, **9** who were receiving steroid enemas had significantly lower IL-6 levels recorded median (range;

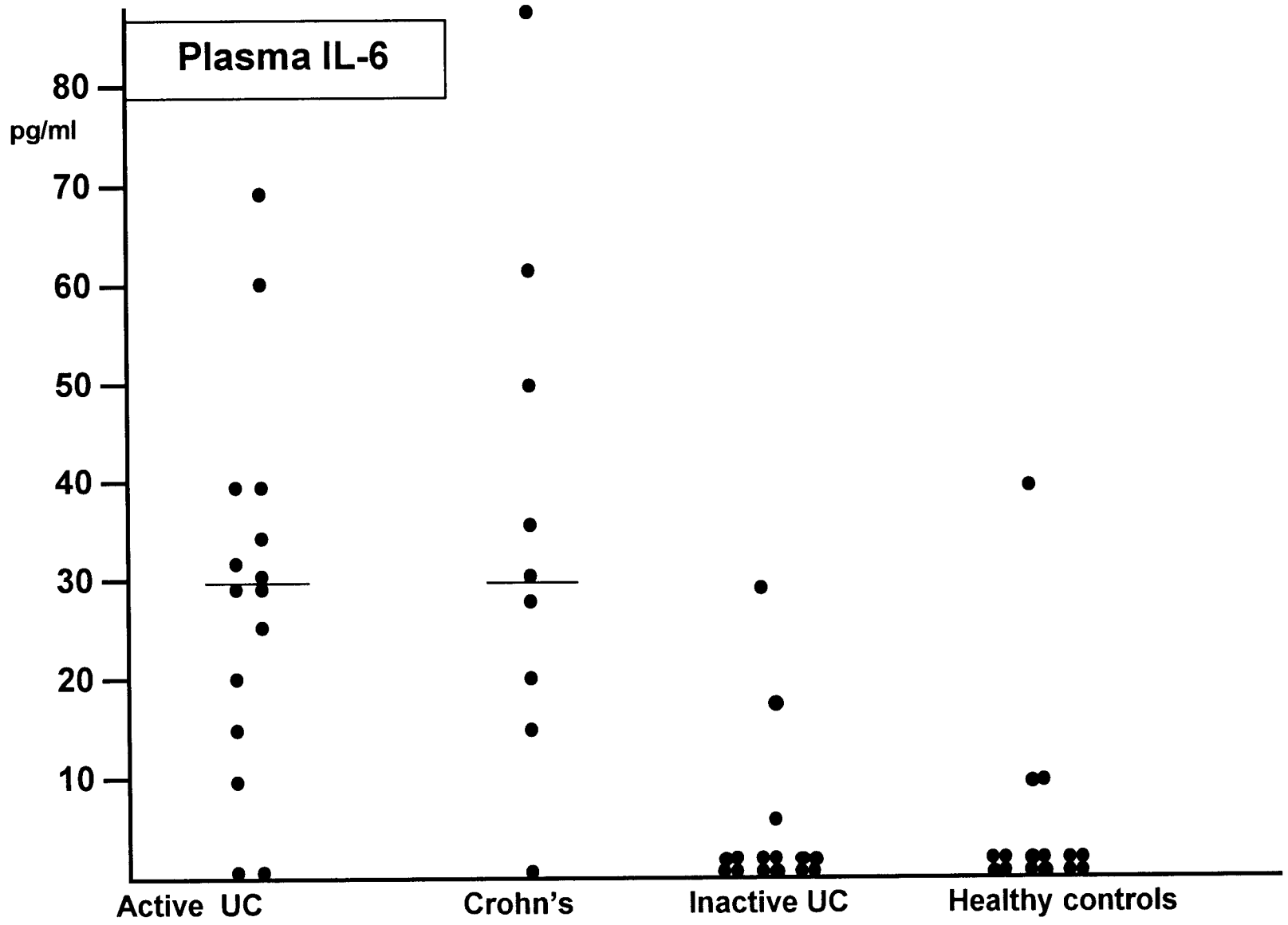



Figure 8.14 Plasma levels of IL-6

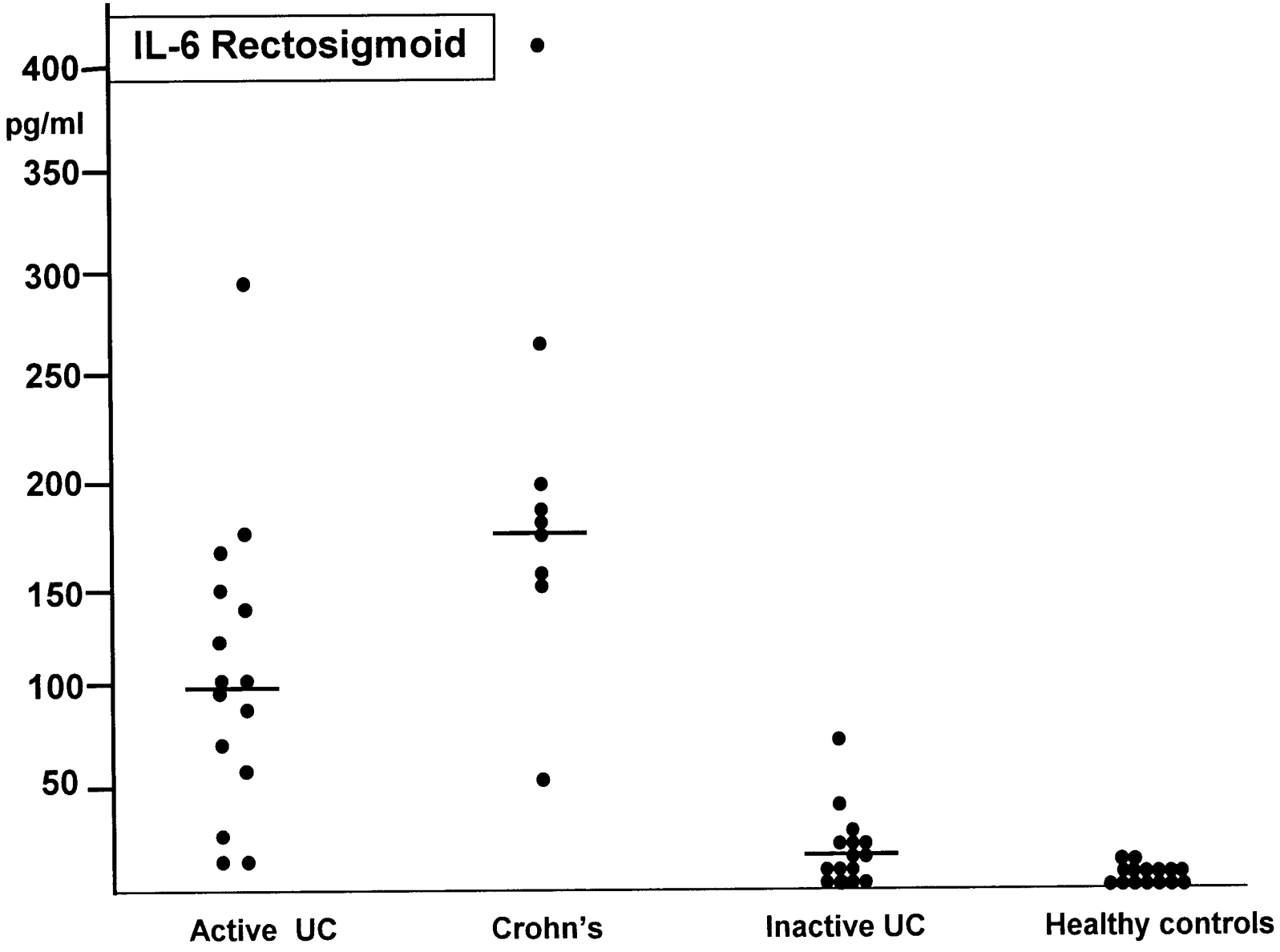



Figure 8.15 IL-6 production from mononuclear cells from rectosigmoid colon

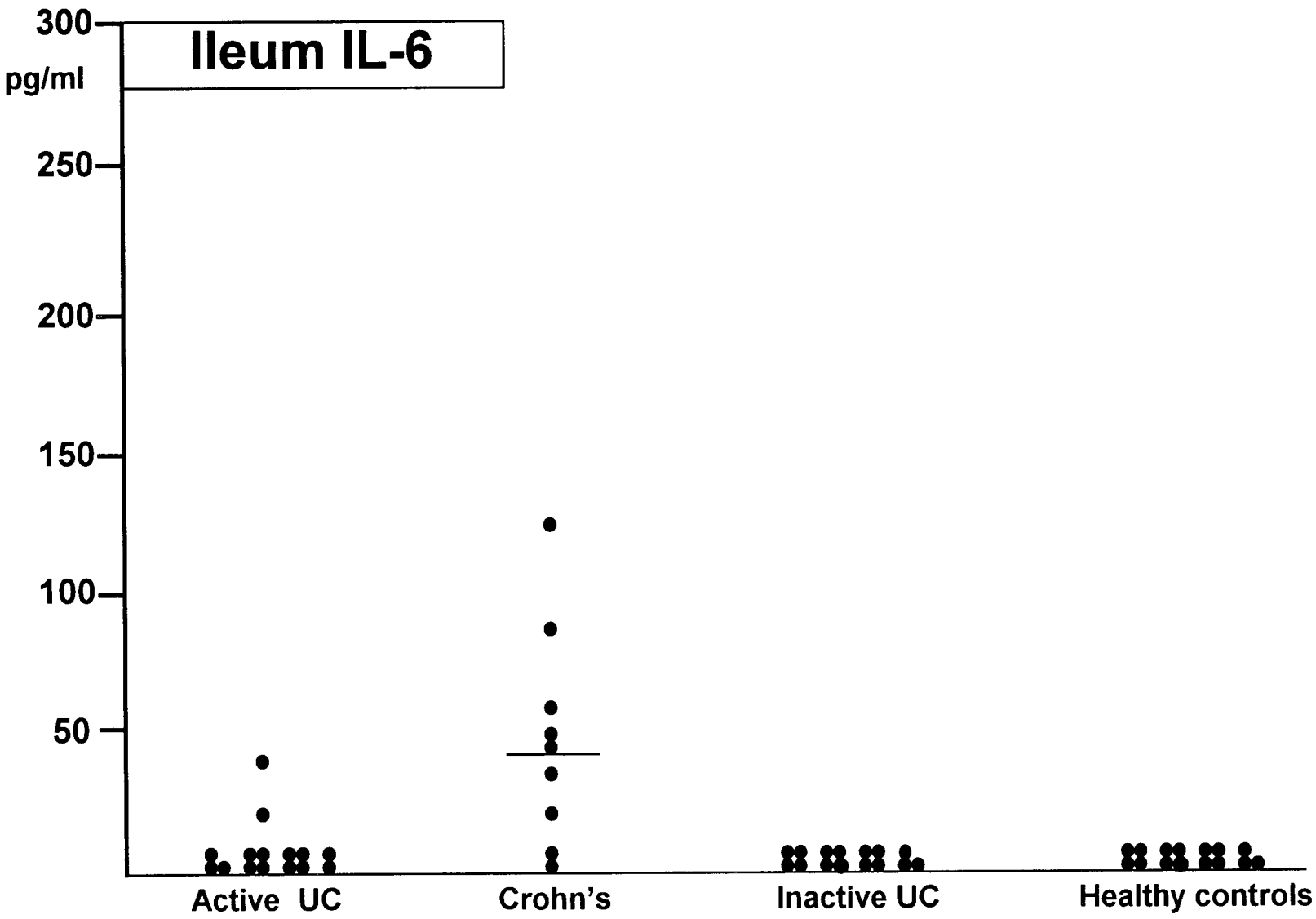



Figure 8.16 IL-6 production by mononuclear cells from ileum

pg/ml) 95 (10-230), than those six who didn't 140 *(50 – 300).*

Table 8.5 P-values comparing IL-6 levels in inflammatory bowel disease with healthy controls ($p<0.05^*$).

| | PLASMA | ILEUM | RECTOSIGMOID |
|---|---|---|---|
| UC(ACTIVE)* | 0.220 | 0.213 | 0.043* |
| CROHN'S* | 0.066 | 0.045* | 0.014* |
| UC(INACTIVE* | 0.332 | 0.340 | 0.120 |

As can be seen from Table 8.6 there is virtually no difference in plasma IL-6 values from healthy controls to patients with IBD. Crohn's ileum again secreted significantly more IL-6 than any of the other groups studied (Figure 8.15) however patients with active UC had levels of spontaneous IL-6 production similar to healthy controls.

Analysis of variance did not reveal any significant difference from the use of systemic steroids and IL-1ß production from isolated mucosal mononuclear cells ($F=0.33$ $P>0.10$).

Stimulation of isolated rectosigmoid mononuclear cells by 1.0 µg/ml of E Coli LPS (Sigma) revealed increased production of IL-6 from [median, range, pg/ml]:- **120 (20-290)** to **150** (20-300) in inflamed UC colon; and increased

production in healthy colon from O(O-2O) to 20(0-100). This difference was statistically significant in the two groups **(P<0.05).**

There was significant correlation between the levels of plasma IL-6 and CRP in Crohn's (r=0.36, P<0.05), and between IL-6 and a-1 glycoprotein in active UC (r=0.43, P<0.05).

The intra-assay and inter-assay coefficients of variation for the IL-6 ELISA assay were 6.5% and 9.3% respectively. Preliminary studies showed that the amount of IL-6 produced by mononuclear cells isolated from inflamed rectosigmoid mucosa in IBD was usually in the range detected by the assay.

## 8.73 Discussion

This study shows that mononuclear cells isolated from colonic mucosa of patients with active IBD, cultured ***in*** vitro, produced much greater amounts of IL-6 than mononuclear cells isolated from normal colonic mucosa.

Increased serum levels of IL-6 have been described after major surgery and in patients with severe burns or acute inflammatory diseases such as bacterial infections or acute pancreatitis [Nishimoto et al, 1989; Shenkin et al, 1989; Waage et al, 1989].

However, in this study increased levels of IL-6 were noted in the plasma of patients with Crohn's disease, but not in the normal controls or patients with UC.

No significant correlation could be found between systemic steroid therapy and levels of IL-6, indeed a certain proportion of patients treated with steroids had higher IL-6 levels than patients with active disease who were not. Steroids have been shown to suppress IL-6 synthesis by mononuclear cells ***in vitro*** [Woloski et al, **1985]**.

Again, I think that once activation of mononuclear cells has occured in vivo, systemic steroids do not exert a strong influence.

