



**THERAPEUTIC MANIPULATION
OF
INFLAMMATORY MEDIATORS**

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

in

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by

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Abstract

Inflammation normally fulfils an important protective role for the host. However, under certain conditions, such as rheumatoid arthritis, the chronic inflammatory responses can be detrimental. Central to the process of inflammation is the complex interaction of different inflammatory cells. They communicate by releasing mediators that target appropriate cells to induce changes in their function. The manipulation of these mediators may provide a way of controlling the progression and tissue damage of chronic inflammation.

The drugs most commonly used in the treatment of both chronic and acute inflammation are the 'Aspirin like' nonsteroidal antiinflammatory drugs (NSAIDs). It is generally accepted their mode of action is the inhibition of prostaglandin (PG) production by inhibiting the enzyme arachidonate cyclooxygenase. This thesis shows that the production and action of inflammatory cytokines, such as interleukin (IL)-1, IL-2 and tumour necrosis factor (TNF), are enhanced with NSAID treatment in vivo and in vitro by reducing PG's which normally suppress IL-1, IL-2, interferon (IFN) γ and TNF. Conversely, IL-6 production is enhanced by PG's.

Like PGE₂, the PGE's₁ and ₃ regulate cytokines and other cell functions. In addition, PGE analogues, such as Misoprostol, have similar effects. All these PG's seem to bind to the same cell surface receptor(s) and effectively raise levels of intracellular cyclic AMP. PGE's enhance IL-6 production by stimulating gene transcription.

Cyclosporin A (CsA) is very effective in preventing the development of adjuvant induced arthritis in rats. CsA inhibits production of the inflammatory cytokines IL-1, IL-2, IFN γ and TNF. IL-6 production is not affected in vitro but enhanced ex

vivo. Assays with monoclonal antibodies indicate that these effects may be mediated by selectively targeting T-helper type 1 lymphocytes.

Overall, this study indicates that PGE's and CsA may have similar modes of action. The findings suggest that therapies that selectively target subpopulations of leucocytes, and manipulate the inflammatory mediators they produce, may be effective in the treatment of chronic immuno-inflammatory diseases similar to rheumatoid arthritis.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best knowledge and belief, contains no material previously published or written by another person, except where due reference had been made in the text.

I give consent to the copy of my thesis, when deposited in the University Library, being available for photocopying and loan.

SIGNED

DATE: 24th June 1993

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Chapter 1

Background: Mediators of inflammation

Concerning inflammation in general

Inflammation is characterised by the movement of fluid and white cells from the circulation into the extravascular tissues. Since classical Greek and Roman times the clinical signs of inflammation have been characterised as rubor, calor, tumor, and dolor (redness, heat, swelling and, pain respectively). These signs may also be associated with loss of function of the affected organ or associated tissues.

Inflammation arises in response to a pathogenic insult and usually represents the host's attempt to eliminate altered cells, foreign particles or microorganisms and their antigens. Under normal conditions the pathogenic insult and any damaged tissue is removed (or isolated). Repair and the return of normal function usually follows. Scar tissue forms when regeneration of specialized tissues is not possible.

Normally there is an orderly progression from the initial pathological insult through the inflammatory response to repair. In these circumstances, the process of inflammation fulfils an important protective role for the host. However, under certain conditions this orderly progression to repair may be impaired. There may be an inability to clear the foreign agents or injured tissue. Immune responses may be directed against the host's own tissue, now recognised as "foreign" due to altered tissue components or aberrations of the host's immune responses. There may also be a perturbation of the regulatory mechanisms which are needed for the resolution/orderly progression of the inflammatory process. Under these circumstances inflammation can be harmful with continued tissue damage leading to loss of function in the affected regions. A common example of this harmful inflammatory response is the chronic disease, rheumatoid arthritis.

Concerning rheumatoid arthritis in particular

Rheumatoid arthritis is a systemic inflammatory disease that involves the joints which, while affecting all age groups, usually has its onset during the third or fourth decade of life. It has varying effects on the individual patient, ranging from transient and limited arthritis through to severe and disabling multi-system disease with occasional life-threatening complications. It is a disease which has severe detrimental social and economic effect on the community.

The pathology of rheumatoid arthritis is the result of complex and interactive inflammatory and immune processes, many of the details of which still are undetermined. Simplified, the arthritis is characterised by two interrelated, but largely functionally separate processes. These are (i) ongoing chronic inflammatory changes in the synovial tissues, and (ii) episodes of acute inflammation dominantly affecting the synovial fluid.

Concerning the relationship between chronic and acute inflammation in rheumatoid arthritis

The chronic inflammation in rheumatoid arthritis is associated with accumulation of macrophages, lymphocytes and plasma cells in the affected tissues with invasive destruction of cartilage, bone and ligamentous structures. This is followed, in some instances, by fibrous repair of damaged tissues. The episodes of acute inflammation are dominated by an accumulation of neutrophil polymorphs and fluid exudate within the synovial fluid (Vernon-Roberts 1983). Generally, acute inflammation is a non-specific local process which can occur in response to active foreign compounds (such as bacterial lipopolysaccharide or carrageenan), activated components of the complement system or mediators (eg histamine, cytokines, platelet activating factor or arachidonate metabolites) released by leucocytes and platelets. In rheumatoid arthritis the acute inflammation is thought

to be mediated by the underlying, immunologically sustained, chronic inflammation occurring within the diseased joint.

Non-steroidal anti-inflammatory drugs (NSAIDs), successful in treating acute inflammatory models, are the mainstay of attempts to suppress acute inflammatory episodes in rheumatoid arthritis, but, practically, have little or no effect on the intensity or progression of the underlying chronic disease.

This thesis acknowledges that chronic inflammation underlies many aspects of the expression of rheumatoid arthritis and therefore investigates some ways of utilising or manipulating naturally-occurring (endogenous) control mechanisms to optimise or replace exogenous drug therapy.

Concerning cell mediators of inflammation

Central to the process of inflammation is the complex interaction of different inflammatory cells. These cells communicate by releasing mediators that target appropriate cells to induce a change in their function. Plasma derived inflammatory mediators are also important in directing inflammatory cell functions. Complement components and kinins are examples of such mediators, recognised as playing an essential role in inflammation due to their chemotactic and vasodilatory effects.

This thesis will concentrate on cell-derived products, particularly those released by activated macrophages and lymphocytes, involved in sustaining and regulating chronic inflammation (see table 1.1). These mediators are usually discrete chemical molecules and can be divided into two classes, protein and non-protein. The central theme of this thesis is the regulation of cytokines, protein mediators derived from leucocyte populations and certain other cells. The other major group of mediators studied in this thesis are the non-protein prostanoids. Many other mediators known to have an active role in inflammation, for example

Table 1.1**Comparison of inflammatory mediators produced by activated macrophages.**

	IL-1	TNF	IL-6	PGE ₂
Biological property				
Endogenous pyrogen (fever)	++	+	+	+
Induction of acute phase proteins	++	++	++	0
Induction of B lymphocyte immunoglobulin synthesis	0	0	++	--
Induction of lymphocyte proliferation	++	+	++	--
Induction of fibroblast proliferation	++	+	0	0
Induction of IL-1 production	++	++	0	-
Induction of TNF production	++	+	0	--
Induction of IL-6 production	++	++	0	++
Induction of PGE ₂ production	++	++	0	0
Induction of IL-2 production	++	+	+	--
Chemotactic for neutrophils	++	+	0	0
Activation of endothelium	++	++	0	0
Vasodilator (odema)	+	+	0	++
Induction of cachexia	0	++	0	0
Induction of cartilage destruction	++	++	0	0
Induction of bone resorption	++	++	+ / 0	++

Information obtained from references described in text and data presented in this thesis.

++ = strong activity, + = moderate activity, + / 0 = conflicting reports, 0 = no effect, - = moderate inhibition and -- = strong inhibition.

the vasoactive amines (eg histamine and serotonin) and the lysosomal derived enzymes (eg cathepsin). Reactive oxygen species, whose role in drug metabolism is investigated in Chapter 2, may also be important local mediators.

Inflammatory cytokines

Inflammatory cytokines comprise a large group of low molecular weight proteins (generally 10,000 - 30,000 kDaltons) that are produced by a wide variety of cell types and usually act in a paracrine or autocrine fashion. Their production is often transient and their release is under the control of complex mechanisms. Different cytokines may share many of the same control mechanisms, and, consequently, a number of different cytokines are usually produced at the same time following a single stimulus. An individual cytokine can also simulate the production of several other cytokines as well as itself, thus generating a network of interactions involving many types of cell.

Over the past two decades, cytokine research has proved to be increasingly relevant to the study of inflammation (Rees 1992). This has been largely due to the advent of recombinant DNA technology which has allowed study of their biological properties *in vivo* and *in vitro* with adequate quantities of highly pure proteins. Until recently, their overlapping biological properties and the pleotropic effects of individual cytokines has made it difficult to analyse the exact role of a particular cytokine in the inflammatory process. In addition, their similar size and chemical nature has made them difficult to isolate from inflamed tissues and inflammatory fluids.

Interleukin-1 (IL-1)

Two forms of IL-1 have been cloned, the more abundant form of IL-1 β was artificially cloned from human blood monocytes (Auron et al 1984), and the less common and usually cell-associated IL-1 α cloned from a mouse macrophage cell line (Lomedico et al, 1984). In macrophages, the biologically active cell associated

IL-1 may be a particularly important in lymphoid tissue where lymphocytes form rosettes around macrophages. Both forms are synthesised from 31 kDalton precursors but share little amino acid homology, 26% in the case of the two human forms of IL-1 (Dinarello 1989). There is more amino acid homology between the IL-1 α (or the IL-1 β) of different species, such as between human and mouse IL-1 α , than between the two forms of IL-1 from the same species. This may indicate that the two forms developed separately, either before or early, in the evolution of mammals. Comparison of the sequences of the two forms suggests that they may have been retro transformed (Dower 1992).

Despite the differences in amino acid sequence, both types of IL-1 appear to bind to the same IL-1 receptor(s) with similar affinities (Dower 1992, Bomsztyk et al 1989, Chizzonite et al 1989). The only other naturally occurring polypeptide known to bind to this receptor is the IL-1 receptor-antagonist whose amino acid sequence is very similar to IL-1 β . Two types of receptor have been identified, type 1 (p80), the predominant type found on B lymphocytes, and type 2 (p60) the predominant type found on T lymphocytes and mesenchymal cells (Dower 1992). IL-1 receptors are widespread on cells from all lineages tested in vitro.

The biological effects of IL-1 are wide ranging both in vivo and in vitro (table 1.1). Before adequate chemical analysis and cloning, the properties of IL-1 were ascribed to many (at least 11) different proteins based on the biological properties of purified preparations. Some of the historic names describe properties of IL-1 that indicate its central role in the process of inflammation, and of rheumatoid arthritis in particular. Some of these names (and functions) given to the protein now recognised as IL-1 are; catabolin (inducing cartilage destruction); endogenous pyrogen (inducing fever); osteoclast-activating factor (inducing bone resorption); lymphocyte-activating factor (stimulation of lymphocyte proliferation and activation); fibroblast-activating factor (stimulating fibroblast proliferation); and, hepatocyte-stimulating factor (induction of acute phase protein release). Most of

these properties were originally demonstrated in vitro. However, in vivo administration has recently been shown to lead to a diverse array of physiological responses that also reflect many of the actions previously recognised in vitro (Dinarello 1989, Dower 1992, Dinarello 1992).

The potent activity of IL-1, and the co-existence of inhibitors of its activity, have made it difficult to detect its activity in vivo. It can elicit responses at femtomolar concentrations (10^{-15} M or <1 pg/ml) in biological assays. In the biological assays described elsewhere in this thesis activity at concentrations as low as 10^{-13} M was measured for standard commercial recombinant preparations. Generally, the biological activities do not show species specificity. The presence of biological inhibitors has made the ELISA the method of choice for detection in biological fluids. However, fluids with levels of IL-1 below the limit of detection by ELISA may still contain biologically active concentrations of IL-1. More sensitive procedures using antisense mRNA specific for IL-1, and PCR techniques have the potential to detect the low levels of IL-1 production in small amounts of tissue or cells.

A wide variety of cells can be induced to produce detectable levels of IL-1 in its precursor forms. However, most cells accumulate IL-1 in their cytoplasm and only a few, such as monocytes/macrophages, release active IL-1 into the extracellular environment. Interestingly, the IL-1 α precursor is active and the IL-1 β precursor is inactive (Mosley et al 1987). Only cells of the myelo-monocytic lineage seem to possess the requisite protease to produce the active form (Black et al 1989). In rheumatoid arthritis high levels of IL-1 are found in the synovial fluid (Fontana et al 1982, Wood et al 1983, Nouri et al 1984) and activated monocytes and macrophages are thought to be the major source of this IL-1.

Tumour necrosis factor (TNF)

TNF was originally defined by its anti-tumoral activity in vivo and in vitro (Carswell et al 1975, O'Malley et al 1962, Old 1985). The same protein was also called cachectin or cachectin/TNF due to its ability to induce weight loss by inhibiting the enzyme lipoprotein lipase (Beutler et al 1985, Mahony et al 1985). A related protein, lymphotoxin was identified as having similar properties after being released from activated lymphocytes (Granger & Williams 1968, Ruddle & Waksman 1968). In recent years these two molecules have been given the names of TNF α and β respectively since their relationship to one another is similar to that described for the two forms of IL-1. TNF α and β have only limited sequence homology but seem to exert the same range of biological properties. The genes for the TNF's are separated by only 1100 base pairs and reside within the major histocompatibility locus (Chromosome 6 in humans) (Nedwin et al 1985). The two cytokines share the same cell surface receptor and bind to it with similar affinities (Schall et al 1990). X-ray crystallography has revealed that TNF α forms trimers which form the specific structures which binds to its receptor(s), (monomers seem to be inactive (Jones et al 1989)

It was originally assumed that TNF α was solely a product of monocytes/macrophages and that TNF β was derived from lymphocytes. With the advent of more sensitive detection techniques (specific immunoassay and mRNA analysis) it is clear that this is not correct. Lymphocytes exposed to particular stimuli can produce TNF α (Cuturi et al 1987) and both forms are also produced by a wide variety of other cells. It is, however, generally true that monocytes/macrophages produce TNF α , lymphocytes produce predominantly TNF β , and that these cells are the prime sources of TNF activity in inflammation.

The natural inhibitor of TNF activity is not a receptor antagonist as is the case for IL-1. It seems that inhibition is mediated by soluble receptor molecules which bind to free TNF and prevent its binding to the receptors on the target cells (Grey et al 1990). At least two forms of TNF receptor exist (p60 and p80) (Hohman et al 1989,

Englemann et al 1990). Both have molecular weights of about 100 kDaltons and both can be cleaved to release the soluble extracellular domain of about 30 kDalton which can be isolated from serum and urine (Englemann et al 1990, Olsson et al 1989, Seckinger et al 1989). These soluble forms of the receptor may be extremely important in regulating TNF and provide a way of therapeutically controlling TNF activity in a number of disorders.

Like other cytokines TNF is extremely potent and exhibits biological activity in vitro in the sub picomolar range. Many biological activities for TNF have been demonstrated which indicate that it has roles in both normal and abnormal pathology other than tumour killing and cachexia. It shares many of the pro-inflammatory functions of IL-1 including activation of neutrophils, macrophages, lymphocytes and fibroblasts (Dinnarello 1989, 1992). TNF α (but not TNF β) has been detected in the synovial fluids of patients with rheumatoid arthritis (Saxne T et al 1988) and, together with IL-1, is considered to be largely responsible for the tissue damage and inflammation of the joints.

In vitro monocyte/macrophages and lymphocytes release biologically active TNF rapidly (peak levels in approximately 3 hours) following a strong stimulus (eg bacterial endotoxin). Other mediators, such as IL-1, PGE₂ and IL-6, may not reach peak levels for 12 hours or more. Since TNF is a strong stimulator of these mediators, it has been postulated that TNF may be a key factor which triggers the overall inflammatory response. This may be a simplistic concept, taking into account the complex interactions that occur amongst inflammatory mediators in chronic inflammation, nevertheless the development of TNF antagonists may be of great benefit for the treatment of arthritis.

Interleukin-6 (IL-6)

IL-6 is another multifunctional cytokine acting on a wide variety of cells. Like IL-1, its pleotropic nature has historically resulted in it being given many names based

on the biological function which each investigating group of workers was interested. What is now recognised as the same 26 kDalton protein (IL-6) was cloned almost at the same time by groups working in separate biological disciplines (Hirano et al 1986, Zilberstein et al 1986, Haegeman et al 1986). IL-6 is recognised to have a positive influence on the maturation and function of B lymphocytes, stimulates the release of acute phase proteins from liver cells, and was once considered to have anti-viral activity. A single gene codes for IL-6 which is a 26 kDalton protein in humans, although variations in glycosylation have led to different isoforms being identified. Like both IL-1 and TNF, its activity is not species specific; the only reported exception being that mouse IL-6 does not act on human cells (Sugita et al 1990).

There is strong evidence that IL-6 and granulocyte colony-stimulating factor have evolved from a common ancestor gene, since their amino acid sequence, tertiary structures and binding proteins are very similar (Nagata et al 1986, Yasukawa et al 1987). The action of IL-6 on hepatocytes is well characterised. Interestingly, the binding of IL-6 to its specific receptor has no effect. It is the association of IL-6 and its receptor on the cells surface that triggers an association of the IL-6 receptor and the protein gp130 (Kishimoto et al 1992). As a consequence, gp130 activates tyrosine kinase activity which, in turn, leads to the activation of the transcription factor NF-IL6 (Akira et al 1992). Other cytokines may also activate the same second messenger (NF-IL6) (Akira et al 1992). It seems that, while the expression of receptors determines the cell's responses, the signal transduction and the second messengers for endocellular signalling, are identical for many cytokines.

IL-6 release can be stimulated in a large number of cell types including lymphocytes (T and B), monocytes, macrophages, and fibroblasts. While IL-6 production is seen in the absence of many stimuli *in vitro*, IL-1, TNF and PGE₂ are strong stimulators. The role of intracellular cyclic AMP in this process is discussed later.

The in vivo half life of IL-6 may be up to 1 hour (Castel et al 1988). Consequently, serum levels can be detected, particularly in patients with inflammation or undergoing graft rejection (Van Oers et al 1988). Its effects may not just be localised to the site of inflammation, although high levels are found in synovial fluid (Houssiau et al 1988, Swaak et al 1988), and its systemic effects account for the acute phase protein production by the liver in such inflammatory diseases. IL-6 seems to play a central role in host defence mechanisms by regulating immune responses, haematopoiesis and acute phase reactions. Its role in inflammation is not certain since it is a poor stimulator of the other inflammatory cytokines (IL-1, TNF) and its other actions could not be considered strongly proinflammatory. Perhaps its role will be better understood when we further understand the role of the acute phase proteins in inflammation.

Interleukin-2 (IL-2)

IL-2 was one of the first cytokines to be identified. It was originally defined as a T-cell growth factor produced by lymphocytes, following antigen or mitogen stimulation, that allowed long-term growth of human T lymphocytes (Morgan et al 1976).

The presence of IL-2 and proliferating lymphocytes in the joints of patients with rheumatoid arthritis suggests a role for this cytokine in the progression of the disease. The presence in the rheumatoid joint of inhibitors of its action (Smith et al 1988) may also be important.

Interferon gamma (IFN γ)

IFN γ is a cytokine with a molecular weight of 17,000 kDalton, released by lymphocytes of the T-helper type one sub-class and is active in the form of a dimer (Ealick et al 1991). Although there is considerable amino acid homology between species, 40% homology between human and mouse IFN γ (Gray & Goeddel 1982),

its activity is species specific (unlike the cytokines discussed previously). Originally identified because of its anti-viral activities, it is now recognised as having a number of other regulant properties.

Its role in inflammation is unclear. Unlike the other cytokines discussed here, only very low levels have been detected in the synovial fluid of patients with rheumatoid arthritis (Firestein & Zvaifler 1987). It also inhibits proliferation of a variety of cells and can inhibit osteoclast activation. Animal studies, in which IFN γ suppressed induction of animal models of arthritis, has led to treatment trials in patients with rheumatoid arthritis (Cannon et al 1990). However, IFN γ may play an important role in initiating and maintaining chronic inflammation (see chapter 5) since it is a strong activator of macrophages (see Chapter 3) and cell-mediated immune functions (Grey 1991).

Other Cytokines

There are many other protein mediators which are known to play important roles in inflammation which were not investigated in the experimental studies reported here. For example the ability of leucocytes to produce chemotactic cytokines has been recognised for some time. Many of these (eg GM-CSF) have also been found in the synovial fluid of patients with rheumatoid arthritis and have been shown to induce unidirectional leucocyte movement in various in vitro assay systems (Xu et al 1989, Bignold et al 1990). These cytokines, together with several non-protein mediators, may be responsible for the rapid influx of polymorphonuclear cells to an inflammatory site.

However, not all cytokines produced during the course of inflammation induce pro-inflammatory responses. Several, such as IL-4, are known to down regulate the production and action of some of the inflammatory cytokines described above (Hart et al 1989). In addition, IL-10 is thought to suppress the production of these and other cytokines that are important in chronic inflammation (Street and

Mosmann 1991). The regulation of these cytokines and their endogenous use in the treatment of inflammation has been considered (Gautam et al 1992).

There is increasing interest in those cytokines which seem to have a role in the repair phase of inflammation following tissue damage. As mentioned previously, the inhibition of the repair phase of the inflammatory process and the continuation of chronic inflammation with damaging acute inflammatory episodes may be an important site to target antiinflammatory therapy in the future. Growth factors, such as platelet derived growth factor, transforming growth factor (TGF) β , and insulin-like growth factor, can stimulate repair of damaged tissue. Although these factors may stimulate directed migration of polymorphonuclear cells and monocytes, they are also potent chemoattractants for fibroblasts and smooth muscle cells, stimulating activities in these cells (eg release of collagenase) essential for tissue repair (Sporin and Roberts 1989). These growth factors are also known to induce bone formation and the regeneration of extracellular matrix in many tissues. These functions could be very important for the repair of an arthritic joint as well as directing healing in a variety of other degenerative disorders.

TGF β , like IL-4 and IL-10, may also down regulate many immune response. This raises the possibility that TGF β may not only induce repair but also play an important role in the switch from tissue-damaging immune-mediated chronic inflammation to tissue repair. TGF β is released as a biologically inactive precursor which, when cleaved, forms active dimers (Roberts and Sporin 1990).

Prostanoids (PG's)

Among the many non-cytokine mediators, products of membrane phospholipids and their component fatty acids are very important. These mediators are derived from arachidonate released from membrane lipids by one of two mechanisms. Firstly through the action of phospholipase A₂ or secondly through the cleavage of arachidonic acid from diacylglycerol, stimulated indirectly by phospholipase C.

Once free arachidonate (20:4) is available, it can be metabolised by one of two pathways: cyclooxygenation to form the prostanoids and thromboxanes; and lipoxygenation to form hydroxyeicosatetraenoic acids and leukotrienes. In addition, other biologically active chemicals such as platelet activating factor may be formed. The prostanoid products of the enzyme cyclooxygenase (prostaglandin G/H synthetase) are the main focus of this thesis.

Unstable endoperoxide derivatives of arachidonate are generated by specific cyclooxygenase enzymes in a variety of inflammatory cells. These are rapidly metabolised to more stable prostaglandins (PGs) such as PGI₂ (prostacyclin), PGF_{2α}, PGE₂, PGD₂ and thromboxane A₂. Different cells may produce different proportions of each metabolite. For example, macrophages produce a range of prostanoids, whereas platelets produce mainly thromboxane A₂. Not all of the PG's have similar actions (see Chapter 4). PGI₂ and PGE₂ are potent vasodilators, while thromboxane A₂ is a potent vasoconstrictor. This is consistent with thromboxane A₂'s role in platelet aggregation. The effects of prostanoids E₂ and I₂ are mediated via specific receptors on the cell surface. When these receptors are bound, adenylyl cyclase is activated which rapidly induces an increase in the levels of intracellular cyclic adenosine phosphate (cAMP) which mediates their effects. The role of cAMP in the regulation of action (and production) of other mediators and cell functions is discussed in more detail in Chapter 4.

The second pathway by which arachidonate is metabolised, lipoxygenation to form hydroxyeicosotetraenoic acids and leukotrienes (LT's), is also important in inflammation. A principal product, leukotriene A₄ (LTA₄), can be further metabolised to other biologically active LT's. LTB₄, which is chemoattractive for, and an activator of, polymorphonuclear cells and macrophages is present in the synovial fluid of patients with rheumatoid arthritis (Davidson et al 1983, Rola-Pleszczynski and Lemaire 1985). Another group of LT's, originally known as slow-reactive substances of anaphylaxis (SRS-As), stimulate symptoms associated with allergic

type reactions. Their ability to induce contraction of smooth muscle and enhance vascular permeability has indicated that they are potential targets for therapy of diseases such as asthma.

Conventional antiinflammatory therapies that affect inflammatory mediators

The most common drugs used in the treatment of both chronic and acute inflammation are the 'Aspirin like' nonsteroidal antiinflammatory drugs (NSAIDs). They are used as a first line drug in the treatment of arthritis. Salicylates, present in the leaves and bark of certain types of trees, have been used for the treatment of inflammation for centuries. Last century, a commercial method for synthesising Aspirin was developed by Felix Hoffmann at the Bayer Pharmaceutical Company. However, its mode of its actions has only been investigated in recent decades. In The early 1970's, Vane showed that Aspirin exerted its anti-inflammatory effect by inhibiting the enzyme cyclooxygenase, thereby inhibiting prostaglandin production (Vane 1971). Since then many other drugs have been produced with similar activities.

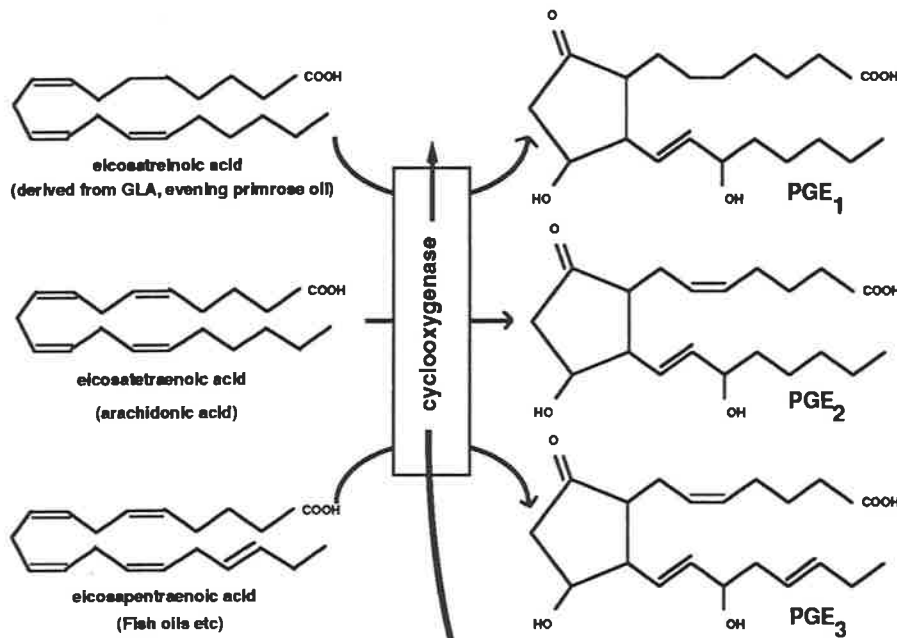
One of the major side effects of NSAIDs is that they not only inhibit PG production at the site of inflammation but also beneficial PG production in the gastric mucosa. This is a important problem in long term NSAID therapy since reduction of gastro-protective PGs promotes ulcer formation and gastric bleeding (Rainsford 1988). The role of PGs as gastroprotective agents is demonstrated by the fact that these adverse side effects of NSAIDs can be partly reversed by the co-administration of PG analogues such as Misoprostol or 16,16-dimethyl PGE₂ (Nicholson 1990) (see figure 1.1 and chapter 4).

Corticosteroids are also known to inhibit PG production but by a different mechanism. This type of drug induces an inhibitor (now designated lipocortin) of phospholipase A₂, and, therefore, the availability of free arachidonate (Flower 1978, Davidson et al 1987). Metabolites of both cyclooxygenase and lipoxygenase

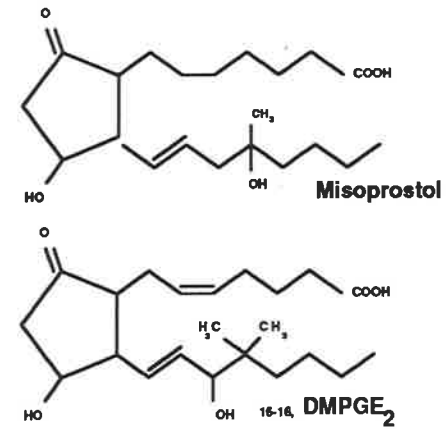
Figure 1.1

EICOSANOIDS.

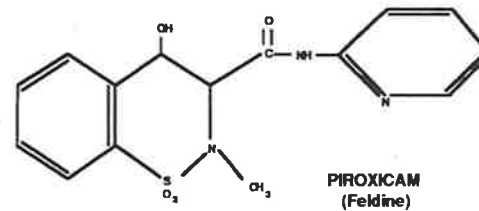
PRECURSOR



ANALOGUE



**Nonsteroidal
antiinflammatory drugs**



are reduced by this mechanism. Recently, it has been recognised that corticosteroids can also regulate the production of cytokines directly (Alison and Lee 1988) and inhibition of IL-1 and TNF may be important component of the beneficial antiinflammatory action of corticosteroids.

Inhibitors of lipoxygenase are another category of drug currently being developed (Marshall and Chang 1989). These may be particularly useful in the treatment of allergic disorders such as asthma.

Other drugs used successfully in the treatment of arthritis may also rely on the regulation of endogenous mediators for their action. Gold compounds, and other second line antiarthritic drugs, are used with variable effects. Recent evidence suggests that gold complexes may regulate the generation of immature leucocytes, possibly by inhibiting the action of cytokines and other colony stimulating factors essential for proliferation (Haynes et al 1988b, Hamilton and Williams 1986). Other drugs, such as the anti-malarials, have also been reported to regulate inflammatory cytokines (Salmeron and Lipsky 1983) and recently, cyclosporin has been used to successfully treat autoimmune disorders because of its ability to suppress cytokines involved in cellular immune responses (see Chapter 5).

Regulation of inflammatory mediators by dietary factors (essential fatty acids)

Recently dietary factors have been trialed for the treatment of arthritis with some success (Kremer et al 1987, Cleland et al 1988). The use of fish oils arose from the observation that Eskimos had a reduced incidence of heart disease, arthritis and other degenerative diseases common to Western societies (Kormann and Green 1980). The role of essential fatty acids in the inflammatory process is thought to be due to their effects as precursor molecules for the eicosanoids, and via their effects on the inflammatory cell membrane. Figure 1.1 shows how, in theory, the availability of precursor molecules other than arachidonate may lead to the production of other PG's, particularly PGE₁ or PGE₃. Similarly, different products

of the lipoxygenase pathway may be formed and affect inflammation. Although the results of human studies have been disappointing in the short term (Hansen et al 1983), beneficial effects have been seen in the longer term (>12 months) (Belch et al 1988). Treatments using dietary augmentation with eicosapentanoic acid in animal models of arthritis have been shown to both decrease (Leslie et al 1985) and augment (Prickett et al 1984) disease. This indicates the complex nature of the metabolism of essential fatty acids. Not only are the effects on the types of PG's and lipoxygenase products important to consider, but also the interactions of these new products may result in unanticipated effects. In addition, essential fatty acids are important in the structure of the cell membrane and any changes to these may greatly affect cell functions (Mead and Mertin 1978).

This chapter indicates that therapeutic manipulation of inflammatory mediators covers a very large and complex field. The aim of this thesis is to investigate a small part of this expanding area of research. Despite each of the following 4 chapters being presented in the form of discrete studies, having their own discussion sections, there are common themes which link these components of the thesis. Initially the studies addressed the actions of the most widely used antiinflammatory drugs, the NSAIDs (Chapters 2 and 3). NSAID's mode of action in vivo and in vitro was shown to depend largely upon their ability to inhibit the production of PG's. Investigations into the means by which different types of PG's regulate inflammatory mediators was then extensively studied (Chapter 4). Since cyclosporin A (CsA) is very effective in preventing the development of adjuvant induced arthritis in rats in vivo and in vitro studies into the effect of CsA on several inflammatory mediators were carried out and revealed that CsA had similar effects to PG's (Chapter 5). Finally the overall findings were addressed and some general conclusions drawn (Chapter 5).

Chapter 2

Stimulation of cytokine-induced lymphocyte proliferation in vitro and in vivo by inhibitors of cyclooxygenase.

INTRODUCTION

Mononuclear phagocytes (MNPs) can modulate many aspects of inflammation by releasing various pro-inflammatory and anti-inflammatory agents. Two examples are lymphoproliferative cytokines (LCs) and prostaglandins (PGs), which have opposing effects on lymphocyte proliferation (Otterness et al 1988). The cytokines, interleukin (IL)-1 and 6, enhance proliferation of activated lymphocytes by stimulating IL-2 production and IL-2 receptor expression (Dinarello 1989, Helle et al 1989). By contrast, PGs (PGE₁ and PGE₂) are known to inhibit lymphocyte proliferation (Baker et al 1981, Lewis 1983, Goodwin 1984). Although PG inhibition of cytokine action and production has been established in vitro, the in vivo stimulation of cytokine activity by PG inhibitors has not been clearly demonstrated.

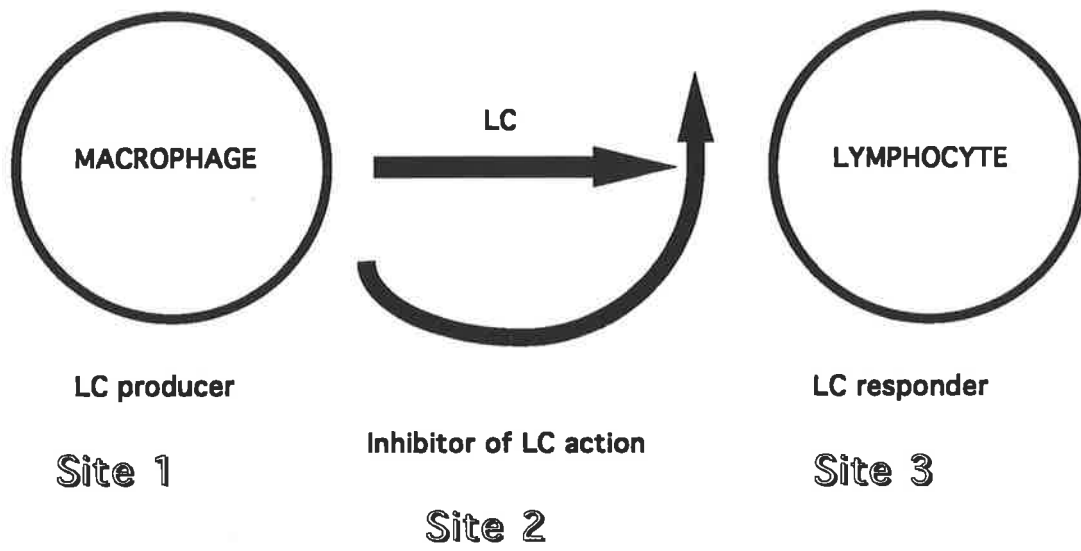
Although the concept of a single mode of action of some non-steroidal anti-inflammatory drugs (NSAIDs) has been challenged (Goodwin 1984, Abrahamson et al 1983), it is widely accepted that their principal mode of action is the inhibition of PG production by inhibiting the enzyme arachidonate cyclooxygenase (Vane 1971). Therefore, as well as inhibiting the proinflammatory effects of PGs, NSAIDs may also stimulate certain aspects of the immune response such as lymphocyte functions (Lewis and Barrett 1986) that are normally subject to PG autoregulation.

The effect of Piroxicam and other drugs on LC production by MNP, and on the action of LCs on lymphocytes was investigated. Three possible sites of action were identified using an in vitro model (Fig 2.1). Human monocytes and rodent

Figure 2.1. Drugs may regulate lymphoproliferation during inflammation by affecting either the production of LC's (lymphoproliferative cytokines) at site 1, the production of a LC inhibitor at site 2, or lymphocyte proliferation at site 3.

Fig. 2.1

3 Possible sites of action



macrophages were used to generate LC and mouse thymocytes (T-lymphocytes) were used as the LC responder (LAF assay). Anti-inflammatory drugs might affect (i) the quantity of LC produced (at site 1), or (ii) simultaneous production by the monocyte/macrophages of an antagonist to the LC (at site 2), or (iii) the response of the lymphocytes to LC (at site 3). The *in vitro* effect of NSAIDs on the lymphoproliferative action of LCs produced by MNP has been previously described (Kunkel and Chensue 1984, Hart et al 1989). Here, this regulation is demonstrated to involve the action and production of lymphoproliferative cytokines and similar effects are shown to occur *in vivo* in an animal model of inflammation induced by oleyl alcohol.

MATERIALS AND METHODS

Chemicals

Drugs and chemicals were obtained from the following sources; PGE₂, Piroxicam, Indomethacin, ^{benzoates, salicylates} and Phenylbutazone from Sigma Chemical Co. USA; Isoxicam from Warner Lambert International; Azapropazone from A. H. Robins UK; Tenoxicam from Roche Products Australia; Naproxen (R and S enantiomers) from Syntex USA; Sulindac and its metabolites from Merck, Sharp and Dome USA; Clozic from ICI Pharmaceuticals UK; CGP drugs from Ciba-Geigy Switzerland; Nimesulide (R-805) from both Riker-3M USA and Boehringer-Biochima Robin Italy; N-Dichlorophenylanthranilic acids were kindly donated by Dr. R. A. Scherrer (St. Paul, Minnesota). All drugs dissolved in RPMI media throughout this study.

Isolation and culture of peritoneal MNPs

Normal mice (C3H/HeJ or LACA Swiss) were sacrificed by cervical dislocation and their peritoneal cavities lavaged with Hank's buffered saline (HBS). The peritoneal cells were washed once by centrifugation. One $\times 10^6$ cells suspended in 1ml of RPMI-1640 medium (supplemented with 10% foetal calf serum, 50 IU/ml penicillin and 50 U/ml streptomycin throughout these experiments) were placed

in 16mm flat-bottomed wells of a 24 well tray (Costar) with 13mm glass coverslips at the bottom of each well. After 1 hour incubation at 37°C in 5% CO₂, the non-adherent cells were removed by washing 3 times with fresh medium. The remaining adherent cells were incubated in 0.5ml of RPMI medium containing concentrations of drug. In some experiments *E. coli* 0111:B4 lipopolysaccharide (LPS) (Sigma Chemical Co.) was added to stimulate the MNPs. After 24 hours the supernatant was harvested and stored at -70°C for subsequent LC assay. More than 96% of the adherent cells stained positively for the presence of non-specific esterase. (Yam et al 1971).

Assessment of MNP spreading

After the supernatant was taken for LC measurement, the MNPs were immediately fixed by immersing the coverslip in 2.5% glutaraldehyde in HBS for 10 minutes. The glutaraldehyde-fixed cells were then stained with Giemsa's stain and the coverslips mounted onto glass slides. With the aid of a microscope graticule at least 5 random fields were sampled (greater than 500 cells), to determine the percentage of adherent cells that were elongated and/or star shaped. A cell was considered to be spread (elongated and/or stellate) if the width was at least twice that of a normal rounded cell.