IL-6 has a relatively short half life in the circulation of about 5 minutes [Castell et al, **1988]**. It is rapidly cleared from the circulation by the liver. Thus a moderate increase in the de ***novo*** synthesis of IL-6 may lead to alterations in the plasma concentration of this cytokine for only a short time. Furthermore, IL-6 produced in the inflamed bowel may be cleared rapidly by the liver, where it induces acute phase protein synthesis before it enters the systemic circulation. Presumably, higher IL-6 concentrations could be found in the portal blood. On the other hand, the circulatory lifetime of serum proteins is in the range of several days [Putnam, **1975]**. Serum concentrations of these proteins are therefore, less variable than that of IL-6, and remain elevated for a longer period.

The stimulus leading to an increased production of IL-6 in IBD can only be guessed, at present, as no hard core evidence exists on the aetiology of IBD.

Stimuli such as bacterial lipopolysaccharide, viruses, and several cytokines such as **IL-1ß**, TNFa, interferon-?, granulocyte colony stimulating factor (GCSF), substance P, and substance K stimulate **IL-6** synthesis by inflammatory cells [Heinrich et al, **1990]**. High circulatory endotoxin concentrations have been found in patients during the acute phase of Crohn's disease [Fink et al, **1988]**. Therefore, one must consider that the increase in **IL-6** seen in the serum might reflect increased endotoxin entering the circulation from increased intestinal permeability [Hollander et al, **1986]**. Considering the relatively short half life of **IL-6**, it seems fair to assume that there is continuos stimulation, which corresponds to a continuous entry of endotoxins into the circulation and/or mucosa. On the other hand, this continuous stimulation of **IL-6** in **IBD** may be propagated by other cytokines.

## 8.8 Summary

The main points are summarised here but further discussion is made in the next chapter.

Plasma levels of pro-inflammatory cytokines did not correlate with disease activity. Possible reasons to explain this are:-

i) the short half lives of the cytokines (5-30 mins)

ii)after release from the gut mucosa, the cytokines are taken via the portal vein to the liver where they are rapidly broken down and thus, are never detected in the systemic circulation. In an earlier study of patients with severe intra-abdominal sepsis [Patel et al, 1994b], I found that levels of plasma TNF and IL-1 did not correlate with the severity of sepsis.

Table 8.6 Plasma levels of the proinflammatory cytokines in the disease study groups (median with ranges).

| Disease | IL-1$\beta$ | IL-6 | IL-8 | TNF$\alpha$ |
|---|---|---|---|---|
| UC active | 0<br>0-30 | 30<br>0-70 | 0<br>0-40 | 0<br>0-40 |
| Crohn's colitis | 0<br>0-20 | 30<br>0-90 | 0<br>0-40 | 20<br>0-80 |
| UC inactive | 0<br>0-20 | 0<br>0-30 | 0<br>0-30 | 0<br>0-20 |
| Healthy controls | 0<br>0-10 | 0<br>0-40 | 0<br>0-40 | 0<br>0-10 |

Plasma levels of the pro-inflammatory cytokines in general do not reflect disease activity in IBD.

In colonic mucosa, high cytokine levels correlated with disease activity in both active UC and Crohn's. It is known that cytokines play a pivotal role in inflammation, therefore, it does not seem surprising that increased levels were found in inflamed colon.