Cytokine-dependent lymphoproliferation (LAF) assay

LC activity was assessed in the LAF assay as previously described (Haynes et al 1988b). Mouse (C3H/HeJ) thymocytes were cultured in the presence of suboptimal (1 ug/ml) concentrations of PHA (Flow Labs), 5×10^{-5} M 2-mercaptoethanol, IL-1 preparations and drugs. After incubation for 68 hours ³H- thymidine was added and 4 hours later the amount of ³H-thymidine incorporated measured. One unit / ml was determined to be the concentration of LC required to give 50% of maximal thymocyte proliferation. This assay will measure both an IL-1 (recombinant IL-1 β donated by Otsuka Pharmaceutical Co., Tokushima, Japan) and IL-6 (recombinant material purchased from Genzyme Corp. Boston, USA) activities.

Interleukin 6 assay

To assay IL-6, 7TD1 hybridoma cells were used, a gift from Dr J. van Snick (van Snick et al 1986). Briefly, these hybridoma cells were grown in RPMI-1640 media described above supplemented with 2-mercaptoethanol (5×10^{-5} M) and 100units/ml rIL-6. These cells were then washed 3 times in HBS, counted and diluted to a concentration of 2×10^4 cells/ml. This cell suspension (100ul) was then added to an equal volume of serially diluted test sample. 72 hours later, proliferation was measured using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide Thiazolyl blue (MTT) dye reaction (Mossman 1983). Serially diluted human recombinant IL-6 (Genzyme) was used as a standard in all these assays. Polyclonal antibodies (Genzyme) directed against human recombinant IL-6 inhibited 95-97% of the activity of both recombinant IL-6 and the IL-6 activity present in the supernatants from human cells.

Interleukin 2 assay

IL-2 activity was measured using a IL-2 dependent cell line (CTLL) (Gillis et al 1978).

PGE₂ assay

PGE₂ concentrations were measured by competitive radioimmunoassay as previously described (Kelly et al 1987). Polyclonal antibodies raised in goats directed against methoximated PGE₂ were a kind gift from Dr R Seamark, Dept of Obstetrics and Gynecology, University of Adelaide. All supernatants were stored at -70°C and methoximated before assay. PGE₂ (Sigma Chem. Co.) methoximated before assay was used to prepare a standard curve.

Experimental inflammation

Inflammation was induced in healthy 8-10 week old C3H/HeJ mice by injecting 0.05 ml oleyl alcohol (OA) in the base of the tail on day 0. Piroxicam (5 mg / kg) or

saline was given intraperitoneally daily, the first injection being given 2 hours prior to the OA injection. This dose of Piroxicam was significantly higher than that given to patients on medication. However the half life of the drug is much shorter in rodents and a previous study in these animals indicated that this dose is required to produce an antiinflammatory effect (Whitehouse 1986). The mice were sacrificed on day 3.

Isolation of peripheral blood, spleen and thymic lymphocytes

Mice were exsanguinated via cardiac puncture after ether anaesthesia, and their peritoneal cavities washed out with HBS as described above. Spleens were removed and homogenised in HBS with a teflon pestle and glass mortar. The thymuses were squeezed through a fine wire mesh. Blood samples from each group were pooled to obtain enough cells for assay. The blood, and individual spleen and thymus cell suspensions, were underlaid with Ficoll and centrifuged (400g for 40 minutes). The buffy coat at the Ficoll-Paque layer interface was washed 3 times with HBS and resuspended to a concentration of 2×10^6 cells / ml in RPMI media.

Lymphocyte proliferation

Lymphocytes isolated from the peripheral blood, spleen and thymus were immediately incubated at a concentration of 1×10^6 / ml (1×10^5 / microtitre well) with 0.5 uCi ^3H -thymidine. 4 hours later the uptake of ^3H -thymidine was measured as described previously.

Statistical analysis

All statistically significant differences between groups were calculated using an unpaired, two tailed student's *t* - test (Snedecor and Cochran 1989).

RESULTS

In vitro studies**a. the effect of NSAIDs on LAF activity produced at sites 1 and 2**

The lymphoproliferative activity of supernatants from normal mouse MNP cultured with Piroxicam and LPS for 24 hours was measured in the co-mitogenic LAF assay. Figure 2.2 shows an apparent increase in the LAF activity from the Piroxicam-treated cultures at higher concentrations of supernatant (< 1/64 dilution). This was highly significant ($p < 0.01$) for Piroxicam concentrations greater than 0.1 μ M. Piroxicam had no direct effect upon the activity of IL-1 in the LAF assay at concentrations below 500 μ M (data not shown). 20 μ M Piroxicam enhanced lymphoproliferation at a 1/4 dilution of supernatant by 10 fold.

Figure 2.3 shows the effect of Piroxicam on PGE₂ production by these same cells. The amount of PGE₂ produced is inversely proportional to the activity of LC produced (DPM) when measured at a 1/16 dilution of supernatant.

b. the effect of NSAIDs mediated by PGE₂ on site 3

The effect of PGE₂ on the LAF activity of 10 units/ml recombinant human IL-1 β in the LAF assay was determined (Fig 2.4). PGE₂ inhibited the proliferation of lymphocytes induced by IL-1 in a dose dependent manner. Since IL-2 production is required for this proliferation, the IL-2 levels in the supernatants of these cultures after 48 hours of the 72 hour LAF assay was also measured. It was found that PGE₂ inhibited (IL-1)-induced IL-2 production at a similar concentrations to those which inhibited overall LAF activity.

c. Comparison of different NSAIDs, salicylate metabolites and immunosuppressants at sites 1, 2 and 3

The ability of different NSAIDs (Table 2.1) to increase apparent LAF activity, as demonstrated with Piroxicam in figure 2.2, was compared by deriving a stimulation index (SI). This was the concentration of drug which increased the LAF activity to twice that of controls at 1/16 dilution of supernatant. Known cyclooxygenase

Figure 2.2. Piroxicam treatment stimulates LAF activity at low dilutions of supernatants from mouse MNPs. Resident C3H/HeJ mouse peritoneal MNPs were adhered to plastic for 1 hour and stimulated with LPS and various concentrations of Piroxicam for 24 hours. Supernatant were removed and the lymphoproliferative activity measured in the LAF assay. Each point represents the mean of at least 3 experiments. The standard error (not shown) was always less than 10%.

Figure 2.2

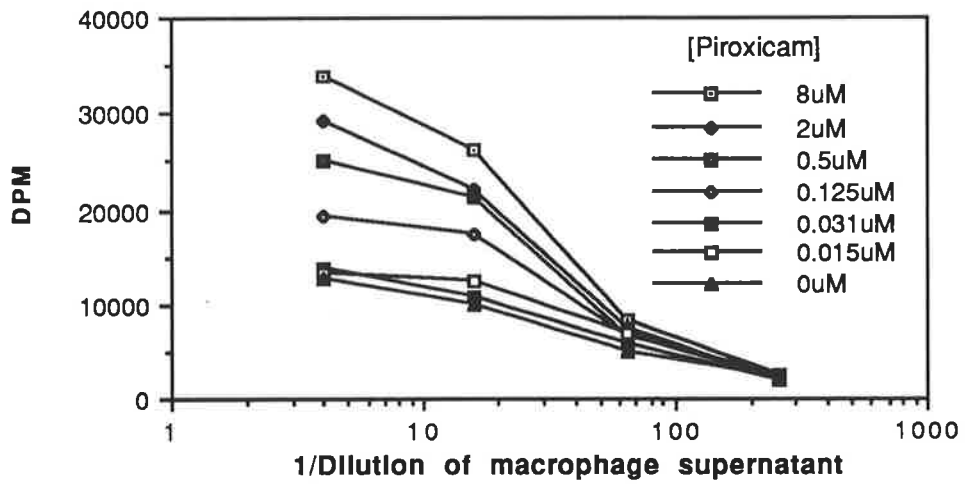


Figure 2.3. The stimulation of LAF activity by Piroxicam is proportional to its inhibition of PGE₂ production. Mouse MNPs were cultured with various concentrations of Piroxicam as described in Figure 1. The LAF activity of a 1/16 dilution of supernatant (closed circles) was compared to the PGE₂ concentration found in the same supernatant (open squares). Each point represents the mean of at least 3 experiments. The standard error (not shown) was always less than 15%.

Figure 2.3

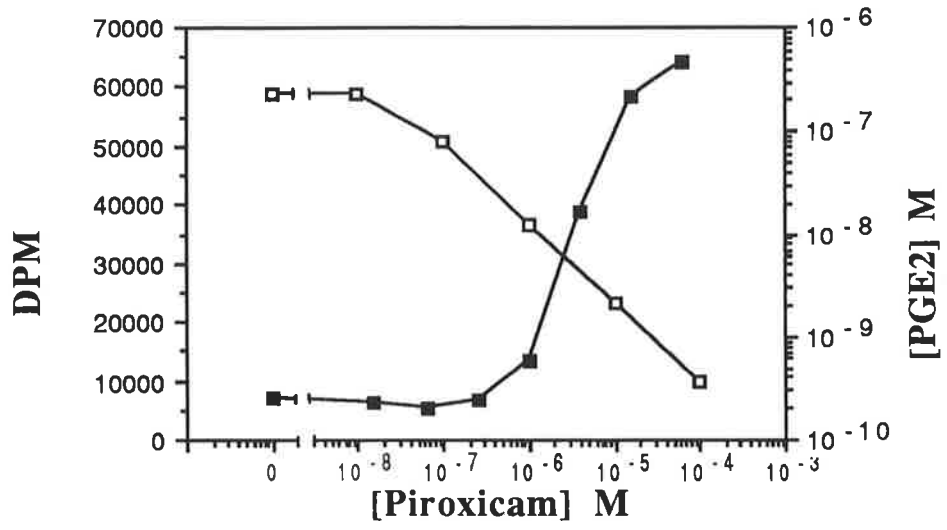


Figure 2.4. Endogenous PGE₂ inhibits the lymphoproliferative activity of recombinant human IL-1 β by its inhibition of IL-2. 10 units/ml of recombinant human IL-1 β was incubated with various concentrations of PGE₂ in the LAF assay with. After 48 hours the supernatants were sampled and their IL-2 activity measured in the IL-2/CTLL assay (closed squares). This was compared to the effect of the same concentrations of PGE₂ on LAF assay (open squares). Activity in the absence of PGE₂ was determined to be 100%. Each point represents the mean of at least 4 experiments +/- standard error.

Figure 2.4

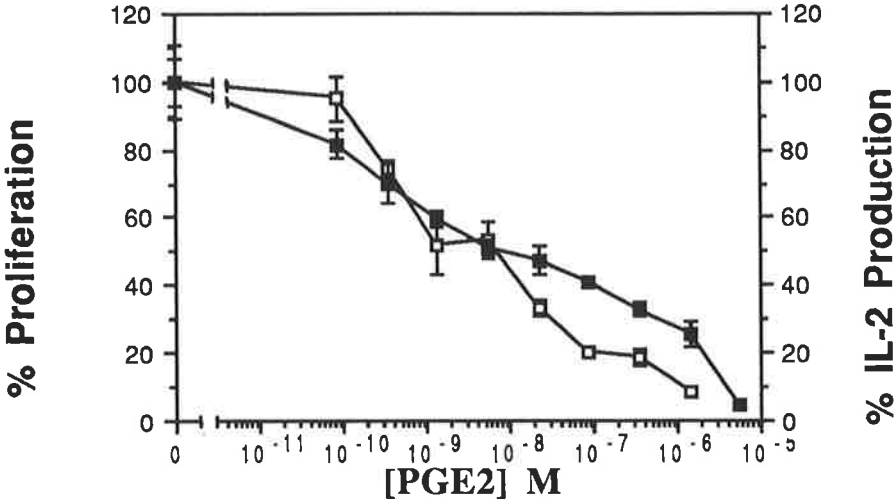


Table 2.1.

The effect of NSAID's on sites 2 and 3

DRUG	SITE 2 SI μM^1	cyclooxygenase inhibition ²	SITE 3 IC50 μM^3	PlasmConc. μM^4
Piroxicam	0.25	++	280	21
Isoxicam	1.0	++	>128	80
Indomethacin	0.14	++	64	14
Phenylbutazone	32	++	>128	325
Aspirin	22	++	510	
Salicylate	100	+	590	1389
Sulindac	16	-	22	
Sulindac sulphide metabolite	0.25	++	22	15
Sulindac sulphone metabolite	>128	-	22	
S-Naproxen	8.0	+	110	110
R-Naproxen	100	-	150	
S-Ibuprofen	0.5	++	100	120
R-Ibuprofen	50	-	380	
CGP-28,237	0.5	++	40	
Nimesulide	3.6	++	3.5	
2,3-DichloroPAA ⁵	0.5		12	
2,4-DichloroPAA ⁵	850		20	
Diclofenac	0.6	++	39	8
CGP 4720/III ⁶	21	-	28	
Meclofenamate	1.5	++	48	
Clozic	>1000	-	130	
BW755c	0.1	++	4.5	

¹ SI = the concentration of drug which increases LAF activity (by suppressing an inhibitor of LC produced by rat peritoneal macrophage treated with 20 $\mu\text{g}/\text{ml}$ LPS by two times (measured at 1/16 dilution of supernatant).-see results section.

² Literature reports (see text) of *in vitro* inhibition of prostaglandin synthesis.

³ The concentration of drug which will inhibit the LAF activity of 1 unit/ml LC by 50%.

⁴ From Lombardino 1985.

⁵ PAA = N-phenylanthranilate.

⁶ 2,4-Dichloroisomer of diclofenac.

inhibitors (Lombardino 1985) had lower SI values than their less active analogues or inactive congeners. For example, the active S(+) enantiomer of Naproxen (Tomlinson et al 1972) had a SI value of 8uM, whereas the inactive R(-) enantiomer had a SI of 100uM. The active metabolite of Sulindac, the sulphide (SI = 0.25uM) (Duggan 1981), was much more active than the sulphone metabolite (SI > 128uM) or sulindac itself (SI = 28uM). 2,3 dichloroisomer anthranilic acid, an active NSAID (Scherrer 1974), had a SI = 0.05uM whereas the inactive 2,3 dichloroisomer had a SI = 85. Clozic was notable, among the clinically active NSAIDs tested, in not stimulating LAF activity. Resting MNP (not stimulated with LPS) produced very little PGE₂ (< 10nM in the supernatants detected in the PGE₂ assay) in contrast to stimulated MNP (see fig 2.3). It was, therefore not feasible to quantify the effects of NSAIDs on LAF activity produced from these unstimulated MNP.

Table 2.2 shows that the metabolites of Aspirin (some of which are shown in figure 2.5) and some related salicylates affected the 3 sites in different ways. None of the compounds tested affected site 1 at concentrations less than 1000uM. Of these compounds aspirin was found to be the most active at site 2, whilst gentisate had no effect at concentrations less than 1000uM. Amongst the structurally related salicylates only 2,5-diacetylgentisate (2,5-DAG) and 5-aminosalicylate (5-AS) had any effect at the concentrations tested, both were effective at low concentrations, having IC₅₀'s of 45 and 25 uM respectively. Of the compounds affecting site 2 all (except Aspirin) could be described as "weak". This meant that they stimulated activity by only slightly more than 2-fold, even at their highest concentrations. High concentrations of Piroxicam and other effective NSAIDs, however, resulted in a greater than 4-fold increase in the stimulation of activity (see figure 2.2). Both gentisate (2,5 DHB) and homogentisate (2,5 DHB) affected IL-1 lymphoproliferative activity (site 3) at low concentrations (IC₅₀'s of 80 and 11 uM respectively) whilst the other hydroxybenzoates tested had little or no effect at the concentrations tested.

Table 2.2.

The effect of Aspirin metabolites and related salicylates on sites 1, 2 and 3
(see fig 2.1).

DRUG	SITE 1 IC ₅₀ uM	SITE 2 SI uM	SITE 3 IC ₅₀ uM
<u>Metabolites</u>			
Sodium salicylate	>1000	100 (very weak)	590
Aspirin (acetyl salicylate)	>1000	22	510
2,3 DHB (dihydroxybenzoate)	>1000	290 (weak)	>1000
2,5 DHB (gentisate)	>1000	>1000	80
<u>Related salicylates</u>			
2,5 DHBS			
(dihydroxybenzene sulphonate)	>1000	>1000	990
2,3 DAB (diacetoxybenzoate)	>1000	>1000	>1000
2,5 DAG (diacetylgentisate)	>1000	45 (weak)	390
2,5 DHG (homogentisate)	>1000	>1000	11
5 AS (5-amino salicylate)	>1000	25 (weak)	>1000
5 AAS (5-acetyl amino salicylate)	>1000	>1000	>1000
ADS (5,5'-azodisalicylate)	>1000	>1000	>1000

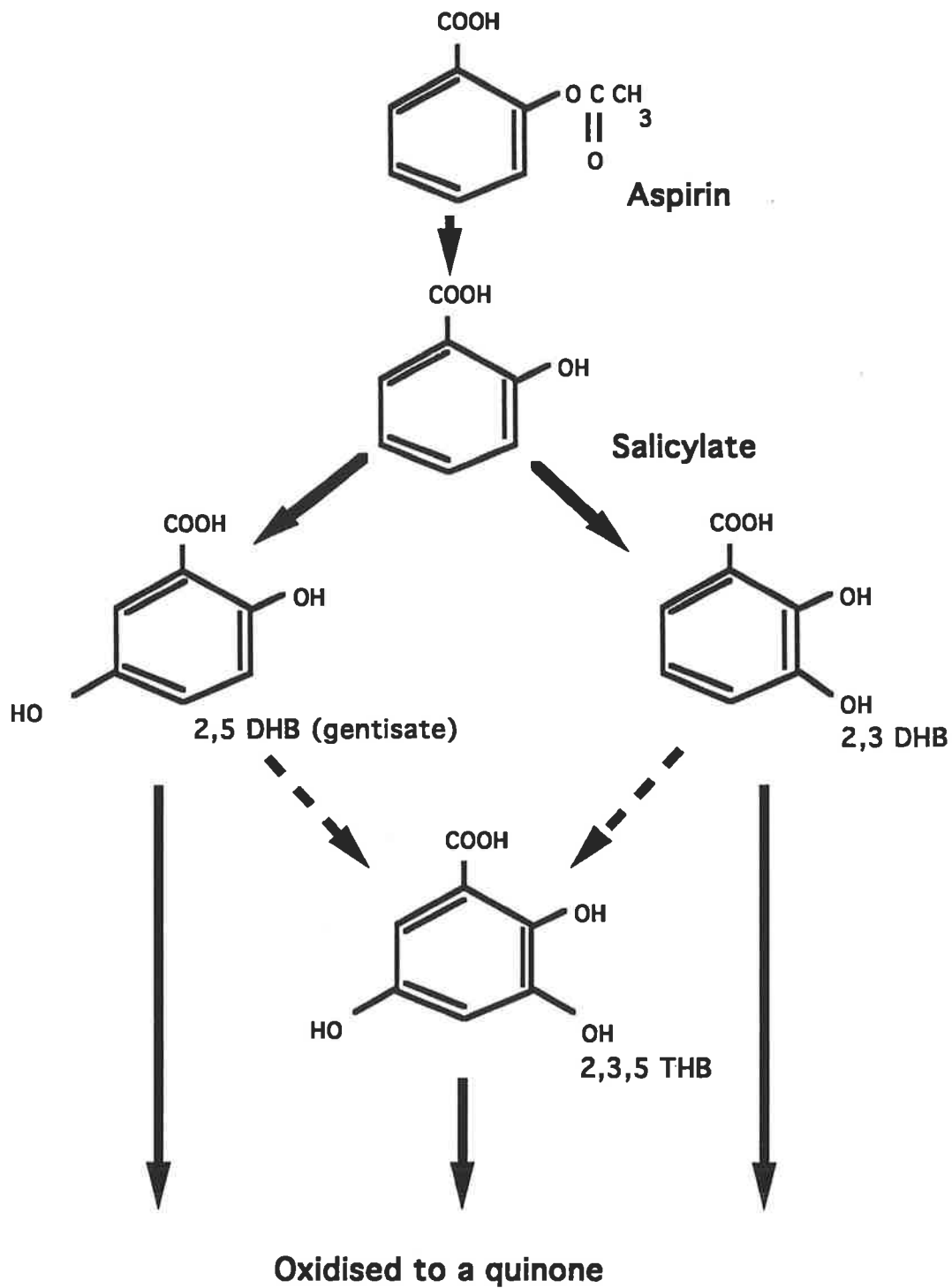
¹ IC₅₀ = the concentration of drug which inhibits the titre of LAF activity by 50%.

² SI = the concentration of drug which increases LAF activity (by suppressing an inhibitor of LC produced by rat peritoneal macrophage treated with 20ug/ml LPS by two times (measured at 1/16 dilution of supernatant)).

³ IC₅₀ = the concentration of drug which will inhibit the LAF activity of 1 unit/ml LC by 50%

Figure 2.5. Some possible products of salicylate oxygenation.

Figure 2.5
Metabolism of Aspirin



The action of steroidal and immunosuppressive drugs at the 3 sites was compared to the NSAIDs (table 2.3). The immunosuppressants had no effect on sites 1 and 2 at the concentrations tested; however all were very effective at suppressing IL-1 action (site 3). Cyclosporin was extremely effective ($IC_{50} = 0.1 \text{ nM}$) while Azathioprine was the least effective ($IC_{50} = 410 \text{ nM}$). The effect of the drugs on ^3H thymidine uptake during the first 3 hours of incubation was measured to determine if these effects were due to the immediate cytotoxicity of the drugs. When drugs were observed to have immediate toxicity, the concentrations were approximately 300 times higher than that which inhibited IL-1 action (site 3). All the steroidal drugs tested inhibited the titre of LAF activity produced. Figure 2.6 shows how the LAF titre is reduced by increasing concentrations of Prednisolone. Compare this to the effects of Piroxicam (Fig. 2.2) where the activity is enhanced at a dilution of 1/16 but the overall titre is not greatly affected. An enhancement of LAF activity at a dilution of 1/16 was noted at concentrations of 0.003 μM of Prednisolone and less, indicating this drug may have similar effects to the NSAIDs at site 2. However, because of its effects at sites 1 and 3 at concentrations of 0.003 μM and above, the full potential of its action at site 2 could not be determined.

In vivo / ex vivo studies

The following four groups of C3H/HeJ or LACA Swiss mice were used: 1) saline-treated (Control), 2) Piroxicam-treated (Px), 3) saline-treated and oleyl alcohol-inflamed (OA), and 4) Piroxicam-treated and oleyl alcohol inflamed (Px/OA) animals. Experiments were carried out with groups of 3-4 animals. Lymphocytes were isolated from 3 separate sites (peripheral blood, spleen and thymus) and their DNA synthesis immediately measured by ^3H -thymidine uptake over 4 hours in vitro (Table 2.4). Increases in DNA synthesis were seen in the spleen cells and blood lymphocytes isolated from Piroxicam-treated mice. This effect was observed in both inflamed and non-inflamed C3H/HeJ mice, but only in inflamed LACA Swiss mice. Piroxicam treatment had little effect on normal high levels of DNA synthesis by thymocytes which was greatly reduced in inflamed C3H/HeJ mice.

Table 2.3

The effect of immunosuppressants and steroids on sites 1, 2 and 3

DRUG	SITE 1 IC ₅₀ nM ¹	SITE 2 SI ₅₀ nM ²	SITE 3 IC ₅₀ nM ³	3 hour SITE 3 IC ₅₀ nM ⁴
<u>Immunosuppressants</u>				
Cyclosporin*	>100	>100	0.1	320
6-mercaptopurine	>1000	>1000	3.0	1000
Azothioprine	>1000	>1000	410	>1000
Methotrexate	>1000	>1000	5.2	>1000
<u>Steroids</u>				
Dexamethosone	1.0	>10	3.0	>1000
Prednisolone	2.0	>10	2.0	>1000
Hydocortisone	4.0	>10	15	>1000

¹ IC₅₀ = the concentration of drug which inhibits the titre of LAF activity by 50%.

² SI = the concentration of drug which increases LAF activity produced by rat peritoneal macrophage treated with 20ug/ml LPS by two times (measured at 1/16 dilution of supernatant).

³ IC₅₀ = the concentration of drug which will inhibit the LAF activity of 1 unit/ml LC by 50%

⁴ IC₅₀ = Inhibition of 3H-thymidine uptake by 50% during the first 3 hours incubation with drug.

* Sources of Drugs ; Cyclosporin A from Sandoz AG Switzerland ;
6-mercaptopurine, Azothioprine, Dexamethosone, Prednisolone, Hydrocortisone
from Sigma Chemical Company, St Louis ; Methotrexate from Lederle Labs USA.

Figure 2.6. The steroidal drug, Prednisolone, inhibits the LAF activity and titre of supernatants from mouse MNPs. Resident C3H/HeJ mouse peritoneal MNPs were stimulated with LPS and treated with various concentrations of Prednisolone for 24 hours. Supernatants were removed and LC activity measured in the LAF assay. Each point represents the mean of at least 3 experiments +/- standard error.

Figure 2.6

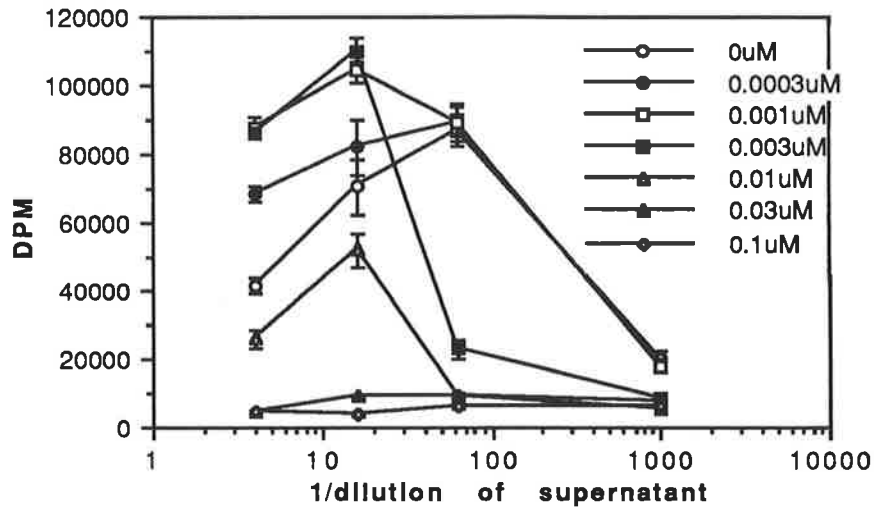


Table 2.4.

Ex vivo ³H-Thymidine incorporation by lymphoid cells from Piroxicam-treated normal and inflamed mice.

Peripheral blood

Group	Exp #1 ¹	Exp #2	Exp #3
Control	341 ± 27 ²	808 ± 32	576 ± 148
Px.	1755 ± 96 **	1225 ± 245	737 ± 220
OA.	327 ± 155	439 ± 13	1093 ± 16
OA/Px.	1398 ± 55 *	741 ± 18 *	2230 ± 393 *

Spleen

Group	Exp #1	Exp #2	Exp #3
Control	23778 ± 10895	30132 ± 3597	20322 ± 1218
Px.	70029 ± 17393 *	67150 ± 15377	12618 ± 2594
OA.	15136 ± 3124	24213 ± 9973	25982 ± 383
OA/Px.	71919 ± 11467 *	51198 ± 2940 *	99073 ± 5254 **

Thymus

Group	Exp #1	Exp #2	Exp #3
Control	66406 ± 779	34324 ± 8466	N.D.
Px.	59133 ± 2961	29534 ± 7562	N.D.
OA.	3006 ± 179	1018 ± 492	N.D.
OA/Px.	8978 ± 419 *	3516 ± 1157	N.D.

N.D. = not determined.

¹ 4 C3H/HeJ mice were used per group for experiment #1, 3 C3H/HeJ mice per group for experiment #2 and 4 LACA swiss mice were used per group in experiment#3. The groups were, Control = saline treated, Px = Piroxicam treated controls, OA = oleyl alcohol inflamed and saline treated, and OA/Px = oleyl alcohol and Piroxicam treated mice (see methods).

²DPM/10⁶ cells +/- standard error after 3 hours incubation.

*corresponds to a p< 0.05 and ** to p< 0.005. Piroxicam-treated groups were compared to the appropriate control (ie Px compared to Control and OA/Px compared to OA).

Adherent cells were isolated from the peritoneal cavity of the same animals. No significant differences in yields of cells were noted between the groups. Staining for non-specific esterase indicated that greater than 96% of the adherent cells were MNPs. No IL-2 activity was detected in the supernatants from cultures of these MNPs. Unstimulated MNPs from Piroxicam-treated animals produced significantly more LC activity than their corresponding controls (Table 2.5). Upon stimulation with LPS, these MNPs produced more LC. Similarly, LPS stimulation enhanced LC production in inflamed animals treated with Piroxicam. MNPs from inflamed animals produced more LC (either unstimulated or when stimulated with LPS) than corresponding cells from normal mice.

Figure 2.7 demonstrates that the LAF assay is sensitive to both IL-1 and IL-6 . Although the LAF assay was not very sensitive to recombinant human (rh) IL-6 alone (approximately 1,800 units /ml IL-6 = 1 u/ml IL-1), low levels of rhIL-6 were found to synergise strongly with rhIL-1 β . For example, a mixture of 250 units of rhIL-6 (as determined in the 7TD1 dependent cell assay) and 10⁻¹⁰ M rhIL-1 β (approximately 150 u/ml as determined on the LAF assay) had a LAF titre (u/ml) 3 fold greater than 10⁻¹⁰ M rhIL-1 β alone. This synergy was even greater with higher concentrations of rhIL-6 (a more than 10 fold increase in titre at 250,000 units /ml) but rhIL-6 had little effect on rhIL-1 β activity at concentrations below 25 units /ml. Since IL-6 activities of greater than 10,000 u/ml are commonly found in stimulated macrophage cultures (Table 2.6), data obtained using the LAF assay must be interpreted with caution.

Even though IL-6 may have potent effects on the LAF assay it is probably not contributing to the elevated LAF activity following Piroxicam treatment. In contrast to LAF activity, IL-6 activity produced by both peripheral blood monocytes and peritoneal macrophages was reduced following in vivo Piroxicam treatment (Table 2.6). Significant reductions in IL-6 activity were observed in inflamed animals.

Table 2.5.

**LAF activity produced by peritoneal macrophages from Piroxicam-treated (Px)
normal and inflamed (OA) C3H/HeJ mice.**

Peritoneal macrophages incubated in the absence or presence of 10 ug/ml LPS for 24 hours and the supernatants assayed for LAF activity measured as units of activity (means of 4 experiments \pm standard error).

LAF ACTIVITY.

units/ml

GROUP.	-LPS		+LPS	
Control	1.13	\pm 0.08	387	\pm 126.0
Px.	32.00	\pm 18.80	453	\pm 71.8
OA.	29.70	\pm 8.41	570	\pm 207.0
OA./Px	2260.00	\pm 1520.00	2900	\pm 802.0*

*P < 0.05. Piroxicam treated groups were compared to the appropriate control (ie Px compared to Control and OA/Px compared to OA).

Figure 2.7. IL-6 enhances the activity of recombinant human IL-1 β . A mixture of recombinant human IL-1 β (1×10^{-9} M, approximately 300 u/ml) and various concentrations of human recombinant IL-6 were serially diluted in the LAF assay and the activity of each dilution determined. Each point represents the mean of at least 3 experiments +/- standard error.

Figure 2.7

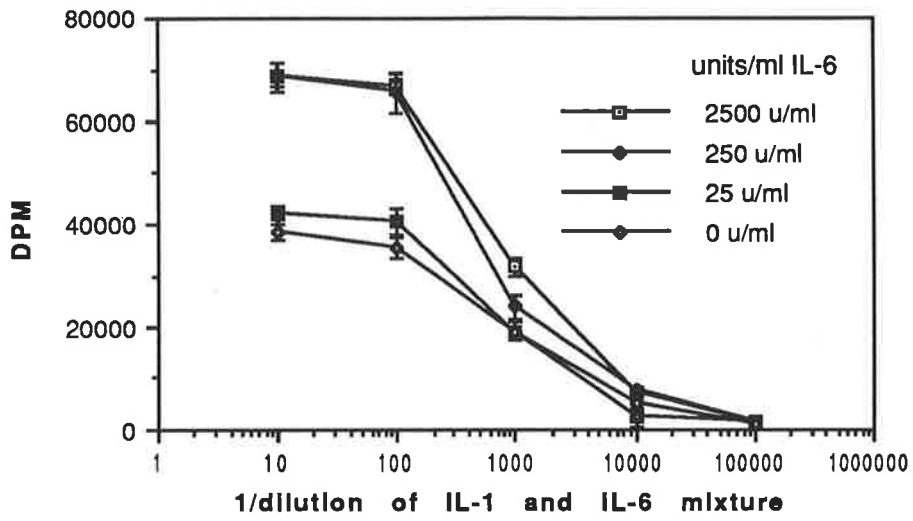


Table 2.6

IL-6 activity produced by peritoneal macrophages and peripheral blood mononuclear cells from Piroxicam-treated normal and inflamed C3H/HeJ mice.

Cells were incubated in the absence or presence of 10 ug/ml LPS for 24 hours and the supernatants assayed for IL-6 activity using the 7TD1 cell assay (measured as units of activity means of 3 experiments \pm standard error).

IL-6 ACTIVITY.
units/ml $\times 10^3$

GROUP.	-LPS		+LPS	
Peritoneal macrophages				
Control	15.00	\pm 2.00	143	\pm 18.6
Px.	2.70	\pm 1.15*	163	\pm 28.0
OA.	4.83	\pm 1.09	513	\pm 118
OA./Px	1.97	\pm 0.15*	247	\pm 33.3
Peripheral blood mononuclear cells				
Control	0.03	\pm 0.01	2.57	\pm 0.73
Px.	0.08	\pm 0.02	3.17	\pm 0.49
OA.	1.07	\pm 0.29	25.7	\pm 7.84
OA./Px	0.08	\pm 0.02*	2.33	\pm 0.33*

*P < 0.05. Piroxicam treated groups were compared to the appropriate control (ie Px compared to Control and OA/Px compared to OA).

Differences were also noted in the morphology of these MNPs after 24 hours incubation. The numbers of spreading MNPs was greater in the cell population isolated from animals treated with Piroxicam (Table 2.7). This enhancement of spreading was most marked in MNPs cultured in the absence of LPS, as compared to those cultured with LPS.

DISCUSSION

There have been many reports suggesting an immuno-regulant role for prostaglandins (PGs) (Lewis 1983, Lewis and Barrett 1986, Goodwin 1985). The majority indicate that PGs, particularly PGE₂, suppress immune functions such as cytokine production, lymphocyte proliferation and antibody production. Since the nonsteroidal antiinflammatory drugs (NSAIDs) are not only commonly used to treat inflammatory diseases but are also potent inhibitors of PG production, they may well enhance or restore immune functions suppressed by PGs (Goodwin et al 1984, Goodwin 1985, Ceupens et al 1986). By contrast, only a few examples of immune enhancement during NSAID treatment have been reported (Ceupens et al 1986, Koga et al 1983).

In vitro studies

a) NSAIDs

In vitro, PGE₂ will inhibit inflammatory cytokine (IL-1)-induced proliferation of lymphocytes in the LAF assay. In part, this inhibition is due to the suppression of IL-2 production needed as a final stimulus for proliferation (Fig 2.4, Baker et al 1981).

MNPs produce a number of lymphoproliferative cytokines (LCs) in response to an inflammatory stimulus. Of these IL-1, IL-6 (Helle et al 1988), and possibly tumour necrosis factor (TNF) (Ranges et al 1988), are detected in the LAF assay. The action

Table 2.7

Spreading of adherent cells isolated from the peritoneal cavity of Piroxicam-treated (Px) normal and inflamed (OA) C3H/HeJ mice.

Peritoneal macrophages incubated in the absence or presence of 10 ug/ml LPS for 24 hours.

GROUP.	-LPS	+LPS
Control	29.7 ¹ ± 3.6	54.6 ± 9.2
Px.	71.7 ± 13.0*	70.9 ± 8.6
OA.	43.6 ± 7.3	50.8 ± 7.7
OA./Px	73.0 ± 11.2	78.4 ± 7.2

¹ Percentage of spread macrophages (elongated or stellar shaped) determined as described in Methods. Means of 4 experiments ± standard error.

* P < 0.05. Piroxicam treated groups were compared to the appropriate control (ie Px compared to Control and OA/Px compared to OA).

of these LCs produced by MNP in vitro is also affected by simultaneous production of PGs. The LAF activity of LCs in the supernatants of LPS-stimulated MNPs is enhanced when Piroxicam is included during the 24 hour incubation. Increasing concentrations of Piroxicam reduce the amount of PGE₂ produced and enhance the lymphoproliferative activity of the LCs present as measured in the LAF assay. However this enhanced activity is only significant at higher concentrations of supernatants from Piroxicam-treated cultures (<1/64 dilution). At lower concentrations, where units of activity are normally determined, no significant difference in activity is observed. This indicates that although the actual amount of LC (detected in the LAF assay) produced is not greatly affected by Piroxicam, the activity of the LCs produced is reduced by simultaneous PG production.

b) Aspirin metabolites

Many natural metabolites of Aspirin have been reported (reviewed Brooks et al 1986a, Rainsford 1984). For example, the metabolite 2,5 DHB (gentisate) has been identified at concentrations of up to 15uM in the plasma and serum of patients receiving salicylate therapy for rheumatoid arthritis (Cleland et al 1985b, Grootveld and Halliwell 1986). Hydroxylation of salicylate to form not only 2,3 DHB but also 2,5 DHB (and possibly 2,3,5 THB) occurs freely in the presence of hydroxy radicals (see fig 2.5) in cell-free systems (Ledvina 1969, Grootveld and Halliwell 1986). This hydroxylation of salicylate and its metabolites may also reduce inflammation by quenching tissue-damaging oxyradicals (Cleland et al 1985a). Studies in which salicylate was incubated with neutrophils pre-activated to produce oxyradicals indicated that 2,5 DHB was the preferred product of hydroxylation by a ratio of about 5:1 (Wright 1989). 2,5 DHB (gentisate) may therefore be an important active metabolite of Aspirin therapy, not only because it may be produced preferentially at an inflammatory site, but also because of its ability to inhibit IL-1 induced lymphoproliferation.

Therefore, the ability of Gentsiate, but not 2,3 DHB, to inhibit the lymphoproliferative activity of IL-1 (site 3) might represent a another important mode of action of Aspirin. Two oxybenzoates related to 2,5 DHB, homogentsiate and, to a lesser extent, diacetylgentsiate, also inhibited at site 3. This may indicate that hydroxyl groups at both 2 and 5 positions on the benzene ring are a key chemical structure for this effect.