Table 8.7 Levels of the proinflammatory cytokines (median with ranges) which were expressed by isolated rectosigmoid mononuclear cells.

| Disease | IL-1β | IL-6 | IL-8 | TNFα |
|---|---|---|---|---|
| UC active | 200<br>20-450 | 100<br>20-290 | 630<br>250-1340 | 90<br>0-710 |
| E coli LPS stim | 240<br>30-500 | 150<br>20-300 | 710<br>200-1200 | 120<br>0-600 |
| Crohn's colitis | 280<br>40-700 | 180<br>50-410 | 600<br>150-1520 | 330<br>90-820 |
| UC inactive | 30<br>0-90 | 20<br>0-70 | 80<br>40-300 | 0<br>0-20 |
| Healthy controls | 0<br>0-10 | 0<br>0-20 | 30<br>20-70 | 0<br>0-10 |
| E Coli LPS stim | 40<br>20-120 | 20<br>0-100 | 40<br>20-100 | 20<br>0-50 |

Stimulation by 1.0 micrograms of E Coli LPS

Levels of pro-inflammatory cytokines in general expressed by cultured mononuclear cells do reflect disease activity

and expression of cytokine from mononuclear cells taken from patients with inactive UC tends to be similar to healthy controls.

Crohn's ileum mononuclear cells express significantly more pro-inflammatory cytokines even when taken from histologically normal ileum. However, mononuclear cells taken from active or inactive UC express similar cytokine levels to healthy ileum.

Table 8.8 Levels of the pro-inflammatory cytokines in the disease study groups (median with ranges) which are expressed by isolated ileal mononuclear cells.

| Disease | IL-1$\beta$ | IL-6 | IL-8 | TNF$\alpha$ |
|---|---|---|---|---|
| UC active | 0<br>0-10 | 0<br>0-40 | 40<br>0-110 | 0<br>0-10 |
| Crohn's colitis | 100<br>30-290 | 40<br>0-120 | 50<br>20-220 | 80<br>20-300 |
| UC inactive | 0<br>0-40 | 0<br>0 | 30<br>0-60 | 0<br>0-30 |
| Healthy controls | 0<br>0-10 | 0<br>0-20 | 20<br>0-40 | 0<br>0-10 |

NB. The results have been rounded off to the nearest 10 to provide clarity.

# Chapter 9

## Cytokine production in pouchitis is similar to that in ulcerative colitis

## 9.1 Introduction

The clinical entity known as pouchitis was first described in continent ileostomies for ulcerative colitis [Kock et al, 1977], and later in pelvic ileal pouches [Handelsman et al, 1983]. Pouchitis remains a well known complication following restorative proctocolectomy, which as the patients are followed up longer, the prevalence actually increases [Lohmuller et al, 1990], and an incidence of 30% has been recently reported [Pemberton et al, 1993]. Like UC, the aetiology of pouchitis is unknown and there remains controversy in the definition, diagnosis and management of this condition.

The 'cytokine explosion' [Durum et al, 1990] has resurged an interest in the intricacies of the inflammatory response in many diseases, in the hope of finding abnormal regulatory events responsible, if not for triggering, at least for the perpetuation of the local tissue damage. If this less than ideal goal could be achieved in UC and pouchitis, a more rational and effective approach to their management could result [Fiocchi et al, 1993]. Pro-inflammatory cytokines are known to play an important role in the activation of lamina propria mononuclear cells in **UC** and increased levels of TNFa, IL-1ß, IL-6 and IL-8 have been reported [Mahida et al, 1992; Mahida et al, 1989; Gross et al, 1992; Raab et al, 1993; Pullman et al, 1992; Satsangi et al, 1987].

However, some investigators have reported decreased levels

[Nielsen et al, 1993; Hyams et al, 1991; Gröttrup et al, 1993] and there has been few studies looking at the pro-inflammatory cytokines in both **UC** and pouchitis [Gionchetti et al, 1993]. The similarities in mucosal morphology between pouch ileum and the colon in **UC**, as well as the rarity of pouchitis (0-6%) after reservoir formation in non-colitics, has led researchers to postulate that the pathogenesis of pouchitis involves reactivation of **UC** [Madden et al, 1990; Scott et al, 1989; Penna et al, 1993]. The aims of this particular study were to assess whether there was increased pro-inflammatory cytokine production by the lamina propria mononuclear cells in pouchitis and if so, are the levels of these cytokines comparable to **UC?**

## 9.2 Patients and methods

### 9.2 1 Mucosal Biopsies

Mucosal biopsies were obtained from the following:-

i)25 patients with non-inflamed ileo-anal pouch (median age 41 years; range 18-57 years) including 5 patients who had had an ileoanal pouch after colectomy for polyposis coli.

ii)9 patients who had a clinical pouchitis (median age 39 years; range 26-62 years) and

iii)20 patients with active **UC** (rectosigmoid region, median age 44 years; range 18-61 years).

Simultaneous plasma samples were also taken from all patients.

## 9.22 Control Groups

Mucosal biopsies were also taken from the following:-

iv)15 patients with normal ileum (median age 48; range 23-64 years),

v)10 patients with non-specific proctitis (inflammation confined to less than 10cm in the rectum) (median age 37; range 27-53 years) and

vi)15 patients with normal colon (median age 49; range 28-70 years). Normal ileal and colonic biopsies were taken from patients undergoing colectomy for non-IBD disorders.

## 9.23 Definition of Pouchitis, active UC and proctitis

Pouchitis, in this study was described as a combination of clinical symptoms of frequency of stools, urgency, passage of blood and mucus, sigmoidoscopic findings of inflammation associated with friability of mucosa and histological criteria of inflammation associated with neutrophil infiltration [Kmiot et al, 1993].

Patients in the active UC group were classified as having moderate-severe UC [Kodner et al, 1990].

Patients with non-specific proctitis were defined as those where inflammation was confined to less than 10cm in the rectum and IBD had been excluded.

The biopsies were taken either in the operating theatre immediately after colectomy together with an ileal biopsy

if this was available, or at the time of pouch endoscopy.

## 9.24 Mononuclear cell isolation

Lamina propria mononuclear cells were isolated from the mucosal biopsies [Bull et al, 1977]. Briefly, the biopsies were placed immediately in cool Hanks buffered saline supplemented with glutamine, penicillin, streptomycin (GPS) and 10% foetal calf serum (FCS). After mincing, washing was carried out. Each of the following steps was preceded by washing which consisted of spinning the cells in a centrifuge at 1600 RPM for 5 minutes and the supernatant would be discarded. 0.75 mM EDTA in Hanks/GPS/FCS was used to lyse the epithelial cells and 1 mM dithiothrietol in Hanks/GPS/FCS was used to remove surface mucous. Following this the tissue matrix was subjected to enzymatic dispersion at 37°C with collagenase (Sigma) at conc 0.5mg/ml and hyaluronidase (Sigma) at conc 1 mg/ml in RPMI/GPS/FCS for 4 hours with pipetting every 20-30 minutes. After filtering, the cells were resuspended. Mononuclear cells were further separated by Percoll, followed by counting in an improved Neubauer chamber. Identity of the mononuclear cells was assessed by flow cytometry and viability using Trypan blue **1:1**, if >95%, further experimentation was performed. The cells were suspended in RPMI/GPS/FCS and incubated for 48 hours at a concentration of $2 \times 10^5$/ml at 37°C in a 5% $CO_2$ chamber. The supernatants were stored at -

80 °c. The cells were quantitated at the end of the culture period and cytokine release corrected for the number of cells alive and viable.

## 9.25 Cytokine Elisa

Plasma cytokine levels were determined, (as previously described in chapter **8** & Helle et al, **1990),** using monoclonal antibody for TNFa (British Biotechnology), **IL-1ß** (Janssen, Belgium) and **IL-6** (Monosan, Belgium) and IL-8 (British Biotechnology). Plasma levels of C-reactive protein (CRP) and ***a1*** acid glycoprotein were also measured by immunoturbidometric assay.

## 9.26 Statistical analysis

Results were not normally distributed and have therefore, been expressed as medians with ranges. The Mann-Whitney U test has been employed to show differences between the cytokine levels in the subgroups. The Spearman rank correlation has been used to show associations.