Unfortunately, the other poly hydroxy metabolite of interest, 2,3,5 THB, could not be tested since synthesis was difficult and, when obtained, (Wright 1989) it was very unstable. All these hydroxy metabolites may undergo further oxidation to form a quinone (Ledvina 1969). It is possible that these quinones may form in vitro (and in vivo), and that it is the quinone, rather than the hydroxy metabolites, that inhibit the action of IL-1 at site 3. These observations challenge the long held view of a single mode of action of NSAIDs and warrant further investigation.

c) Immunosuppressants and steroidal drugs

Steroid drugs are recognised as potent antiinflammatory drugs. It has long been thought that they reduce inflammation by inhibiting the release of arachidonic acid and thus reduce the production of proinflammatory prostanoids and leukotrienes (see chapter 3). If this were so, these drugs might be expected to enhance LAF activity at site 2 like the NSAIDs. However, this is not the case. While not stimulating LAF activity, steroid drugs inhibited its production (at site 1). This is consistent with recent findings that these drugs inhibit the production of IL-1 and other cytokines (Allison and Lee 1988). In addition, the steroidal drugs, like the immunosuppressants, inhibited the action of IL-1 (site 3). This may be mediated by inhibition of IL-2 production which is essential for proliferation following IL-1 stimulation in the LAF assay. Cyclosporin-A, for example, a potent immunosuppressive drug, inhibits IL-1 induced lymphoproliferation in this way (see Chapter 5). Whilst the steroid drugs may, like NSAIDs, still inhibit PGE

synthesis at site 2, their potent action at sites 1 and 3 will mask this effect and make it difficult to detect in this assay system.

In vivo/ex vivo studies

a) Lymphoproliferation

The effect of applying Piroxicam in vivo on ex vivo lymphoproliferation and LC production was investigated using oleyl alcohol as an inflammatory stimulus. In accord with the in vitro findings, lymphoproliferation ex vivo of peripheral blood and spleen cells was enhanced by Piroxicam treatment of both inflamed and noninflamed mice. However, thymocyte proliferation was not affected by Piroxicam treatment. This may indicate that in vivo lymphocyte proliferation within the thymus is not subject to PG suppression. The considerable reduction in lymphocyte DNA synthesis in the thymus induced by inflammatory stress might be due to the action of natural corticosteroids, so that Piroxicam treatment only slightly increased proliferation in the inflamed mice.

Surprisingly, in the absence of an inflammatory stimulus, Piroxicam treatment considerably increased proliferation of peripheral blood and spleen lymphocytes. This may indicate that, in these C3H/HeJ mice, either PG has a role in regulating normal lymphocyte proliferation, or the mice may have had a low grade inflammation even though they otherwise appeared healthy. Genetic factors may also be involved since, when these experiments were repeated using another strain, (LACA Swiss), the Piroxicam treatment of non-inflamed mice did not significantly increase proliferation of peripheral blood and spleen cells. C3H/HeJ mice are known to have some suppressed immune functions (Beutler et al 1986). These results might indicate that this strain of mice is either more sensitive to the effects of normal low levels of PG or they have higher basal levels of PG than other strains of mice.

b) Lymphoproliferative cytokines

Prior in vivo Piroxicam treatment resulted in a large increase in the LAF activity (units/ml) produced ex vivo by peritoneal MNPs isolated from inflamed mice, whilst IL-6 production by the same cells was reduced. Both basal and stimulated LAF production was enhanced, and IL-6 production reduced, by this Piroxicam treatment; probably due to the inhibition of PG production in vivo by Piroxicam. A recent report has indicated that IL-1 β production by peripheral blood mononuclear cells is also enhanced following in vivo treatment with the cyclooxygenase inhibitor, Indomethacin (Endres et al 1989). However, these authors did not observe a significant increase in IL-1 β production following Indomethacin treatment in vitro.

The LAF assay is not specific for any one cytokine as IL-1, IL-6 (Fig 2.7), IL-2 and TNF are reported to act positively in this assay (Helle et al 1988, Ranges et al 1988). However, there are two reasons why the enhanced LAF activity is probably due to increased IL-1 production. Firstly, no IL-2 or TNF was detected in the supernatants tested (the C3H/HeJ strain of mice is deficient in producing TNF (Butler et al 86). Secondly, although IL-6 can be detected in this assay (Fig. 2.7), it was reduced by Piroxicam treatment (table 2.6). Therefore, if IL-6 is contributing significantly to the LAF activity in these assays, the enhancement of IL-1 activity following Piroxicam treatment is underestimated.

There was no similar dramatic increase in the titre of LAF activity (site 1) produced by peritoneal MNP treated with Piroxicam in vitro (Fig.2.2). This may indicate that Piroxicam's inhibition of PG synthesis either i) does not directly affect IL-1 production by MNPs but inhibits the production of other cytokines produced by lymphocytes (such as interferon gamma) required to maintain IL-1 production by MNPs; or , (ii) Piroxicam treatment for more than 24 hours is needed to directly affect IL-1 production. This aspect is discussed in detail in Chapter 4 and is in direct contrast to the stimulation of IL-6 production by macrophages by Piroxicam in vitro (also Chapter 4).

c) Macrophage morphology

Piroxicam treatment also enhanced cell spreading, which is another marker of MNP activation. This is probably mediated by the inhibition of PGE₂ by Piroxicam since PGE₂ inhibits the spreading of MNPs in vitro (Cantarow 1978). This could indicate that the effects of in vivo Piroxicam treatment on MNPs (ie. inhibition of PGE₂ production) are still maintained ex vivo 24 hours after isolation and culture in Piroxicam-free media. Further investigation into the effects of NSAIDs and PGE₂ on MNP morphology (Chapter 3) indicated these effects are also seen after in vitro Piroxicam treatment.

These findings indicate that PGs may indeed be potent regulators of immune functions at concentrations comparable to those found in vitro, within inflammatory sites (Bray and Gordon 1978, Robinson and Levine 1974). Not only do PGs suppress proliferation of lymphocytes directly, but they may also suppress the production of mitogenic cytokines which precede and trigger lymphoproliferation. Similar regulation is observed in vitro with the more potent steroid and immunosuppressive drugs. Consequently, an inhibitor of PG production (Piroxicam) can stimulate some immune responses in mice with chronic inflammation. NSAID therapy may, therefore, enhance immune responses in patients with chronic inflammation. This could be beneficial as a heightened immune response might facilitate the removal of a persistent inflammagen. However enhanced production of some inflammatory cytokines might prove counter productive. For example, IL-1 not only stimulates the immune response but also could induce detrimental effects such as fever and bone resorption. NSAID therapy, via its effects on PG production, may enhance a variety of immune functions during inflammation.

Chapter 3

The effects of some anti-arthritic drugs, prostanoids, cyclic nucleotides and cytokines on the shape and function of rodent macrophages in vitro.

INTRODUCTION

Many cell types undergo the active process of cell spreading, by which normally round cells change shape and flatten onto a solid substrate. Cells undergoing this process are generally adherent and this shape change is often associated with the expression of motile structures, such as pseudopods. The spreading of a cell is an essential prerequisite for movement of amoeboid cells on surfaces (Vasiliev, 1982, 1985). The relationship between spreading, adhesion and locomotion is complex since too much spreading may also be associated with excessive adhesion and decreased motility (Keller et al 1979).

Cells of the macrophage/monocyte lineage play a central role in the immune and inflammatory responses. They are directly involved in the removal of bacterial and other foreign material as well as releasing many mediators that regulate the immune and inflammatory responses (reviewed chapter 1). Macrophages are adhesive and motile cells. Therefore, control of their spreading may play an important role in determining the resolution or exacerbation of many immune and inflammatory disorders. In addition to controlling macrophage movements, spreading may also indicate their activation for a wide variety of functions (Adams and Hamilton 1987).

Supernatants from endotoxin(LPS)-stimulated macrophages treated with NSAIDs exhibited enhanced IL-1 activity in vitro, as measured in the LAF assay (Chapter 2). This was probably due to inhibition of prostaglandin(PG)-E₂ production. These

macrophages readily attached to, and spread over, plastic or glass coverslips. This chapter describes the increased cell spreading of NSAID-treated macrophage cultures when compared to untreated (no drug) cell cultures. Enhanced spreading was also noted when the macrophages were treated with corticosteroids and some of the immunosuppressant drugs used to treat chronic inflammation. This enhanced spreading is one marker of macrophage activation. Macrophage activating factor (MAF) or interferon gamma ($\text{IFN}\gamma$) will activate macrophages in a variety of ways and increase their spreading (Schreiber et al. 1985).

Since the surface morphology of glass-adherent macrophages is well preserved by critical point drying (Pollack & Gordon 1975), it is possible to study the changes in surface structure (as revealed by scanning electron microscopy (SEM) induced by various drug/cytokine treatments. This study confirms that PGE_2 inhibits macrophage spreading (Cantarow et al 1978) and presents evidence to show that macrophages may autoregulate their own spreading by producing PGE_2 . This increased spreading may indicate enhanced motility and/or cell-cell adherence.

MATERIALS AND METHODS

Chemicals

Drugs and chemicals were obtained from the following sources: PGE_1 , PGE_2 , PGA_2 , $\text{PGF}_{1\alpha}$, $\text{PGF}_{2\alpha}$, PGD_2 , bt_2cAMP , bt_2cGMP and Piroxicam were purchased from the Sigma Chem. Co. (St Louis Mo. USA); and PGE_3 from Cayman Chem. Co. (Ann Arbor Mi. USA). Misoprostol was a gift from the G. D. Searle and Co. (Skokie, Il. USA); Naproxen (R and S enantiomers) from Syntex USA; Ibuprofen (R and S enantiomers) from Boots Drug Co UK.; Cortisol, Prednisolone, Dexamethasone, 6-mercaptopurine and Indomethacin from the Sigma Chemical Company St Louis; Methotrexate from Lederle Labs USA; Cyclosporin-A from Sandoz AG Switzerland.

Cytokines

The following cytokines were used; recombinant human IL-1 β donated by Otsuka Pharmaceutical Co., Tokushima, Japan; recombinant human IL-1 α , recombinant human IL-6, recombinant murine and human IFN γ , and recombinant murine TNF β from Genzyme, Boston; human recombinant TNF α and β from British Biotechnology; recombinant human IL-2 from Boehringer Mannheim GmbH. All recombinant cytokines were produced in E. coli.

Isolation and culture of rodent peritoneal macrophages

Normal rats (Dark Agouti) or mice (C3H/HeJ, C57/BL, CBA or Balb/C) from the central animal house (University of Adelaide) were sacrificed by cervical dislocation and their peritoneal cavities lavaged with Hank's buffered saline solution (HBS). The peritoneal cells were washed once by centrifugation. One $\times 10^6$ peritoneal cells suspended in 1ml of RPMI-1640 medium with 10% foetal calf serum were placed in 16mm flat-bottomed wells of a 24 well tray (Costar) with 13 mm glass coverslips at the bottom of each well. After 1 hours incubation at 37°C in 5% CO₂ the non-adherent cells were removed by washing with HBS. The adherent cells were then incubated in 0.5 ml of RPMI medium containing the appropriate concentration of drug. In some experiments E. coli 0111:B4 lipopolysaccharide (LPS) (Sigma Chemical Co.) at concentrations between 1-10 ug/ml was added to stimulate the macrophages. After 24 hours the cells were fixed for 10 minutes in a 4% glutaraldehyde/HBS solution.

Histochemical procedures

Cell preparations were stained for myeloperoxidase by the Kaplow method (Kaplow 1965). Briefly, cells fixed in glutaraldehyde (2%) were covered with freshly prepared peroxidase stain for 1 minute as described (Kaplow 1965) then counterstained with neutral red stain before drying and mounting.

For non-specific esterase (NSE) staining the method described by Yam et al (1971) was used. Briefly, glutaraldehyde-fixed cells were covered with fresh NSE stain for 30 minutes as described (Yam et al 1971). The cells were briefly counterstained with methyl green before drying and mounting. Rodent macrophages stained lightly with this NSE stain when compared to human monocytes.

Assessment of macrophage spreading by light microscopy

The glutaraldehyde-fixed cells were stained with Giemsa's stain and the coverslips mounted onto glass slides. With the aid of a microscope at least 5 random fields were sampled (greater than 500 cells) to determine the percentage of adherent cells that were elongated and/or star-shaped. A cell was considered to be 'spread' (ie elongated or stellate) if its width was at least twice that of a normal (rounded) cell. There have been many previous reports, mainly in studies with polymorphonuclear cells, using various techniques for estimating cell spreading (reviewed Rogers and Bignold 1990). Most have relied on visual assessments of spreading, whilst others used quantitative techniques such as using microscopic graticules or computerised methods to determine cell area. This study has relied on subjective visual assessment to quantitate spreading. However these assessments were carried out "blind" and where possible independent observers were used. Variation found between and by the same observer(s) was small (less than 8% and 5% respectively). An ED₅₀ was determined as the concentration of drug/cytokine which produced a two-fold increase in macrophage spreading computed from a dose-response plot.

Preparation of samples for scanning electron microscopy (SEM)

The glass coverslips with adherent cells were fixed as for light microscopy. The coverslips were then rinsed twice. In HBS and dehydrated in a graded series of aqueous/acetone solutions (up to 100% acetone) for 5 minutes at each step. After two washes in 100% acetone the specimens were quickly transferred to a precooled high pressure chamber of a critical point drying apparatus (Blazers

Union, Furstenum Liechtenstein) and dried using liquid N₂. All samples were then coated with gold/palladium and observed under a Phillips 505 SEM. Photographs were taken on Kodak T-MAX 100 film with an accelerating voltage of 20kv at magnifications from 1-50 x 10³ times. Experiments were performed in triplicate and appearances were within the range of variation one would expect with SEM.

Actin staining

Cells were stained with rhodamine-labelled phalloidin, a cyclic peptide that specifically binds to F-actin (Wulf et al 1979). Staining of macrophages was carried out as described (Amato et al 1983). Briefly, 100ul of 0.16 ug of rhodamine-phalloidin/ml in PBS was applied to the coverslips after their 24 hour treatments described above. After 20 min the coverslips were rinsed twice and viewed immediately under a fluorescence microscope (Leitz).

PGE₂ assay

PGE₂ concentrations were measured by competitive radioimmunoassay as described in chapter 2.

Statistical analysis

All statistically significant differences between groups were calculated using an unpaired, two tailed student's *t* - test (Snedecor and Cochran 1989).

RESULTS

Morphology and spreading induced by NSAIDs

Of the adherent cells isolated from the peritoneal cavity of healthy rats and mice, more than 96% stained positively for non-specific esterase and less than 10% for myeloperoxidase. This indicates the population consisted mainly of mature macrophages since peroxidase is lost as they mature whilst positive non-specific esterase staining remains characteristic of this cell type. After 24 hours of culture

macrophages isolated from C3H/HeJ mice appeared to be a heterogeneous population of mostly round cells with some spread cells present (Fig 3.1a). The percentage of peritoneal cells spreading varied from 20 to 50% depending upon (a) the age (mice older than 12 weeks spread less) and strain of mouse (Fig 3.9) and (b) the environmental conditions in which they resided before harvesting (macrophages from infected mice or mice in poor health spread poorly). If a NSAID (such as 20 μ M Piroxicam) were included in the culture medium, there was a significant increase in the proportion and numbers of cells spreading (Fig. 3.1b).

All the NSAIDs tested, that are known to inhibit PGE₂ production, enhanced the spreading of peritoneal macrophages (Table 3.1). For example the inactive R-isomer of Naproxen was approximately 100 times less effective than the active S-isomer. The relative concentrations at which the drugs enhanced macrophage-spreading correlated with the effective plasma concentrations of these drugs in patients undergoing therapy with the drug. This in vitro effect of the NSAID in enhancing macrophage-spreading was noted in both the presence and absence of 10 μ g/ml lipopolysaccharide (LPS).

Spreading induced by corticosteroids/immunosuppressants

Dexamethasone was the most potent of the drugs tested, inducing a two fold increase in macrophage spreading at concentrations as low as 1.9×10^{-9} M (Table 3.1). The other steroids tested had similar effects at higher concentrations. The immunosuppressive drugs, Methotrexate and 6-mercaptopurine were more than 1000 fold less effective. The spreading induced by all these drugs was stellar in shape, similar to that induced by the NSAIDs. Cyclosporin-A had no effect upon macrophage spreading and was rather surprisingly toxic at concentrations greater than 1μ M.

PGE₂ production and its effect on spreading

Figure 3.1. Peritoneal macrophages from C3H/HeJ mice cultured for 24 hours in the presence (a) and absence (b) of 20uM Piroxicam. This shows the enhanced spreading of macrophages treated with Piroxicam. Cells were fixed and stained with Giemsa's stain. Similar results were obtained using macrophages from different strains of mice (see Fig 3.9). Magnification ~ 200x.

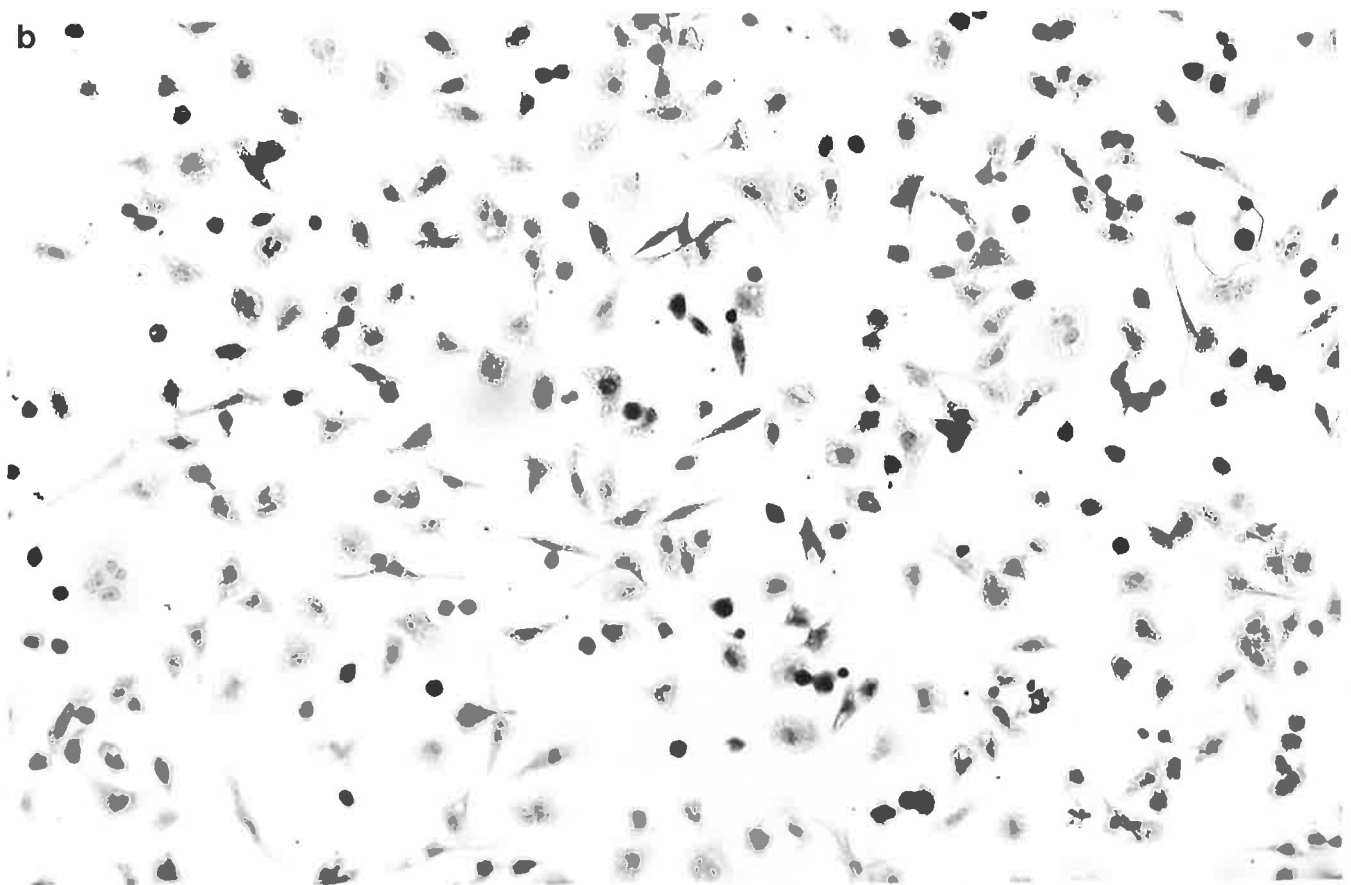
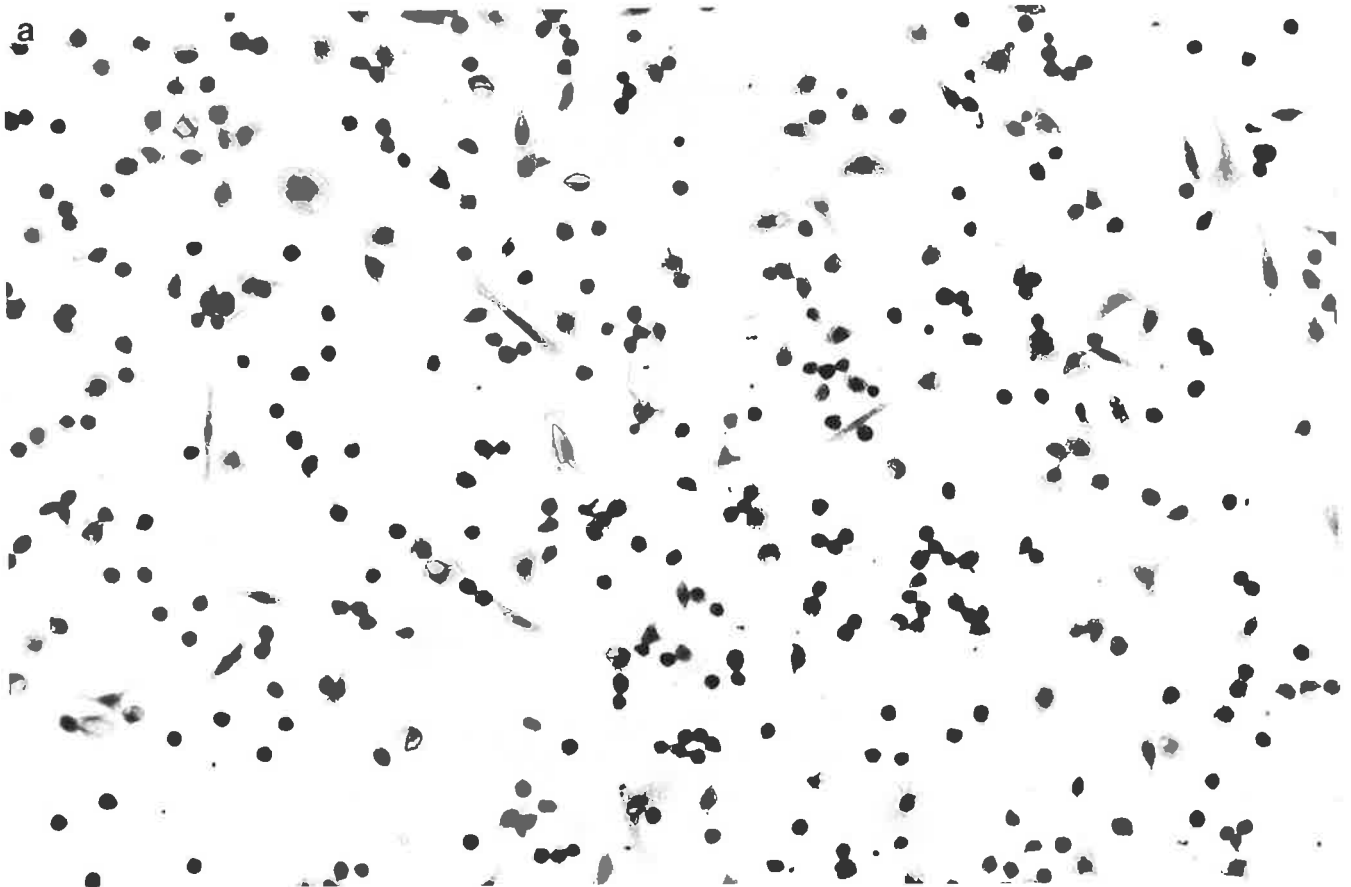


Table 3.1

The effect of some A) antiinflammatory and B) immunosuppressant drugs on macrophage spreading.

<u>Group</u>	<u>Drug</u>	<u>ED₅₀ (μM)*</u>
A)	Indomethacin	5.7
	(S)Ibuprofen	9.9
	(R)Ibuprofen	2300
	Piroxicam	25
	(S)Naproxen	30
	(R)Naproxen	1700
	Aspirin	12000
B)	Dexamethasone	0.0019
	Prednisolone	0.0054
	Cortisol	0.011
	6-mercaptopurine	0.47
	Methotrexate	3.1
	Cyclosporin-A	>10 **

* The concentration of drug which induced a two fold increased the number of cells spreading (see Methods).

** Toxic at 1μM.

The ability of Piroxicam to enhance the spreading of peritoneal macrophages correlated closely with this drug's ability to inhibit the cells own production of PGE₂ (Fig. 3.2). It seemed likely therefore that endogenous PGE₂, normally produced by adherent macrophages, might inhibit the cells own spreading.

The effect of prostanoids and their analogues on spreading

Most of the prostanoids affected the morphology of mouse (C3H/HeJ) macrophages (Fig.3.3). They inhibited the spreading of glass-adherent macrophages with ID₅₀'s of 31nM for PGE₁, 20nM for PGE₂, 610nM for PGE₃ and 590 nM for Misoprostol. Of the non-E series prostanoids tested only PGA₂ inhibited spreading at concentrations below 10,000 nM (ID₅₀=222nM) (Fig. 3.4).

The effect of cyclic nucleotides on spreading

Lipophilic (dibutyryl) analogues of cAMP (bt₂cAMP) and cGMP (bt₂cGMP) were used to assess the effects of cyclic nucleotides on cell shape. Like the E-series prostanoids, bt₂cAMP also inhibited spreading (ID₅₀ = 63 uM) whereas bt₂cGMP enhanced the already high levels of background spreading in the presence of 20 uM Piroxicam (Fig. 3.5).

Spreading induced by cytokines

Of the cytokines tested, namely IFN γ , IL-1, IL-2, TNF α and TNF β (lymphotoxin), only IL-1 and IL-2 had no effect on normal peritoneal macrophage spreading. IFN γ of mouse origin only induced mouse macrophages to spread and did not affect rat cells. Recombinant human IFN γ did not alter the morphology or spreading of rat or mouse macrophages. Surprisingly rTNF α (mouse and human) and rTNF β (human) increase the spreading of mouse macrophages (Table 3.2), which was inhibited by adding PGE₂ (data not shown).

Scanning Electron Microscope (S.E.M.) studies of various cell populations

Normal cells (Fig. 3.6 a.b)

Figure 3.2. Peritoneal macrophages from C3H/HeJ mice were cultured in the presence of 10ug/ml LPS and increasing concentrations of Piroxicam. After 24 hours the supernatants were harvested to determine the PGE₂ produced and the cells fixed and stained in Giemsa's stain to determine the percentage of cells spreading. Each point represents the mean of at least 3 experiments.

Figure 3.2

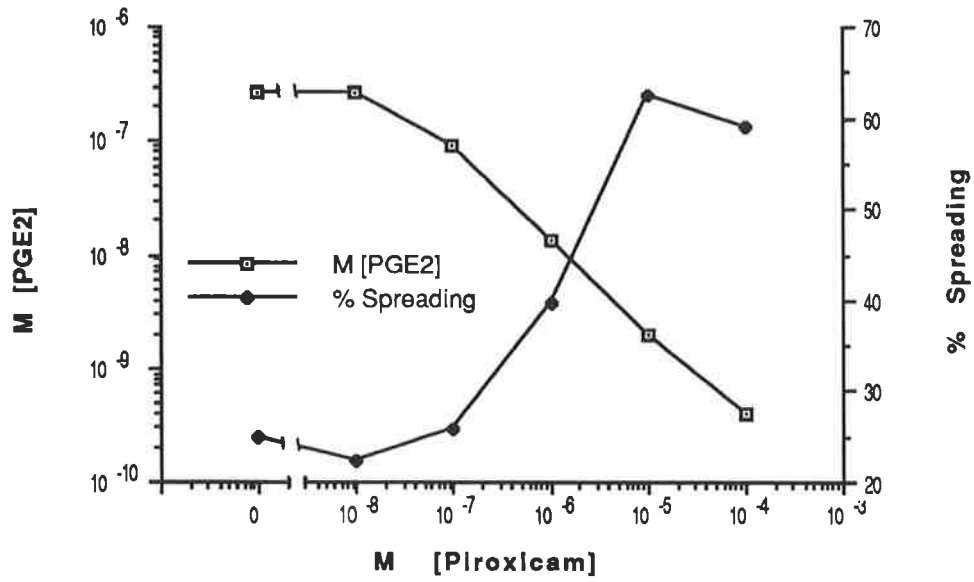


Figure 3.3. The effect of E-series prostaglandins on spreading of murine peritoneal macrophages cultured in the presence of 20uM Piroxicam and test compounds for 24 hours. The cells were fixed, stained and the percentage of cells spreading determined. Each point represents the mean of at least 3 experiments.

Figure 3.3

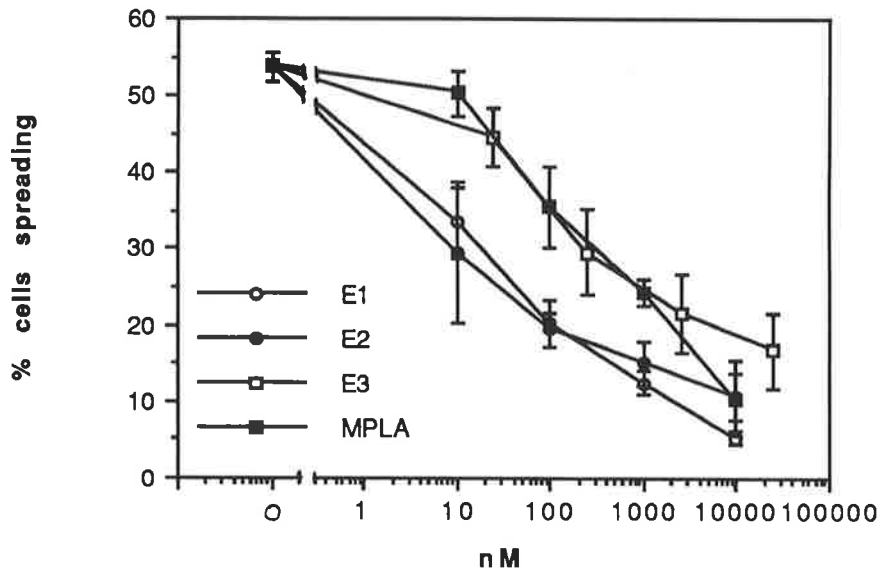


Figure 3.4. The effect of some non-E-series prostaglandins on spreading of murine peritoneal macrophages cultured in the presence of 20uM Piroxicam and test compounds for 24 hours. The cells were fixed, stained and the percentage of cells spreading determined. Each point represents the mean of at least 3 experiments.

Figure 3.4

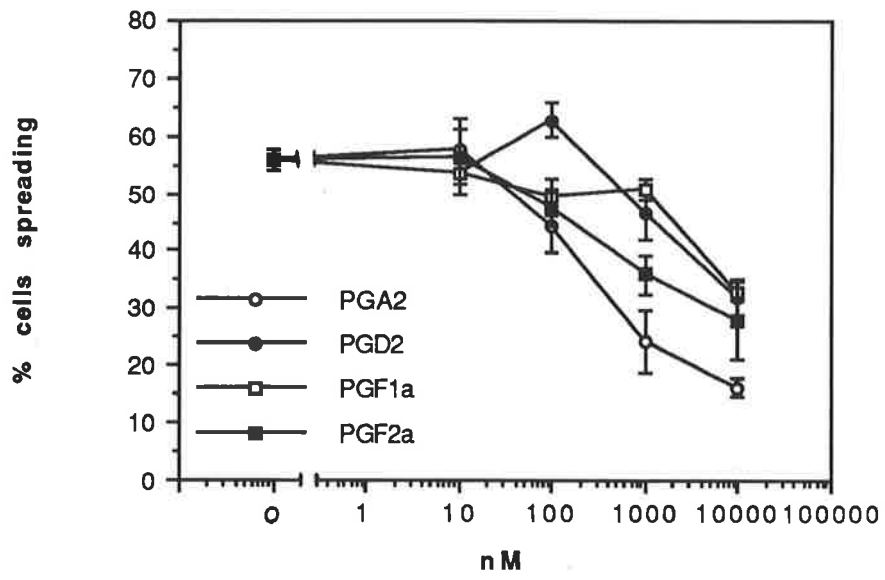


Figure 3.5. The effect of btcAMP and btcGMP on spreading of murine peritoneal macrophages cultured in the presence of 20uM Piroxicam and test compounds for 24 hours. The cells were fixed, stained and the percentage of cells spreading determined. Each point represents the mean of at least 3 experiments.

Figure 3.5

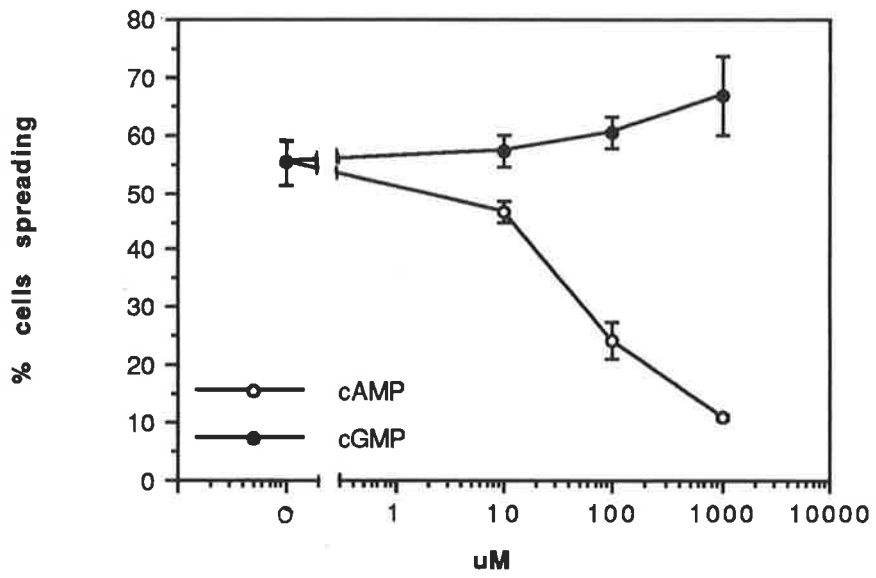


Table 3.2

The effect of cytokines on C3H/HeJ mouse macrophage spreading

Cytokines	ED ₅₀ (pM) ¹	
murine rIFN γ	86.5	(11) ²
human rIFN γ	>100,000	(>10,000)
human rTNF α	60.1	(16)
human rTNF β	1280	(960)
murine rTNF α	87.0	(23)
human rIL-1 β	>100,000	(>3.33 $\times 10^5$)
human rIL-2		(>1 $\times 10^5$)
human rIL-6		(>1 $\times 10^6$)

¹ The concentration of drug which induced a two fold increase in the number of cells spreading (see this chapter Methods).

² Values in parentheses are in units / ml.

After 24 hours, approximately half of the cells were still spherical in shape. Under the S.E.M. the rounded (approx. 10 μm dia.) cells displayed irregular contours with ridge-like profiles and ruffled membranes. Very rarely (<2%) small round cells of less than 0.6 μm in diameter were seen with smoother membranes: these were probably lymphocytes. Other cells (up to 50% in number) had begun to spread out by extending cord-like processes which attached to the glass coverslips. Occasionally a thin veil of cytoplasm extended from beneath the cell. When this veil extended beyond the area covered by the rounded cell, the cells were flattened leaving a raised dome-shaped nuclear pole. The ridge-like profile of the membrane was considerably smoother on the spread out cells.

NSAID-treated cells (Fig. 3.6 c.d)

Cells treated for 24 hours with 20 μM Piroxicam were generally more spread out and flattened than the untreated controls. A significant proportion (approx. 20%) of cells remained rounded, resembling those noted in the untreated population. However the majority of the cells (which did spread with Piroxicam treatment) were considerably flatter than those cells showing spontaneous spreading in the untreated population. With Piroxicam the central nuclear pole was only slightly raised and the ridge-like processes more flattened. These Piroxicam-responsive cells were usually irregular in shape with radiating filopodia extending from two or more poles. A prominent ruffling membrane was often noted at one or more sides of the spread cells. Among this heterogeneous population we noted not only an increase in the numbers of cells spreading but also in the degree of spreading.

Cytokine-treated cells (Fig. 3.7)

There were clear differences in the type of spreading induced by the various cytokines and drugs. The spreading induced by TNF more closely resembled spreading induced by NSAIDs and the corticosteroids/immunosuppressants (Fig. 3.7 a,b). The spreading was polar with the cytoplasm extending in two, three or

Figure 3.6. Scanning E.M. was carried out on macrophages from C3H/HeJ mice cultured in media alone (fig 3.6a,b) or media supplemented with 20uM Piroxicam (fig 3.6c,d). Figures 3.6a and 3.6c were taken at a magnification of 500x. Higher magnifications (2000x) are of a typical rounded cell from the untreated cultures (fig 3.6b) and a typical spread cell from the Piroxicam-treated cultures (fig 3.6d).