## 9.3 Results

The distribution of the findings for the individual cytokines TNFa, **IL-1ß, IL-6** and **IL-8** are shown in Figures 9.1-9.4 respectively. Pro-inflammatory cytokine production was significantly increased ($P < 0.05$) in the inflamed

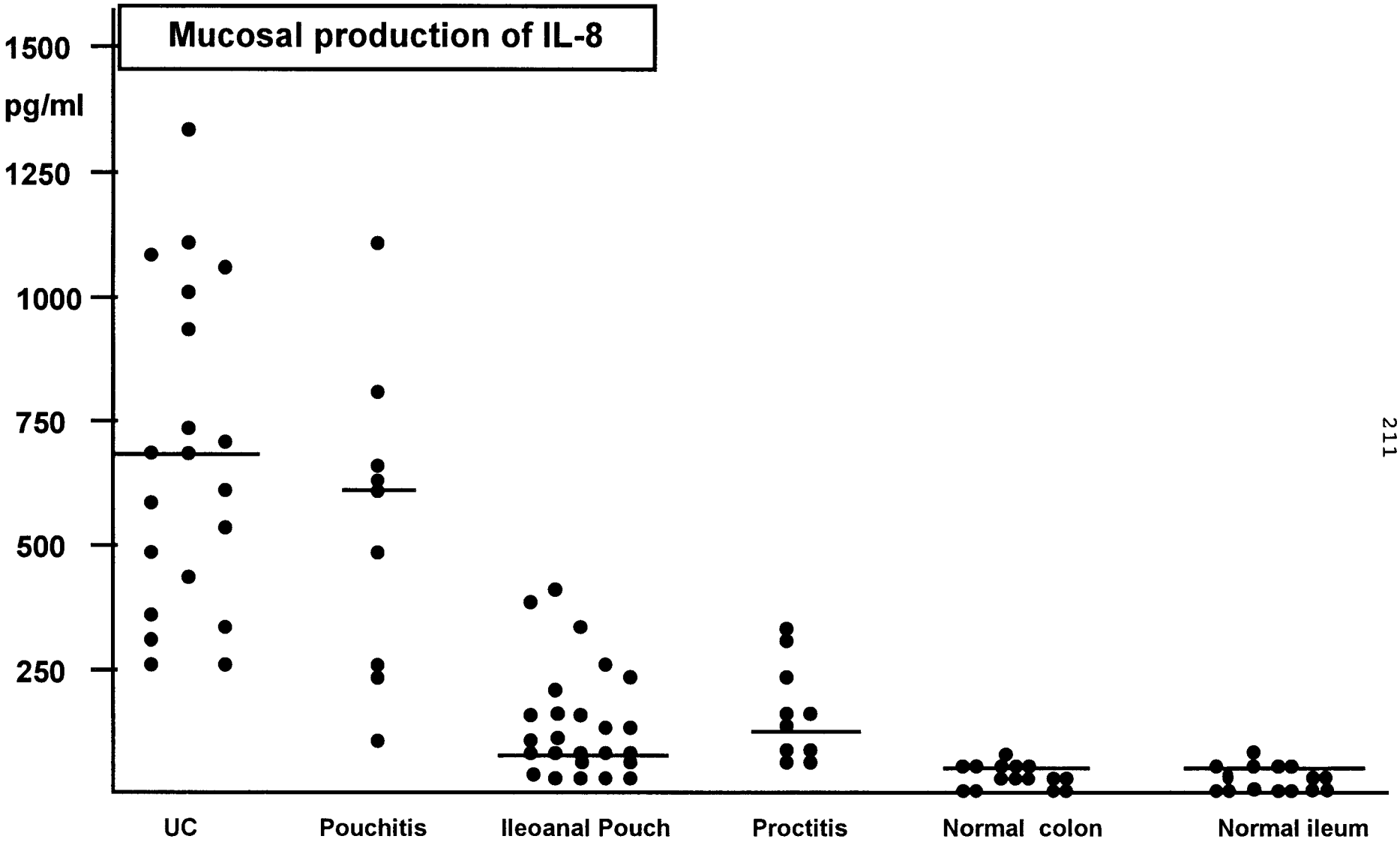



Figure 9.1 IL-8 production by mononuclear cells

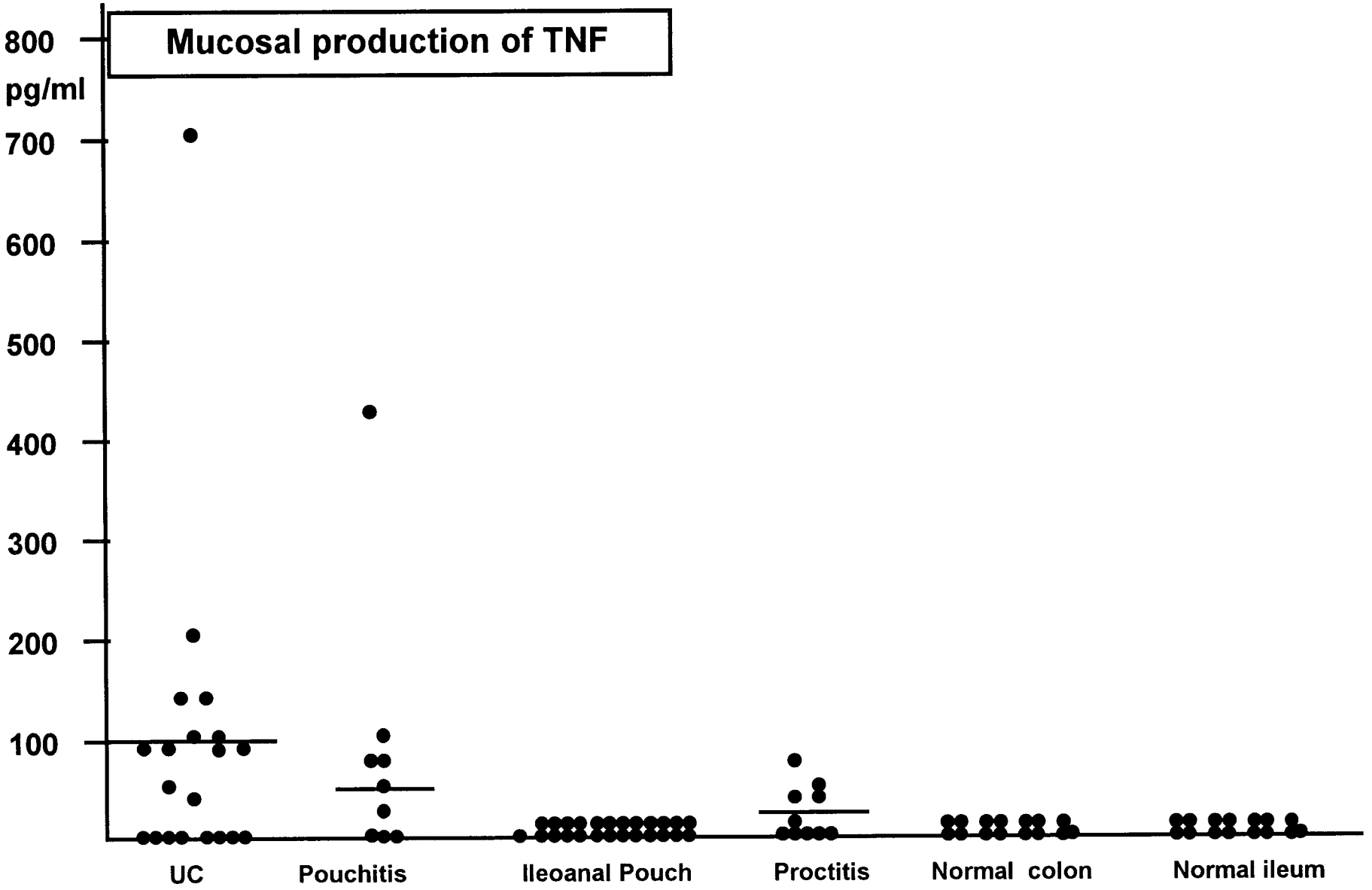



Figure 9.2 TNF production by mononuclear cells

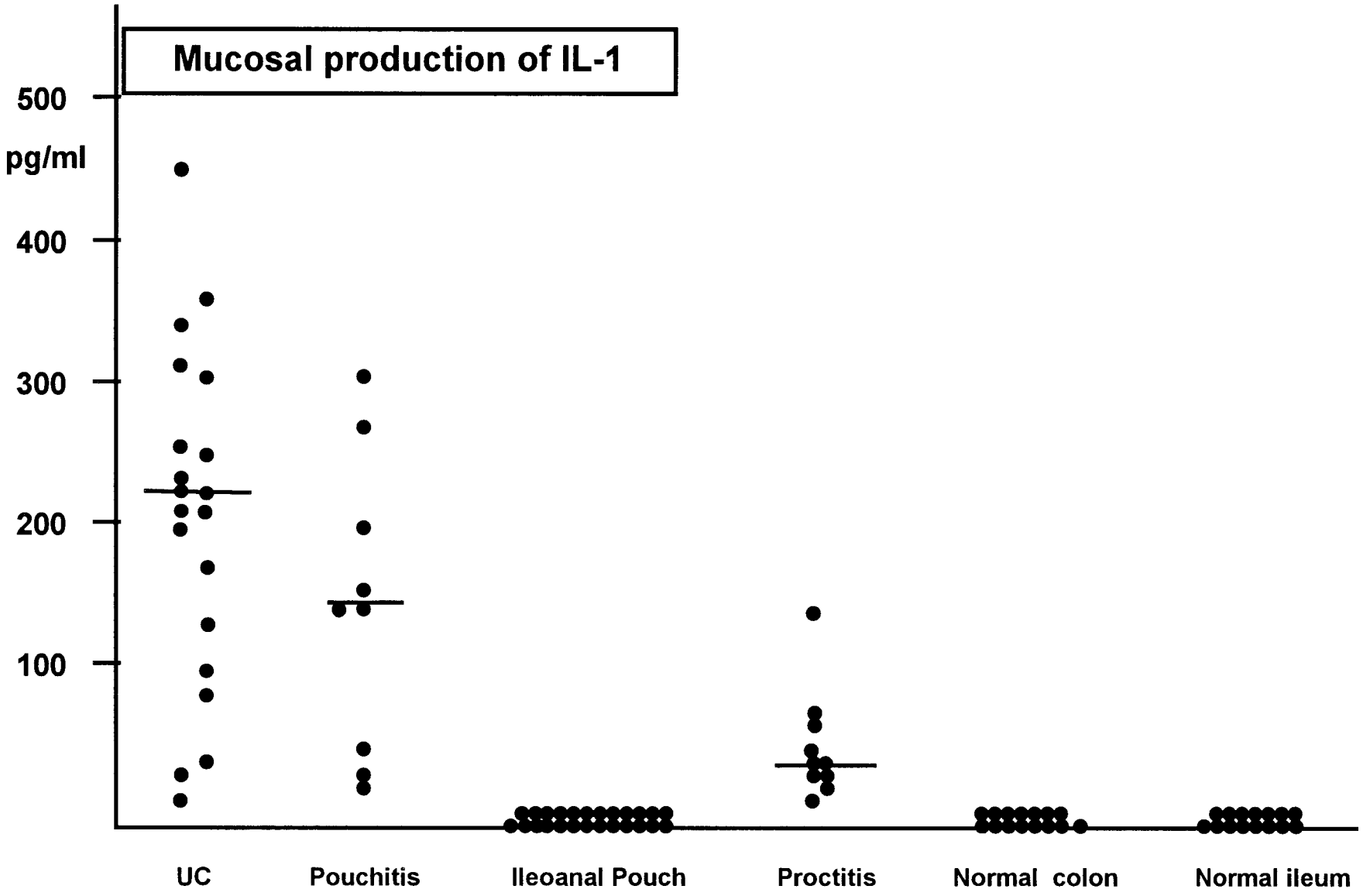



Figure 9.3 IL-1 beta production by mononuclear cells

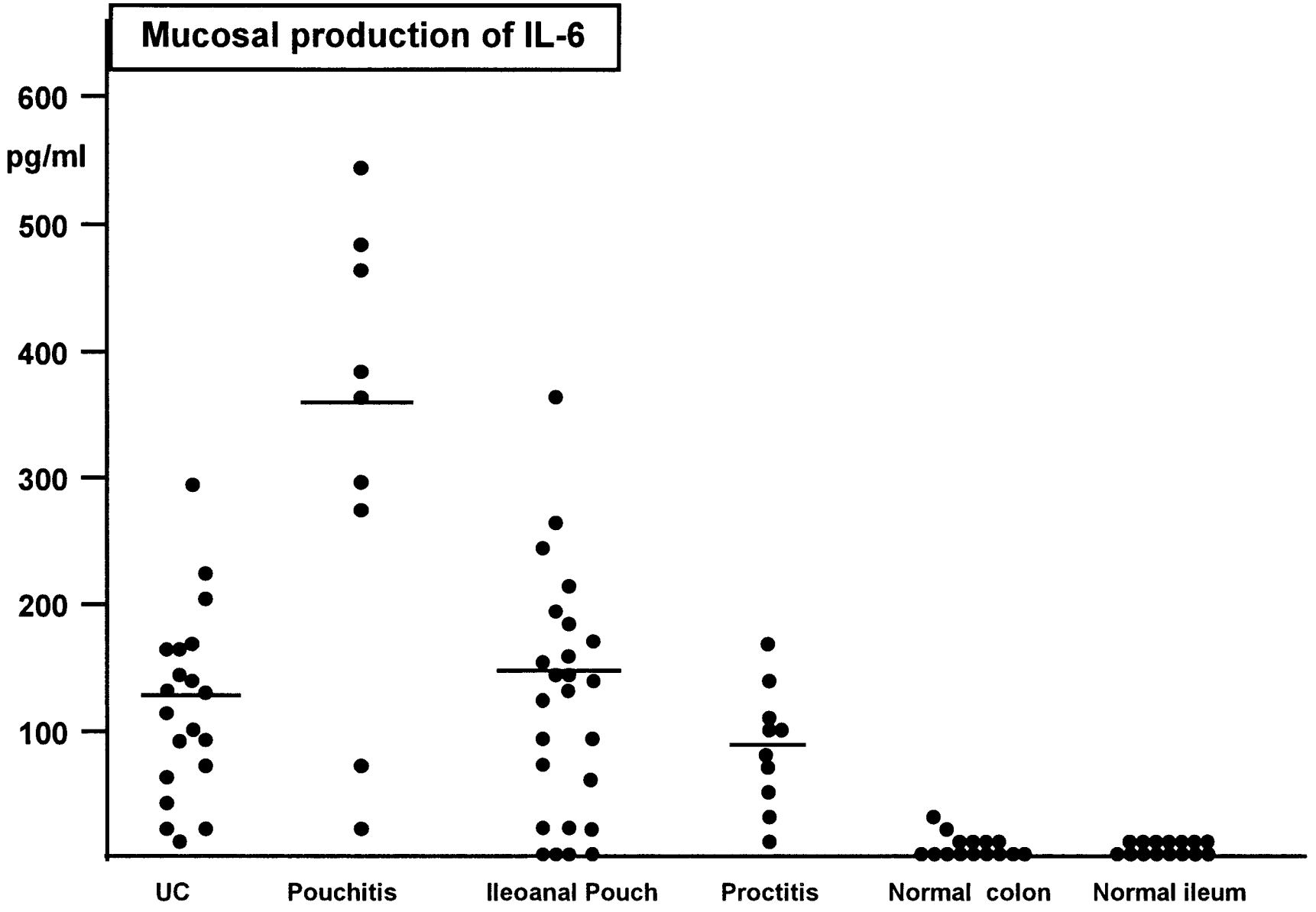



Figure 9.4 IL-6 production by mononuclear cells

ileoanal pouch (pouchitis) compared to healthy control ileal biopsies (Table 9.1).

Table 9.1. Cytokine levels in the various disease groups

| | IL-1β | IL-6 | IL-8 | TNFα |
|---|---|---|---|---|
| Ileoanal pouch | 0 (0-10) | 160 (0-370) | 120 (30-420) | 0 (0-40) |
| Pouchitis | 150 (20-310) | 360 (20-540) | 590 (50-1100) | 70 (0-430) |
| UC | 220 (20-450) | 120 (20-290) | 670 (250-1340) | 100 (0-710) |
| Normal ileum | 0 (0-10) | 0 (0-10) | 50 (0-120) | 0 (0-10) |
| Proctitis | 50 30-170 | 100 (20-190) | 140 (50-370) | 30 (0-100) |
| Normal colon | 0 (0-10) | 0 (0-30) | 60 (30-130) | 0 (0-10) |

(median with ranges pg/ml).

Patients with a non-inflamed pouch who were relatively symptom free also had significantly raised IL-6 and IL-8 levels compared to healthy controls which was statistically significant ($P<0.05$).