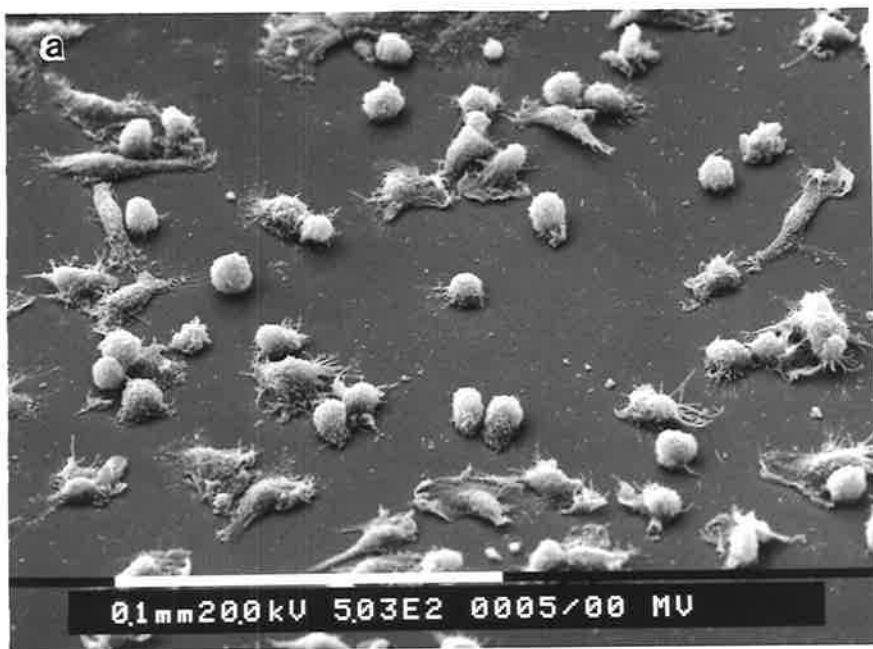
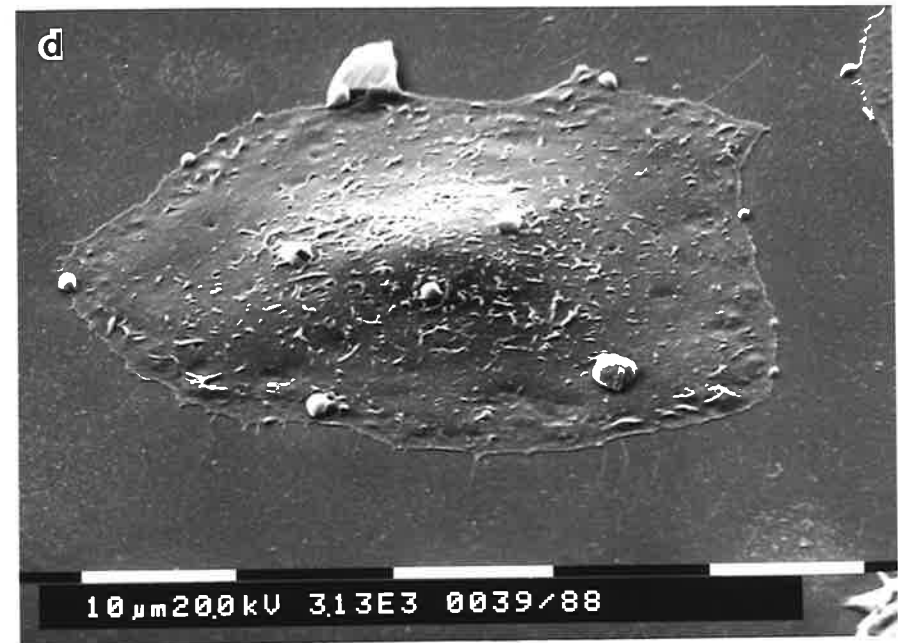
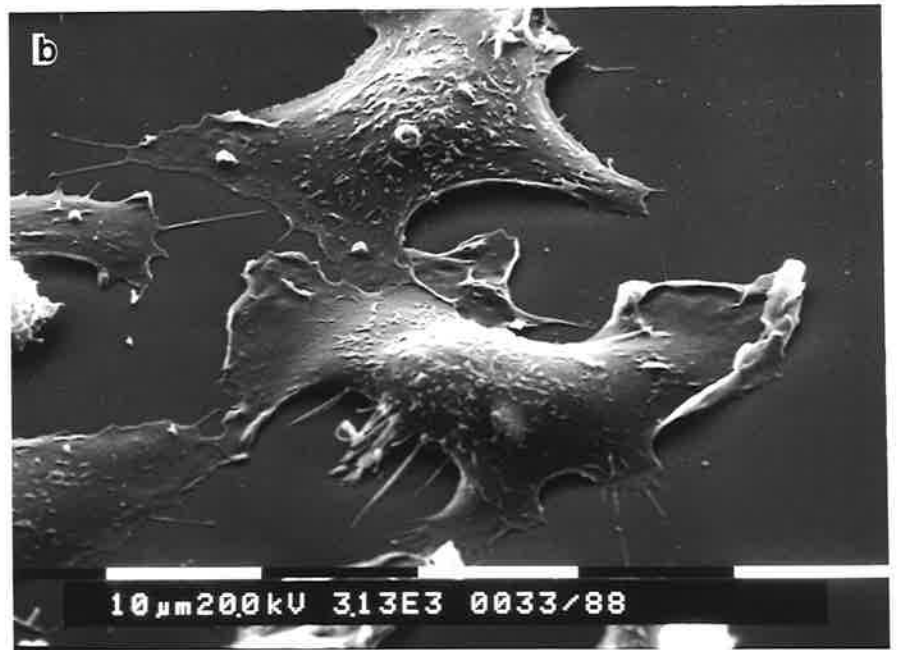
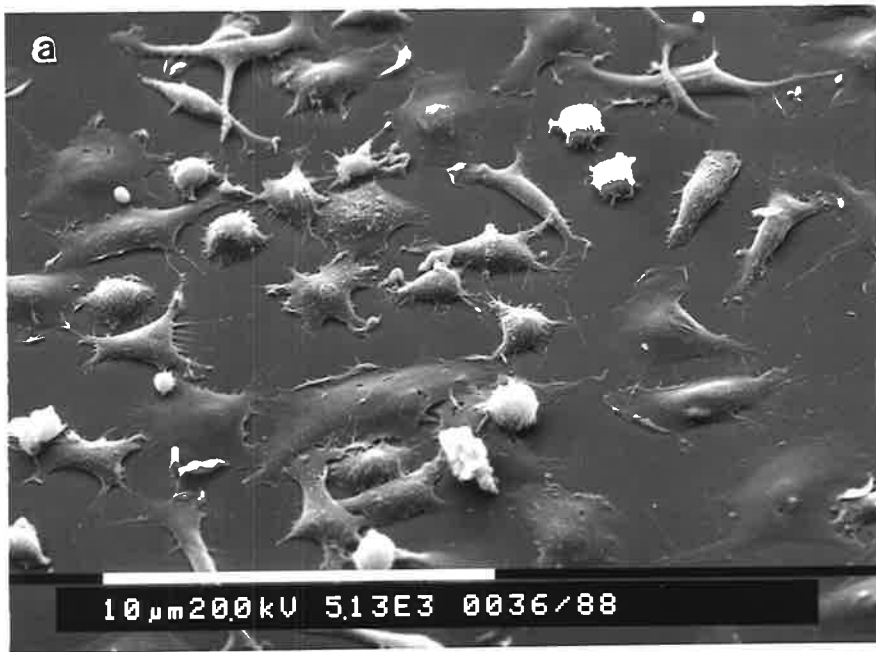


Figure 3.7. Scanning E.M. was carried out on macrophages from C3H/HeJ mice cultured in media supplemented with 1000 units/ml murine recombinant TNF α (fig 3.7a,b) or media supplemented with 1000 units/ml murine recombinant IFN γ (fig 3.7c,d). Figures 5a and c were taken at a magnification of 500x. Higher magnifications (2000x) are of a typical spread cell from the TNF treated cultures (fig 3.7b) and a typical spread cell from the IFN γ treated cultures (fig 3.7d).



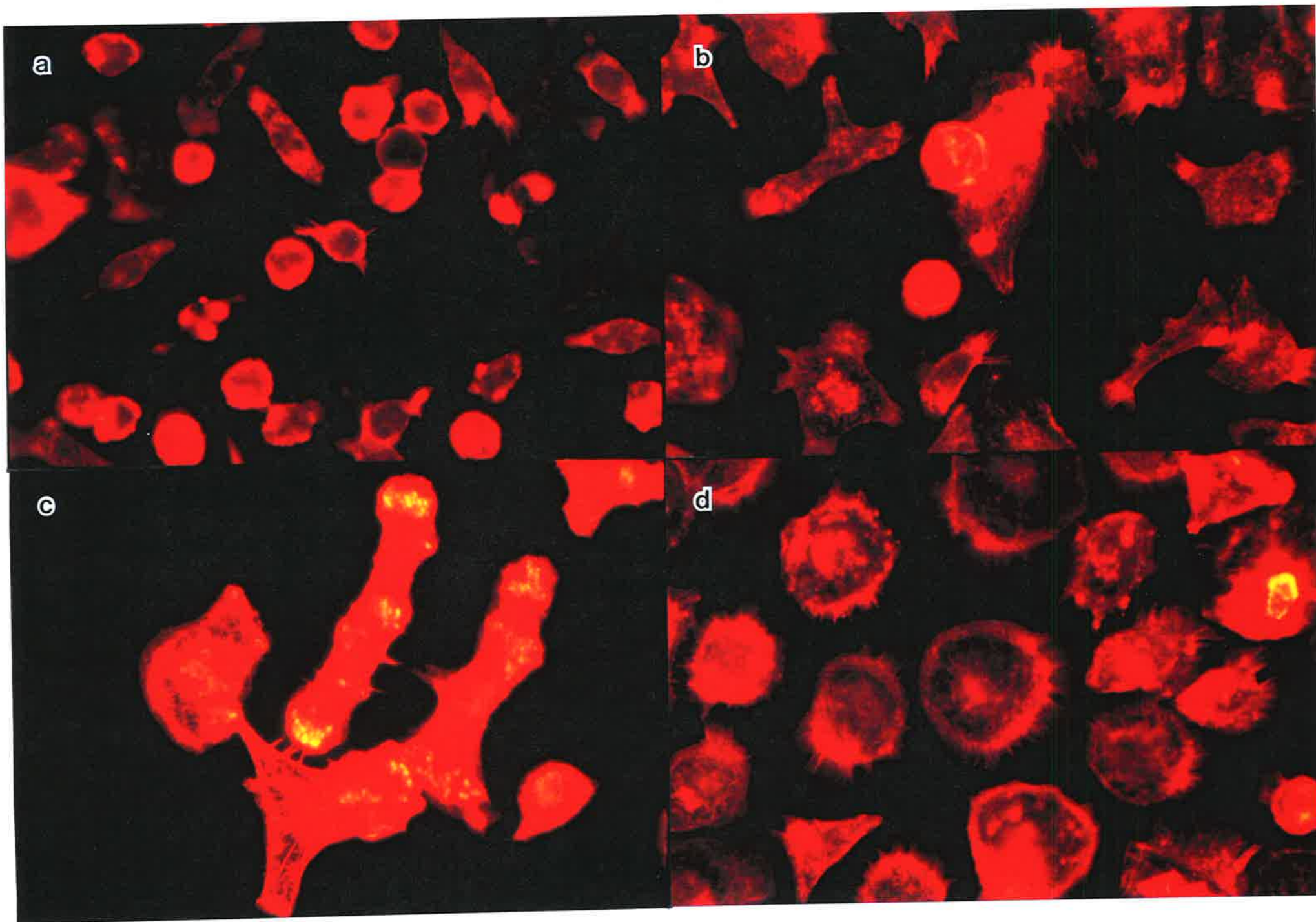
more directions. A ruffling edge was usually seen at the end of each "cytoplasmic arm". As seen with NSAID-induced spreading, cord-like processes attached the cell to the glass coverslips. Unlike IFN γ -induced spreading, the cytoplasm was often raised in places leaving a gap between the cell and glass coverslips. The cells seemed to be only attached to the coverslip at the end of each spreading arm with the centre of the cell being slightly raised.

By contrast, cells induced to spread by IFN γ were usually circular with an even distribution of cytoplasm extending around a slight dome shaped nuclear-pole (Fig. 3.7 c,d). No gaps were seen between the edge of the cell and the glass coverslip. Ruffling or rolled edges usually extended most of the way around the cell. Many of the cells had raised cytoplasmic spheres.

The effect of treatments on the pattern of actin staining

Rhodamine-phalloidin was used to stain the macrophages after various treatments as described above. Typically rounded cells found in the untreated populations (Fig. 3.8a) showed diffuse staining throughout the cytoplasm with dense staining around the circumference. At a high plane of focus, a diffuse cortical band of fluorescence was seen, possibly associated with the nucleus. Elongated cells, such as those seen following Piroxicam or TNF treatment (Fig. 3.8b and 3.8c) also showed a cortical band of fluorescence at a high plane of focus. At a low plane of focus linear fluorescence structures running throughout the cytoplasm were observed. Strong staining was seen toward the ends of the polar cytoplasmic arms and these extended thin 'spiky' fingers into the protruding cytoplasm. Strong staining structures were seen throughout the cytoplasm; the more dense ones seemed to be associated with the linear fluorescent fibres running throughout the cell. In IFN γ treated cells (Fig. 3.8d) the cortical band of fluorescence was observed at a low plane of focus. The pattern of staining found at the ends of the cytoplasmic arms (shown in Fig. 3.8b and c) was noted around the circumference of the IFN γ treated cells. The fluorescent structures throughout the cytoplasm

Figure 3.8. Rhodamine-phalloidin staining was carried out on macrophages from C3H/HeJ mice cultured in media alone (fig 3.8a), media supplemented with 20uM Piroxicam (fig 3.8b), media supplemented with 1000 units/ml recombinant murine TNF α (fig 3.8c) or media supplemented with 1000 units/ml recombinant murine IFN γ (fig 3.8d).



were not linear, as noted following NSAID and TNF treatment, but arranged in a mesh or crosslinked structure. A few dense staining punctate structures were occasionally seen randomly throughout the cytoplasm.

The effect of drugs and cytokines on macrophages from different strains of mice

The percentage of macrophages spreading was assessed after 24 hours culture with recombinant murine TNF α (1000 units/ml), Piroxicam (20 μ M), Dexamethasone (0.1 μ M) and a mixture of TNF (1000 units/ml) and Piroxicam (20 μ M) (Fig. 3.9). Peritoneal macrophages from 4 strains of mice were used (C3H/HeJ, CBA, Balb/C and C57/Bl) and experiments were carried out in the presence and absence of 2 μ g/ml LPS. In the absence of drugs and cytokine, the numbers of C3H/HeJ macrophages spreading was significantly less than that seen in cultures of macrophages from other strains. LPS treatment reduced the numbers of cells spreading from all strains except C57/Bl. In general, in all strains of mice tested, Piroxicam and Dexamethasone treatment enhanced spreading; both being more effective in the presence of LPS. Similarly TNF enhanced spreading. However it was less effective in the presence of LPS. Simultaneous treatment of macrophages with TNF and Piroxicam stimulated more spreading than with either drug/cytokine alone. All mice were 8-12 weeks old, nominally pathogen free and healthy. When older, possibly infected mice, were used as a source of macrophages we noted greater variation in the effects of drugs and cytokines. However similar trends were generally noted.

The effect of NSAIDs on macrophages from inflamed rats

Peritoneal macrophages were isolated from rats with adjuvant-induced arthritis (14 days after injecting a mycobacterial adjuvant). These cells attached and spread onto glass and plastic as described previously (Fig. 3.10a). Their spreading also increased after 24 hours treatment with Piroxicam. However unlike macrophages from normal animals, these adjuvant-activated macrophages often aggregated into

Figure 3.9. Peritoneal macrophages from C3H/HeJ, CBA, Balb/C and C57/BI mice were cultured in media alone (Cont) or in the presence of 20 μ M Piroxicam (Px). 1000 units/ml murine recombinant tumour necrosis factor (TNF) α , 20 μ M Piroxicam and 1000 units/ml TNF α (Px + TNF) and 10^{-8} M Dexamethasone (Dex). Experiments were carried out in the presence and absence of 10 μ g/ml LPS. After 24 hours the cells were fixed and stained and the percentage of cells spreading determined. Each bar represents the mean \pm standard error of at least 3 experiments.

Figure 3.9

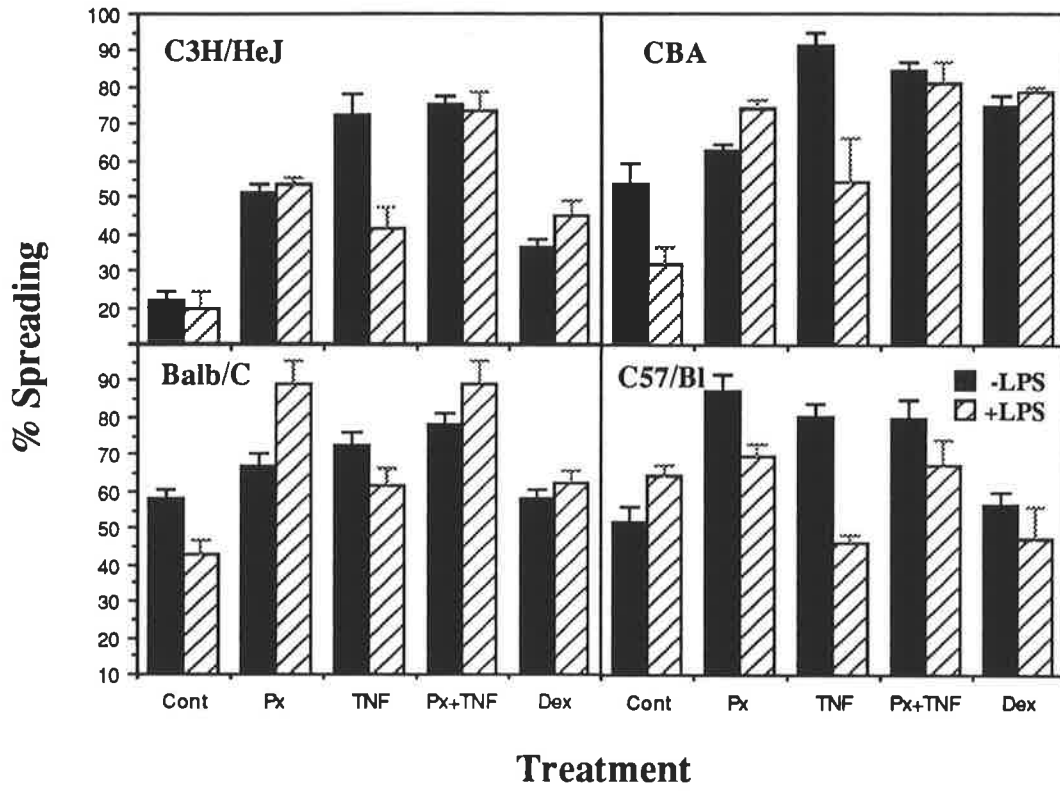
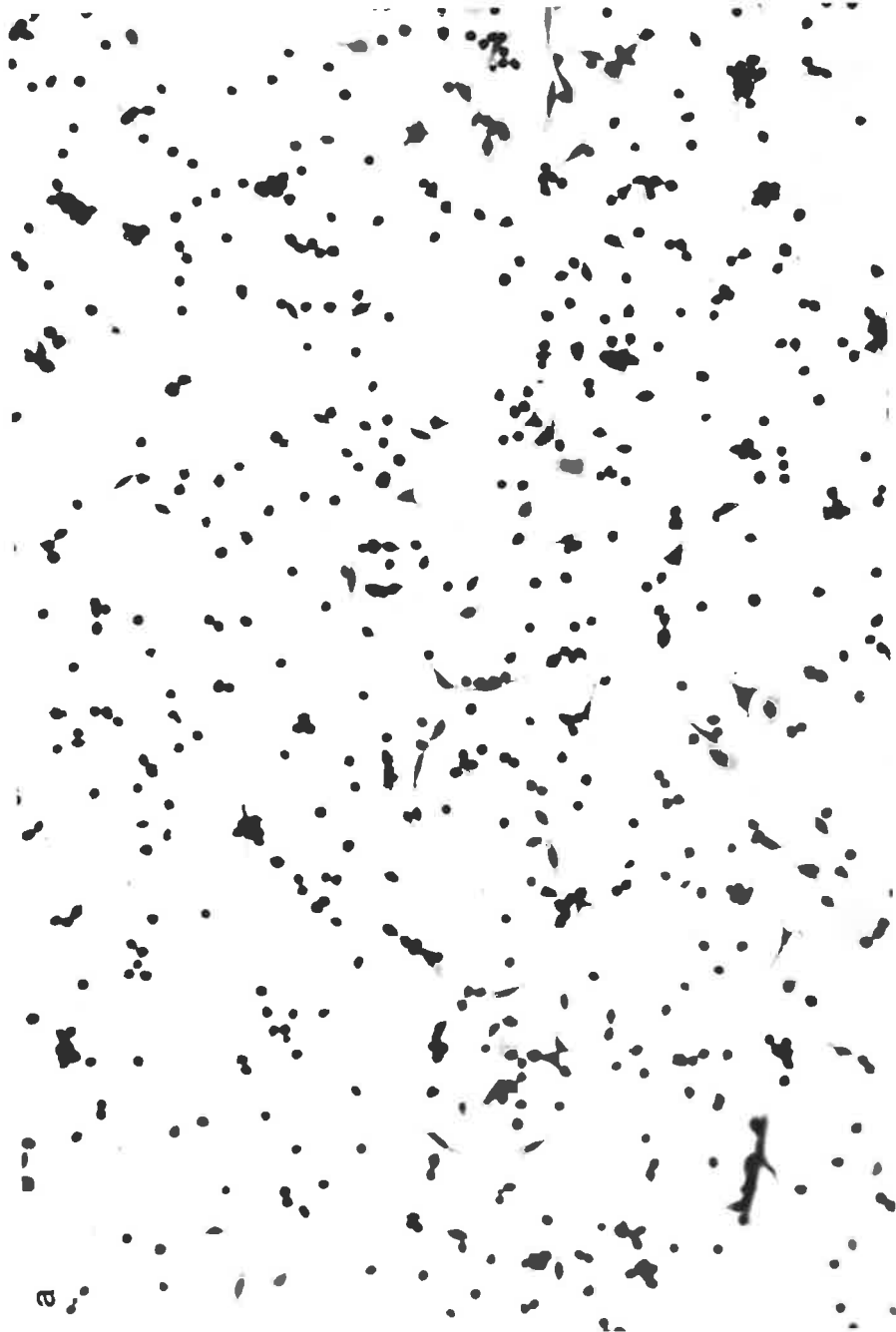
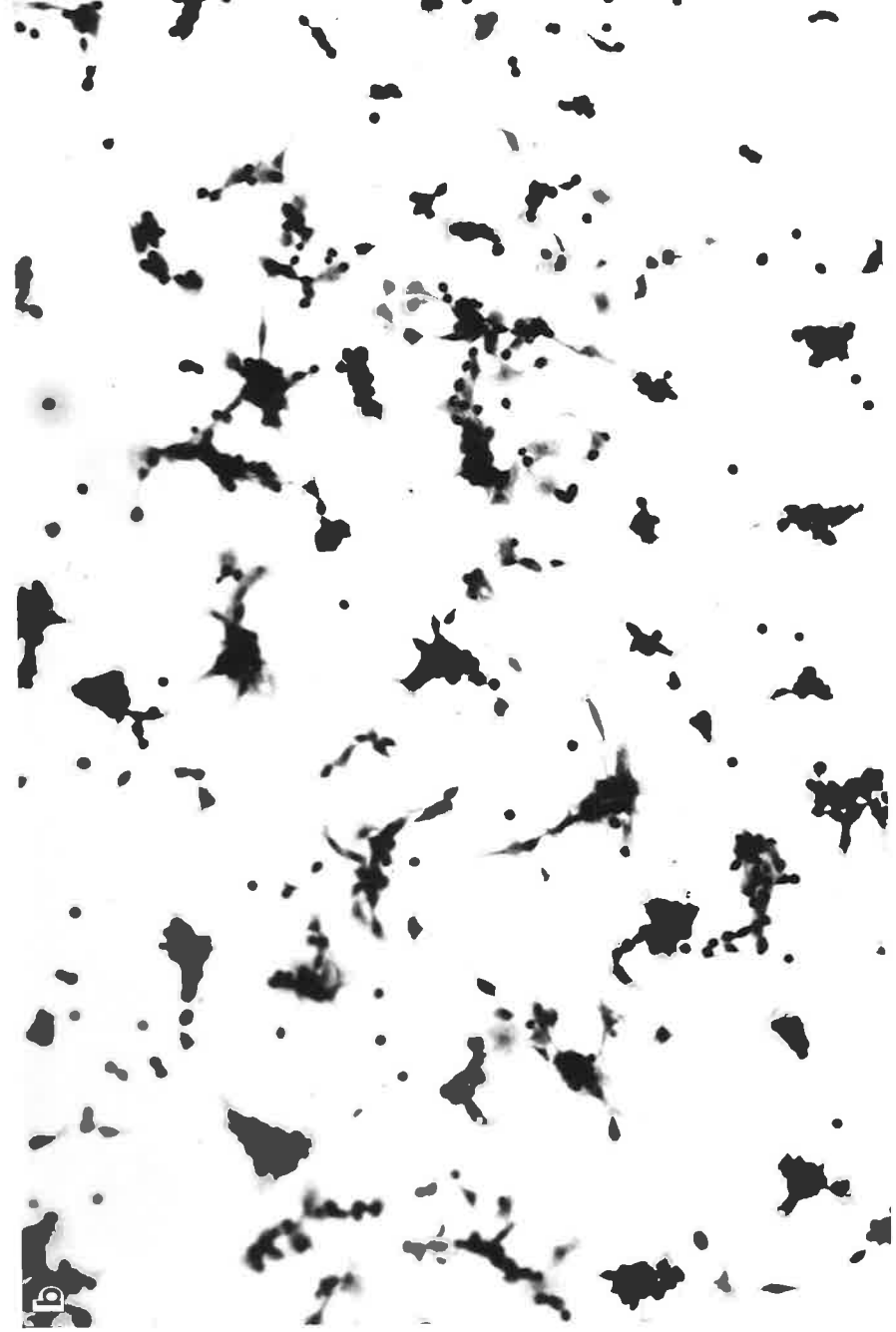