Subgroup analysis of the non-inflamed ileo-anal pouch patients (n=20) revealed that with a long standing pouch (>2years; n=9) significantly greater levels of mucosal IL-6 and IL-8 [190 (0-370) and 150 (40-420) pg/ml respectively] were found compared to recently constructed ileo-anal pouches (<2 years ; n=11) [130 (0-260) and 110 (30-300) pg/ml, respectively. However no significant differences were noted for IL-1ß and TNFa! in this subgroup analysis.

There was significant correlation between the CRP and IL-1ß, IL-6, IL-8 (Table 9.2) and between al-glycoprotein and IL-6.

As previously shown in chapter 8 plasma levels of cytokines again did not differ significantly from controls.

Table 9.2. Correlation matrix showing comparison between C-reactive protein, al-glycoprotein and pro-inflammatory cytokines.

| | IL-1 | IL-6 | IL-8 | TNF | CRP |
|---|---|---|---|---|---|
| IL-6 | 0.039 | ------- | ------ | ----- | ----- |
| IL-8 | -0.155 | 0.413* | ------ | ----- | ----- |
| TNF | -0.015 | 0.060 | 0.060 | ----- | ----- |
| CRP | 0.292# | 0.498* | 0.423* | 0.110 | ------ |
| α1-gly | 0.112 | 0.273# | 0.187 | 0.050 | 0.196 |

*P<0.01 # P < 0.05

For patients with proctitis, higher levels of the pro-inflammatory cytokines were noted, compared to normal colon controls. A greater level of cytokine expression was noted in active UC compared to proctitis, even though the grade of inflammation for some patients, in the two groups, was comparable histologically.

Stimulation of isolated mononuclear cells from inflamed (active UC) and non-inflamed mucosa with 1.0 μg/ml E. Coli LPS (sigma) revealed increased cytokine expression in both groups, as described in Chapter 8.

## 9.4 Discussion

Levels of the pro-inflammatory cytokines have been shown to be elevated in a variety of chronic inflammatory 'autoimmune' diseases such as rheumatoid arthritis, Wegener's granulomatosis, systemic lupus erythematosis, polyarteritis nodosa and also recently in ulcerative colitis and Crohn's disease [Mahida et al, 1992; Mahida et al, 1989; Raab et al, 1993; Patel et al, 1993; Brynskov et al, 1992].

This study has shown that mucosal levels of the pro-inflammatory cytokines IL-1ß, IL-6, IL-8 and TNFa are not only elevated compared to healthy controls, but are also comparable in active chronic UC and pouchitis. One can argue that this, in vitro finding may not reflect the **in** vivo mucosal environment, and also that these findings simply reflect the local inflamation seen in both conditions. However, on the other hand could pouchitis represent a reactivation of the immunological mechanisms which led to active UC. In favour of this hypothesis, is the finding that some patients with pouchitis, have resurgence of the extra-intestinal manifestations of UC in the same way as patients who have active UC [Lohmuller et al, 1990]. RFD9 macrophages have been found to be present in the mucosa of patients with active UC, but do not appear as a non-specific response in infectious colitis. However, they are also present in pouchitis, which suggests that similar effector mechanisms for triggering the original UC

may be operating in pouchitis [Gionchetti et al, 1993].

In this study, mononuclear cytokine production is compared in ileum and colon, on the premise that pouchitis may represent a reactivation of ulcerative colitis in mucosa which has undergone colonic metaplasia and villous atrophy. The levels of cytokines expressed in some patients with active IBD were much greater quantitatively than those expressed in non-specific proctitis which histologically had the same degree of inflammation noted. When pouchitis is diagnosed endoscopically, histological evidence of acute inflammation is invariably present, ie. neutrophil infiltration, crypt abscesses, and ulceration [Tytgat et al, 1988]. These findings are very similar to the features seen in active **UC** and is far more common for **UC** than for familial adenomatous polyposis [Nicholls et al, 1985; Shepherd et al, 1987].

A typical feature of the acute inflammatory response in active **UC** and in pouchitis is mucosal ulceration and neutrophil infiltration [Goldman, 1984; Moskowitz et al, 1986].

If the neutrophil is considered to be the main inflammatory cell, then by what mechanisms is it activated? The mechanisms responsible for the recruitment and activation of neutrophils in the mucosa In **UC** and pouchitis are probably multifactorial and the mediators of the inflammatory changes are cytokines.

IL-8 is a powerful neutrophil chemoattractant [Baggiolini

et al, 1989]. Our findings with those of others [Mahida et al, 1992; Raab et al, 1993; Izzo et al, 1992] of increased mucosal IL-8 in **UC** and in pouchitis make it very likely that this cytokine is an important mediator in the activation, diapedesis through vascular endothelium and focal recruitment of neutrophils at the site of inflammation [Striter et al, 1988]. What factors are responsible then for inducing increased IL-8 production? Immune complexes, endotoxins and several cytokines including IL-1ß and TNFa have been shown to be potent inducers of IL-8 [Baggiolini et al, 1989; Striter et al,1988; Striter et al, 1989; Colditz et al, 1989].

This study together with another recent study which found an increased TNFa concentration in colonic-rectal perfusate fluid in **UC** which significantly correlated with increased IL-8 release suggests that TNF is one possible mediator in the increased IL-8 synthesis in active **UC** [Raab et al, 1993].

The following flow chart is a hypothesis of the sequence of events which may occur:-

Chronic UC-Total Colectomy

Normal ileum

Ileo-anal pouch operation (normal ileum)

Pathological changes in the ileum resulting from a change in the physiological role of the ileum :–

1. stasis

2.perturbation of the bacterial flora in the ileum (3.nutritional deficiences)

(4.ischaemia)

Changes in the morphological structure of the ileum as a result of one or a combination of these factors leading to villous atrophy and colonic metaplasia

Histological change in the ileum to colon and leading to immunological recognition of the ileum as colon and subsequent reactivation of the mechanisms which led to ulcerative colitis

Pouchitis

Successful treatment with metronidazole

Decrease of gram-ve bacteria and anaerobic bacteria from the ileum or an immunosuppressive as yet undefined action of metronidazole leading to reversal of the ileal changes and disease remission.

In Figure 9.5 is shown a proposal of cytokine mechanisms operating in active ulcerative colitis and pouchitis. Endothelial cells and fibroblasts have also been shown to produce increased amounts of both IL-6 and IL-8 in response to IL-1 and TNF [Striter et al,1988; Striter et al, 1989; Schroeder et al, 1989]. This could explain the increased IL-6 levels noted in this study and those of others [Gross

et al, **1992]**. IL-6 is the most potent inducer of acute phase protein synthesis by the liver whereas IL-1ß and **TNFa** have been shown to play a relatively minor role [Castell et al, **1989**; Castell et al, **1988]** therefore it doesn't seem surprising that in this study IL-6 correlated significantly with CRP in both pouchitis and UC.

Investigation into the role of cytokines in general which lead to inflammation in UC are at an early stage. Perhaps by studying patients with pouchitis we can unravel some of the intricacies of the inflammatory response which occurs in UC in the hope of finding new therapeutic modalities [Youngman et al, **1993]**.

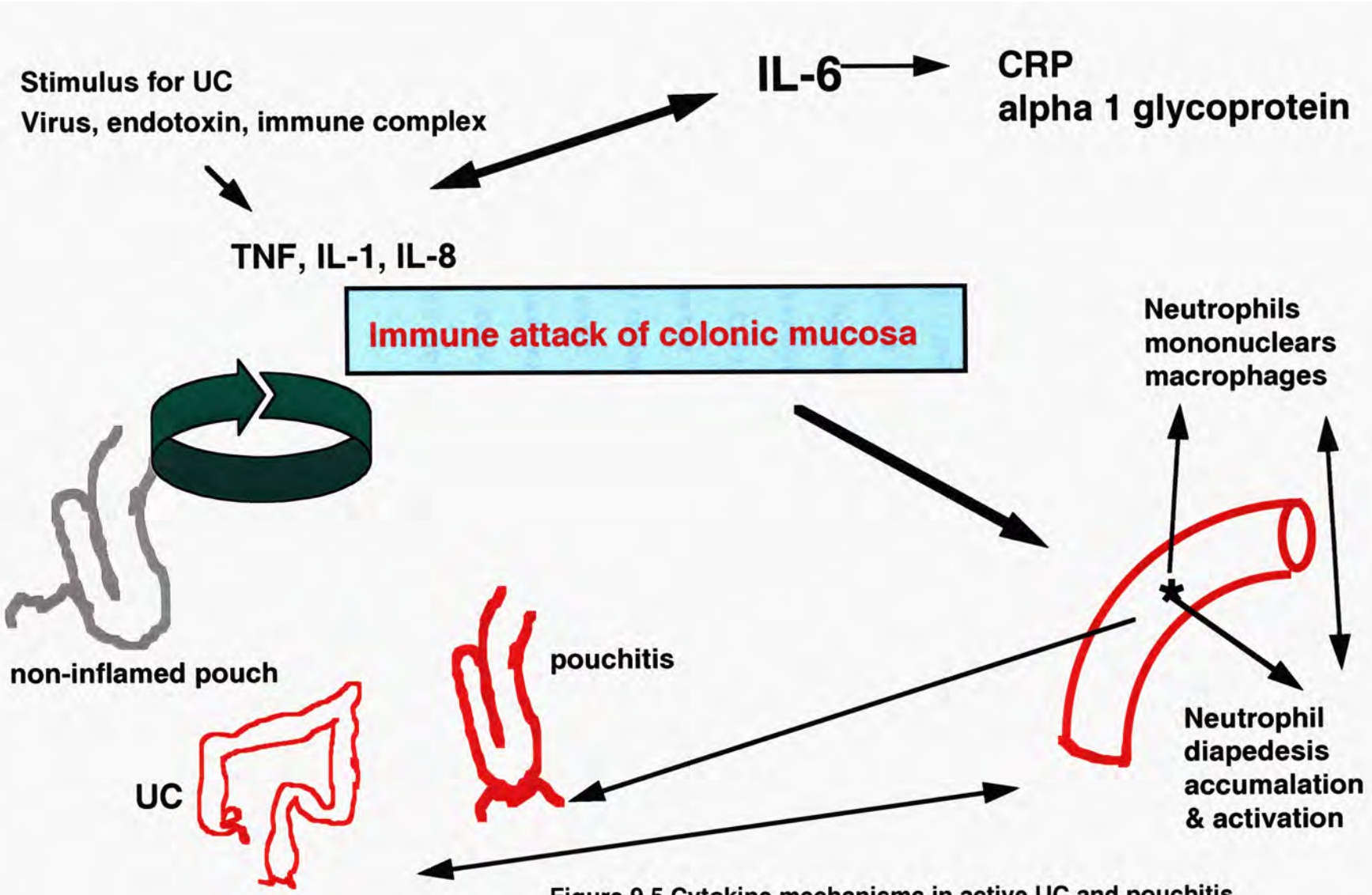



Figure 9.5 Cytokine mechanisms in active UC and pouchitis

## *9.5* Summary

Controversy exists as to whether pouchitis represents a reactivation of the immunological mechanisms which led to ulcerative colitis (UC). The aims of this study were to determine local levels of the cytokines:- interleukin-1ß (IL-1ß), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumour necrosis factor alpha (TNFa) in the mucosa of patients with normal ileum (n=15), ileoanal pouch for UC (n=20), pouchitis (n=9) and active ulcerative colitis ( n=20). Lamina propria mononuclear cells were isolated from mucosal biopsies, by enzymatic dispersion, and cultured for 48 hours. Pro-inflammatory cytokine levels were measured in the supernatants. IL-10, IL-6, IL-8 and TNFa secretion was significantly greater in pouchitis and active UC then in the non-inflamed ileoanal pouch and normal ileum **(P<0.001).** There was significant correlation (r=0.63, **P<0.05)** between levels of cytokines expressed in pouchitis and active UC. Perhaps pouchitis represents a reactivation of UC in mucosa which has undergone colonic metaplasia and villous atrophy.

# CHAPTER 10

## Summary, conclusions and prospects for further research

## 10.1 Summary

These studies were designed to assess the role of autoantibodies, soluble adhesion molecules and pro-inflammatory cytokines in patients with ulcerative colitis and following restorative proctocolectomy.

The findings are summarized below:-

***CHAPTER 2:-*** ANCA were originally described in patients with systemic vasculitis and they are found in the majority of patients with ulcerative colitis. ANCA persist in high titre in the sera following total colectomy for severe UC. The prevalence of ANCA in pouchitis was 100%.

***CHAPTER 3:-*** Sera from patients with ulcerative colitis recognise a variety of antigens on the neutrophil namely lactoferrin, cathepsin G, enolase and elastase. These antigens are not the commonly encountered antigens of ANCA in sytemic vasculitides, which are proteinase 3 and myeloperoxidase.

***CHAPTER 4:-*** Confocal laser microscopy localised the ANCA antigens to the intra-nuclear portion of the neutrophil, and not the peri-nuclear or cytoplasmic portion. This has been attributed to the method of fixation of the neutrophils using ethanol. There are no other studies to date relating to these findings.

***CHAPTER 5:-*** AECA is also found in sera from patients with ulcerative colitis and Crohn's disease, thus providing further serological proof of vasculitis in UC as well as Crohn's disease.

***CHAPTER 6:-*** Other autoantibodies including AECA, anti-EPI and anti-tropomyosin also persist, despite total colectomy, suggesting that immunological mechanisms are not halted by removal of the target organ (the colon) in UC. The cross-reactivity of ANCA with anti-EPI antibodies using adsorption studies may help to explain the high prevalence of ANCA in UC.

***CHAPTER 7:-*** Soluble adhesion molecules ICAM-1, E-Selectin but not VCAM-1, may be used as markers of disease activity in UC.

***CHAPTER 8:-*** Pro-inflammatory cytokines, namely **IL-1ß, IL-6**, IL-8 and TNFa, produced by lamina propria mononuclear cells, are increased in active UC and Crohn's disease, when compared to healthy controls and inactive ulcerative colitis. The levels of cytokines in the plasma do not increase in UC, but slight increases in Crohn's disease have been noted, suggesting that there might be a greater expression of these cytokines locally, which pass through the liver and spill over into the plasma.

*CHAPTER* 9:- There is similar quantitative expression of pro-inflammatory cytokines in both active UC and pouchitis but not in non-specific proctitis. These findings seem to suggest that perhaps, pouchitis is not merely a complication of ileoanal pouch surgery, but reactivation of UC in ileal mucosa which has undergone villous atrophy and colonic metaplasia.

## 10.2 Hypothesis A

'Ulcerative colitis is a 'gut limited' vasculitis which bears immunological resemblances to systemic vasculitis'.

The following have been found to occur in both groups of diseases:

1. ANCA
2. AECA
3. Autoantibodies persist following target organ removal such as the colon, which suggests that immunological mechanisms are not halted by colectomy in UC.

Why should these autoantibodies persist?
eg. Taking tetanus as an example, immunity wanes with time and we need to have a booster. So were the samples to measure titres taken too early, does one need to wait for 10 years to see if the titres will fall?
Secondly, if the immune system is to blame for this disorder are we to assume that the colon is entirely normal

in **UC?**

If the latter is true, than by manipulation of the immune system, one may be able to control the disease in the future without the need arising for colectomy.

The neutrophil has a central role in bringing about the inflammation in **UC.** Increased soluble adhesion molecules in the sera may not only be used as markers of disease activity but also, inhibitors of these molecules could be employed to dampen the transmigration of neutrophils and subsequent inflammation.

## 10.3 Hypothesis B

Pouchitis bears immunological resemblances to **UC,** and represents reactivation of **UC.**

**ANCA** have been found in a large majority of patients with active **UC** and all patients with pouchitis, but are not found in infective colitis.

Pro-inflammatory cytokine production, quantitatively is similar in both active **UC** and pouchitis but not in non-specific proctitis, even though histological similarities have been reported.

## 10.4 Further Research and future prospects.

The in vivo role of autoantibodies in systemic vasculitis is thought to be a cytotoxic role. However, so far there is no convincing evidence for such a role in **UC** and further

study is needed to assess any cytotoxicity both *in vitro* and *in vivo*.

*In vitro*, cytotoxicity could be assessed using Chromium 51 assays after isolating the antibodies by HPLC.

Finally, the mechanisms by which the pro-inflammatory cytokines exert their action *in vivo* are poorly understood. One of the main reasons for this is that there are so many of them - up to 140 have been described and the number is growing every day. They have a pleiotropic mode of action and this makes their pathophysiological complexity immense.

## 10.5 New Treatments

Increasing knowledge of intestinal inflammatory processes in the inflammatory bowel diseases has been obtained in the last 5 years, based on clinical, immunologic and biochemical studies. The inflammatory response in the the colonic mucosa may evolve in 3 stages:-

1. Activation of the immune system
2. Production of inflammatory mediators with subsequent amplication of this response
3. Destructive interactions of activated inflammatory cells with gut epithelium.

Pharmacologic intervention at each of these stages is regarded a desirable therapeutic goal in the absence of a known aetiology.

Phospholipase-A2, lipoxygenase inhibitors, radical

scavengers, mediator (cytokine) receptor antagonists or modification of mediators with less active derivatives are examples of recent developements [Allgayer et al, **1990].**

Vasculitis has been well described in rheumatoid arthritis but not in UC. It is interesting to note that patients with rheumatoid arthritis are now being treated with a drug which is commonly used in IBD, namely salazopyrine [Hilliquin et al, **1992**; Murphy, **1992].**

Pooled immunoglobulin have been shown to be of benefit in patients with systemic vasculitis who have relapsed on conventional steroid and cyclophosphamide therapy [Jayne et al, **1991].** Recently, some benefit has been reported in the treatment of IBD [Levine et al, **1992).** Currently, pooled immunoglobulins are undergoing evaluation in the Departments of Renal medicine and Rheumatology at the Queen Elizabeth Hospital, Birmingham for the treatment of systemic vasculitis.

It seems interesting also to note that there appears to be nothing documented in the literature, on the use of cyclophosphamide in IBD, which is the standard first line treatment in systemic vasculitis.

*Systemic vasculitis and UCrarely occur together. Anecdotally, I came across such a young man, who had polyarteritis nodosa and went on to develop* ***UC.*** *His major symptoms were chronic non-healing leg ulcers, despite*

*multiple skin grafts, since 10 years and colitis since 2 years. His symptoms of colitis had shown improvement on only steroids and cyclophosphamide therapy. Following withdrawal of cyclophosphamide treatment because of a herpetic penile ulcer, his UC became uncontrollable on medical management, and he underwent total colectomy for severe UC. Skin grafts on his leg ulcers which had initially taken well a few months previously,, also broke down simultaneously at this time. An ileoanal pouch procedure was then carried out.*

*Eighteen months following the procedure, he developed pouchitis several times, which has been successfully treated with metronidazole. Of interest, is the fact that during episodes of pouchitis, his leg ulcers increased in size and severity. Serological investigation during an acute episode of pouchitis revealed presence of ANCA, AECA and anti-EPl autoantibodies. The ulcers on the legs became quiescent again, once his symptoms of pouchitis improved. This case serves to illustrate several of the points discussed in this thesis.*

**Cyclophosphamide is a standard immunosuppressant usually used in systemic vasculitis and malignancy. It may be of benefit in certain cases of UC. However, its use in this disease needs to be evaluated further, bearing in mind all its side effects.**

**Other immunosuppressants/cytotoxics such as *6*-mercaptopurine have shown some benefit in inducing**

remission in UC [Adler et el, **1990]**. Potential benefits have also been documented from cyclosporin and azathioprine use, but their use has not yet been universal.

**IL-1ß** receptor antagonist has been found to have some benefit in experimental colitis [Cominelli et al, **1992]** and clinical trials are currently underway to assess its role in ulcerative colitis.

This thesis has looked at and developed immunological methods which are relatively new, in the study of patients with ulcerative colitis, and used two immunological models for comparison namely pouchitis and systemic vasculitis.

The results, thus far, look encouraging and there is hope for future studies looking at the mechanisms of inflammation, in order to develop new treatments for this disease.

# APPENDIX 1

## REAGENTS

### Dulbecco,s B phosphate buffered saline

| | g/l | mM |
|---|---|---|
| NaCl | 8.0 | 137.0 |
| KCl | 0.2 | 2.7 |
| $Na_2HPO_4$ | 1.15 | 8. |
| $KH_2PO_4$ | 0.2 | 1.5 |
| $CaCL_2$ | 0.1 | 0.9 |
| $MgCl_2.6H_2O$ | 0.1 | 0.5 |

### PBS-Tween 20

| | |
|---|---|
| NaCl | 8.0g |
| KCl | 0.2g |
| $Na_2HPO_4.12H_2O$ | 2.8g |
| $KH_2PO_4$ | 0.2g |
| Tween 20 | 0.5ml |

Make up to 1 litre with distilled water

### Substrate Buffer for alkaline phosphatase

| | |
|---|---|
| Diethanolamine | 97ml |
| $MgCl_2.6H_2O$ | 100mg |
| $NaN_3$ | 200mg |
| Distilled water | 900ml |

Add concentrated KCl (10M) until pH *of* solution is 9.8

### White Cell Staining Fluid

20% acetic acid + Gentian violet ( 2 drops per ml)

### Methyl Green

| | |
|---|---|
| Methyl green (Sigma) | 0.025g |
| Glycerol | lml |
| Distilled water | 9ml |

The dye is added just before end of the electrophoresis using a spinal needle. Great care is taken not to contaminate the dye solution with running buffer after application as the alkaline nature of the buffer tends to turn the dye colourless.

## Coomassie Blue Stain

2.5g Brilliant Blue R (Sigma)

Dissolve in 250ml ethanol, then add 250 ml distilled water
and **50ml** glacial acetic acid. Stir for 30 minutes then filter with Whatman No 1 paper. Stain for 3 hours.

## Gel Destaining Solution

70% distilled water

20% ethanol

10% glacial acetic acid

Destain for 2-3 days.

## Substrate AEC preparation for immunostaining

Dissolve 1 AEC tablet (3amino 9ethyl carbazole, Sigma) in 2.5ml of dimethyl foramide (DMF, Sigma). Add 2.5 ml of AEC-DMF to 47.5 **ml** of 50mM acetate buffer pH 5.0 with

stirring. Add 25 µl of fresh 30% hydrogen peroxide immediately prior to use. Filter the solution through a 0.2 µicron filter.

Acetate buffer :- add 74 ml of 0.2M acetic acid (11.55ml of glacial acetic acid) and 176 ml of 0.2M sodium acetate buffer (27.2 g sodium acetate trihydrate/l) to 1 litre of deionized water and mix.

## Reagents for **SDS-PAGE**

### Stock Reagent Preparation

### A. Acrylamide/Bis (30% T, 2.67% C)

Acrylamidel46.0g

**N'N** - bis-methylylene-acrylamide 4.0g

Distilled water to 500ml. Filtered and stored at 4°C in the dark. Maximum shelf life under these conditions is 30 days.

### B. 1.5M Tris-HCl, pH 8.8

54.45 g Tris base

150 ml distilled water

Adjust to **pH** 8.8 with 10 N HC1. Distilled water to 300ml.

Store at 4°C.

### C. 0.5M Tris-HC1, pH 6.8

6g Tris base

60ml distilled water

Adjust to pH 6.8 with 10N HC1. Distilled water to 100ml. Store at 4°C.

D. 10% (w/v) SDS

Dissolve 10g SDS in water with gentle stirring. Distilled water to 100ml.

E. 10% Ammonium persulphate (w/v)

100 mg ammonium persulphate.

Distilled water to 1 ml. Make fresh daily.

F. Sample buffer (SDS reducing buffer: *62.5* mM Tris-HC1, pH *6.8,* 10% glycerol, **2%** SDS, *5%* Mercaptoethanol)

| | |
|---|---|
| Distilled water | 3.8ml |
| 0.5M Tris-HC1, pH 6.8 | 1.0ml |
| Glycerol | 0.8ml |
| 10% SDS | 1.6ml |
| ß-mercaptoethanol | 0.4ml |
| 0.05%(w/v)Bromophenol blue (in water) | 0.4ml |
| Total | 8.0ml |

Dilute the sample at least 1:4 with sample buffer. Heat at 95-99°C for 4 minutes.

*G.5* x Electrode (Running) Buffer (25 mM Tris, 192 mM glycine, 1% SDS, pH 8.3)

| | |
|---|---|
| Tris base | 45.0g |
| Glycine | 216.0g |
| SDS | 15.0g |

Distilled water to 3 litres. Store at 4°C. Warm to 37°C before use if precipitation occurs. Dilute 300 ml 5 x stock with 1.2 L distilled water for one electrophoretic run.

## H.TEMED - N,N,N',N'-Tetramethylethylenediamine

Molecular formula $C_6H_{16}N_2$.

Store at room temperature

# Appendix 2

Viability of the cells was assessed with Trypan blue 1:l and if >95% further experimentation was performed. The cells were incubated in Nunc cell culture plates for 48 hours with nothing or with 1 μg/ml of E.Coli lipopolysaccharide (Sigma) at a concentration of 2 x $10^5$/ml at 37°C in a 5% CO, chamber.

The maximal secretion of IL-8, TNFa, IL-1β and IL-6 by addition of lipopolysaccharide to the mononuclear cells was determined on biopsies obtained from 5 healthy patients. Lipopolysaccharide at concentrations of 0.001, 0.01, 0.1, 1.0, and 10 micrograms/ml was used.

Interleukin-8

| | 0.00 | 0.01 | 0.10 | 1.00 | 10.00 |
|---|---|---|---|---|---|
| PT 1 | 20 | 20 | 30 | 150 | 160 |
| PT 2 | 0 | 0 | 30 | 40 | 30 |
| PT 3 | 0 | 0 | 0 | 0 | 0 |
| PT 4 | 50 | 40 | 110 | 120 | 100 |
| PT 5 | 20 | 20 | 30 | 50 | 50 |

TNFα

| | 0.00 | 0.01 | 0.10 | 1.00 | 10.00 |
|---|---|---|---|---|---|
| PT 1 | 0 | 0 | 0 | 50 | 110 |
| PT 2 | 0 | 30 | 20 | 60 | 30 |
| PT 3 | 0 | 0 | 20 | 20 | 20 |
| PT 4 | 0 | 20 | 20 | 100 | 140 |
| PT 5 | 0 | 20 | 70 | 70 | 70 |

Interleukin-1β

| | 0.00 | 0.01 | 0.10 | 1.00 | 10.00 |
|---|---|---|---|---|---|
| PT 1 | 30 | 30 | 30 | 40 | 30 |
| PT 2 | 0 | 30 | 30 | 30 | 20 |
| PT 3 | 0 | 0 | 0 | 0 | 0 |
| PT 4 | 20 | 20 | 20 | 20 | 20 |
| PT 5 | 20 | 20 | 20 | 20 | 20 |

Interleukin-6

| | 0.00 | 0.01 | 0.10 | 1.00 | 10.00 |
|---|---|---|---|---|---|
| PT 1 | 0 | 0 | 30 | 30 | 30 |
| PT 2 | 0 | 0 | 20 | 30 | 20 |
| PT 3 | 0 | 0 | 0 | 0 | 0 |
| PT 4 | 0 | 20 | 20 | 30 | 40 |
| PT 5 | 20 | 30 | 20 | 30 | 30 |

*1.0 microgram of LPS caused maximal stimulation of mononuclear cells isolated by the techniques described and this dose has been also used by other investigators.*

## Anatomical Site and cytokine production

**Preliminary studies looking at** TNFa **production by mononuclear cells from 4 different anatomical sites in the colon were undertaken:**

**a.ascending colon**

**b.transverse colon**

**c.descending colon**

**d.rectosigmoid colon**

**Study of mononuclear cells in 6 patients did not reveal any significant difference in the amount of cytokine produced**

by anatomical site in healthy colon.

## Coating Buffer

Add 1.59g $Na_2CO_3$ to 500 ml distilled water

Add 2.93g NaHCO, to 500 ml distilled water

Add together until pH 9.6

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