Figure 3.10. Peritoneal macrophages were isolated from male Dark Agouti rats with adjuvant arthritis (14 days after injection of mycobacterial adjuvant). These cells were cultured for 24 hours in the presence (a) and absence (b) of 20uM Piroxicam. These cells not only spread more in the presence of Piroxicam but also formed aggregates or clumps of cells. Cells were fixed and stained with Giemsa's stain. Magnification ~200x.



a



b

clumps of about 10 or more cells (Fig. 3.10b). The structure of these aggregates changed little after 24 hours of culture. Culturing these cells for up to 96 hours with various NSAIDs failed to reveal any fusing of cell membranes or the formation of multinucleated giant cells. This aggregation of macrophages was inhibited by adding PGE₂ to the culture medium. We only occasionally noted this spontaneous clumping with cells isolated from normal animals.

DISCUSSION

Drugs

NSAID treatment of macrophages attached to glass or plastic not only increases the numbers of cells spreading but also the extent to which these cells spread. Under normal circumstances macrophages produce PGE₂, either in small amounts upon attaching to a substrate or in larger amounts in response to an inflammatory stimulus (eg LPS). NSAID treatment reduces the amount of PGE₂ produced by inhibiting arachidonate cyclo-oxygenase (Vane 1971). Since PGE₂ inhibits macrophage spreading induced by a variety of treatments (NSAIDs, IFN γ and TNF in mice) it is not surprising that NSAID treatment enhances the spreading of both unstimulated and stimulated (LPS treated) cells.

All the prostaglandins of the E series as well as the PGE₁ analogue Misoprostol were very effective at inhibiting cell spreading. However some non-E series prostanoids were much less effective, suggesting that receptor binding specificity may regulate this and other functions such as cytokine production (see chapters 2 and 4). The implications of this conjecture, including the effects of diet and prostanoid therapy, are discussed in detail in Chapter 4.

The effects of cyclic nucleotides upon cell morphology indicate these second messengers may mediate the actions of prostanoids, drugs and cytokines. The

lipophilic analogue of cAMP, bt₂cAMP, inhibited cell spreading like the E-series prostanoids. Prostanoids, ^{though not as potent} particularly those of the E-series, raise the levels of intracellular cAMP (Table 4.3, discussed in Chapter 4) that may be responsible for changes in the cytoskeleton which cause cells to round up. Conversely bt₂cGMP (the lipophilic analogue of cGMP) enhanced spreading, indicating that intracellular cGMP levels may be involved in the organisation of actin and/or other structural proteins. Intracellular cGMP is reported to stimulate pseudopodium formation and motility in the slime mold *Dictyostelium discoideum* (Liu & Newell 1991). It is thought that cGMP exerts its effect in this organism by regulating myosin association with the cytoskeleton, possibly through Ca⁺⁺ and calmodulin. This suggests there may also be complex interactions of cyclic nucleotides and calcium-dependent processes taking place in the cytoplasm of macrophages that regulate the functions described here and elsewhere in this thesis.

Corticosteroids were also potent stimulators of macrophage spreading. The relative potencies of the steroids tested was in accord with their published glucocorticoid potencies (Brooks et al. 1986b). Corticosteroids may thus also affect macrophage spreading by inhibiting PGE₂ synthesis as a consequence of reducing the availability of arachidonic acid by inhibiting phospholipase A₂ (PLA₂) (Flower 1978, Kaplan et al. 1978). These steroids were considered to induce certain proteins (lipocortin/macroscortin/calpactins) that inhibit PLA₂ (Hirata et al 1980). However it has been recently suggested that these particular proteins are not normally PLA₂ inhibitors (Allison & Lee 1987, Davidson et al. 1987) but relatively abundant cytoskeletal-associated proteins. Since NSAIDs may also inhibit PLA₂ (Vigo 1987) it would of interest to know if they can also induce these, or other, cytoskeletal proteins that might be involved in the changes in cell morphology induced by both NSAID and corticosteroids.

Cytokines

IFN γ stimulates a number of macrophage functions, including cell spreading in vitro (Schultz 1980). Mouse IFN γ (either recombinant or purified) selectively stimulated the spreading of mouse peritoneal macrophages but not rat peritoneal macrophages. Human IFN γ did not affect mouse or rat macrophages. This would indicate that unlike other cytokines (eg interleukin 1 and 2, TNF) IFN γ is only effective on autologous macrophages ie. of the species from which it is derived.

Both TNF α and TNF β (lymphotoxin) were effective stimulators of peritoneal macrophage spreading. Unlike IFN γ , mouse and human TNF (α and β) were equally effective on rat and mouse cells, indicating their effects are not species-specific. Some previous reports indicated that TNF treatment of polymorphonuclear leucocytes can increase their adherence to endothelial cells (Gamble et al 1985). The effects of TNF (and IFN γ) on the microfilament network of endothelial cells (Stolpen et al 1986) may have functions in common with the effects of TNF on macrophages described here. It is possible that promotion of macrophage spreading and endothelial cell adherence by TNF could involve similar mechanisms of action. TNF may not only kill tumour cells directly but also indirectly by stimulating the spreading of macrophages, thus increasing macrophage/tumour cell contact and subsequent tumour cell necrosis.

The spreading responses to TNF and a NSAID appeared very similar in morphology. When mouse macrophages were treated with TNF and a NSAID (Piroxicam) simultaneously in the presence of LPS, the increase in spreading was the sum of their individual effects. This might indicate that, although the types of spreading induced are similar, the actual mechanisms by which they induce spreading may differ. NSAIDs most probably stimulate spreading by inhibiting the production of an inhibitor (endogenous PG) whereas TNF may directly induce macrophage spreading. However these two mechanisms may not be unrelated since NSAIDs enhances production of TNF (by suppressing PGE₂, an inhibitor of TNF production, see Chapter 4) which in turn can promote spreading.

Genetic influences

The variation in the spreading of macrophages obtained from different strains of mice suggests there may be a genetic component in this phenomenon. The percentage of C3H/HeJ macrophages spreading was certainly lower when compared to the other strains tested. This is not unexpected since macrophages from C3H/HeJ mice are defective in their response to LPS (Beutler et al 1986) and in other macrophage functions (Ruco & Meltzer 1978, Vogel & Rosenstreich 1979). Drugs and TNF could restore the normally defective spreading of these cells. As a consequence the stimulation of spreading by both drugs and TNF was more marked in cells from this strain of mice.

Conversely macrophages from C57/Bl mice responded poorly to drugs and cytokines in the presence of LPS. C57/Bl was also the only strain in which LPS treatment increased the percentage of cells spreading. This may indicate that LPS induces lower levels of PGE₂ production by macrophages from C57/Bl mice compared with other strains.

Morphology

For a cell to demonstrate spreading it must, (i) adhere to the substrate and (ii) be able to change its shape (Gustafson and Wolpert 1967). Cell adhesiveness may be influenced by changes in the cell membrane, substrate or medium (Rabinovitch and DeStefano 1973). Various treatments (eg cytokines and PGE₂) might modify the expression on the cell surface of those adhesive components involved in the attachment of the cell to its substrate. For example, IFN γ increases the expression of several membrane components including Fc receptors (Becker 1983) and Ia antigens (Fertsch & Vogel 1984). IFN γ treated macrophages were particularly flat with no gaps between the cytoplasm and the substrate being seen under S.E.M. By contrast, cells treated with TNF or NSAIDs often had gaps between the cytoplasm and substrate. This might indicate that IFN γ increases the numbers of attachment

sites which are more evenly spread underneath the cell. Cells induced to spread (by drug or TNF treatment) only attached to the substrate at the ends of the stellar arms, indicating that the membrane components involved might not be distributed so evenly under the cell. IFN γ , but not TNF or the tested drugs might therefore increase the numbers of adhesive components on the cell surface. It is also possible that cytokines might enhance spreading by inhibiting PGE₂ release (Boraschi et al 1984).

If PGE₂ inhibits spreading by reducing the numbers of cell-anchoring components, one might expect cells to detach from the substrate when treated with PGE₂. However we found PGE₂ treatment only slightly increased the numbers of cells detaching after 24 hours culture. It is unlikely that PGE₂ blocks cell attachment by binding to the substrate or affecting anchorage components present in the medium.

Previous SEM studies have also noted that the cell surface of macrophages became smoother as the cell spread out (Pollack and Gordon 1975, Rabinovitch and DeStefano 1973). Although the area taken up by spread cells may be 10 times greater than that of the rounded forms, the actual surface area might not change. The numerous folds and ridges of the rounded forms might represent a reserve of membrane only used as the cell spreads to maximise its adherence (Follett and Goodman 1970, Rabinovitch & DeStefano 1973).

Previous studies with electron and immunofluorescence microscopy have revealed that actin plays an important role in cell adhesion and spreading (Amato et al 1983, Allison et al 1971). SEM studies indicate the spreading lamellipodia of macrophages are largely made up of a dense foci, interconnected by radiating filaments and filament bundles, which may link actin to the substratum (Trotter 1981). This is consistent with observations showing strong actin staining at the ends of the extended arms of the stellar spreading cell (Fig 3.8c), ie the same site at

which SEM revealed close attachment of the cell membrane to the glass coverslip. Other studies have also shown that the fluorescent punctate structures described here may act as actin-substratum attachment sites (Berlin and Oliver 1978, Painter et al 1981). Following NSAID and TNF treatments, fully spread macrophages showed clearly orientated actin fibres running, often longitudinally, throughout the cell. However, in contrast to these more stellar spread forms, these fibres were absent in the rounder spread cells following IFN γ treatment. It would seem that the type of treatment stimulating macrophage spreading can determine if these fluorescent actin fibres form. This may explain why some (Berlin and Oliver 1978, Amato et al 1983) but not others (Phaire-Washington et al 1980, Oropeza-Rendon et al 1979) have observed fluorescent actin fibres in fully spread macrophages.

Function

Unless a cell can anchor itself to a fixed substrate it will be unable to control its own movements. In the blood stream it will follow the flow of blood until it attaches to the endothelial wall. If the cell is to move through the extracellular tissue it must become motile. Locomotion of cells, like other vehicles, requires traction and this can only occur when good contact is made with the surface across which it is moving. Cells which spread may then respond more readily to chemoattractants facilitating their congregation within sites of inflammation. NSAIDs may enhance this process in more than one respect. Firstly by inducing ruffling edges, an indication of motility. Secondly peritoneal macrophages from inflamed rats treated with NSAIDs in vitro not only spread more readily but also spontaneously aggregated into clumps. Presumably these NSAID-treated cells can now respond to a chemoattractant, move towards the site of its production (possibly another adherent cell) and adhere to one another. NSAIDs may also promote cell-cell adherence and/or enhance production of chemoattractant.

This clumping of macrophages may represent the first stages of multinucleate giant cell (MNGC) formation. Subsequent cell fusion, which we did not observe,

may require the presence of other cytokines such as those produced by T lymphocytes and known to induce MNGC formation (McInnes and Rennick 1988, Galindo et al 1974). PGE₂ might therefore regulate the formation of MNGC associated with chronic inflammations and the tissue damage they cause.

Motile macrophages would be attracted to the inflammatory site but upon arrival, the high local concentrations of PGE₂ therein could cause the cells to lose their motility by reducing their capacity to spread. In this way PGE₂ might act like a trapping agent or a migration inhibiting factor at the site of inflammation. The PGE₂ may be produced by the macrophages themselves in response to either the inflammatory stimulus or to various inflammatory cytokines (IFN γ , TNF etc). NSAID therapy would reduce the endogenous PGE₂ thereby restoring motility to these macrophages. Motile cells may be better able to resolve the inflammatory processes, being free to move away from the centre of an inflammatory site perhaps taking phagocytosed inflammagenic cell/tissue debris with them. Thus the enhancement of macrophage spreading may be yet another facet of the anti-inflammatory effects of NSAIDs which has not yet been given due consideration.

Chapter 4

The prostaglandin E₁ analogue, Misoprostol, regulates inflammatory cytokines and immune functions in vitro like the natural E-prostaglandins (1,2 and 3.).

INTRODUCTION.

The products of arachidonate (20:4 ω 6) oxygenation, in particular the monocyclic prostanoids, are known to play a central role in inflammation. Furthermore a variety of multifunctional cytokines, such as interleukin-1 (IL-1), tumour necrosis factor (TNF) and interleukin-6 (IL-6) are considered to be important in the development and maintenance of inflammation (Dinarello 1989, Akira et al 1990). It is, therefore, not surprising that antiinflammatory therapies are often directed towards suppressing the production of prostanoids (PG's) and/or inflammatory cytokines.

Therapy directed towards modifying the PG production largely depends on nonsteroidal antiinflammatory drugs (NSAIDs). More recently, dietary supplements have been studied to attain the same goal, with the possible advantage that they might provide the same benefits as NSAIDs but have less noxious side effects. Fish oil supplements, rich in eicosapentaenoic acid (20:5 ω 3) and docosahexaenoic acid (22:6 ω 3), have been under trial for the treatment of rheumatoid arthritis (Kremer 1987, Cleland 1988). Diets enriched with gamma-linolenic acid (22:3 ω 6) have also been suggested to be beneficial (Horrobin 1988). These diets raise the levels of precursors of PGE₁ and PGE₃, namely dihomo-gamma-linolenic acid (20:3 ω 6) and eicosapentaenoic acid (20:5 ω 6) respectively, which compete with arachidonate for the enzyme cyclooxygenase (see figure 1.1).

The net effect would be to increase the production of PGE₁ and PGE₃ possibly at the expense of PGE₂. Such changes in the relative levels of PG's may have profound effects on the course of inflammation (Horrobin 1988).

The biologically related cytokines IL-1, TNF and IL-6 are also targets for drug therapy. These cytokines have many activities in common, can antagonise the action of one another, and further induce the production of each other and themselves (Dinarello 1989). Cells of the monocyte/macrophage lineage not only produce IL-1, TNF α and IL-6 but also PG's (normally PGE₂) following an inflammatory stimulus. It is important that the interrelationships between these cytokines and PG's be clarified to understand how to maximise the beneficial effects from both novel and traditional drug therapies.

Dietary supplements can affect IL-1 and TNF α production (Endres et al 1989). We have compared the effects of PGE's 1,2 and 3 on (i) the production by mononuclear phagocytes, and (ii) the action, of each of the three inflammatory cytokines IL-1, TNF and IL-6. We have also compared their effects with the immunoregulatory/antiinflammatory action of the more stable methyl ester of a PGE₁ analogue, Misoprostol. The parent acid of this analogue has a much longer half-life in vivo than natural E-prostaglandins (acids), approximately 20 minutes compared to less than 1 minute for PGE₁. Misoprostol is currently employed as an anti-ulcer drug to treat gastric injury caused by NSAIDs.

MATERIALS AND METHODS.

Chemicals.

PGE₁, PGE₂, PGA₂, PGF₁ α , PGF₂ α , PGD₂, bt₂cAMP, bt₂cGMP and Piroxicam were purchased from the Sigma Chem. Co. (St Louis Mo. USA); and PGE₃ from Cayman Chem. Co. (Ann Arbor Mi. USA). Misoprostol was a gift from the G. D. Searle and Co. (Skokie, Il. USA)

Cytokines.

Human recombinant TNF and IL-6 were purchased from Genzyme Corp. (Cambridge Ma. USA) and Boehringer Mannheim GmbH (Mannheim Germany). Human recombinant IL-1 was a gift from Otsuka Pharmaceutical Co. Ltd. (Tokushima Japan).

Isolation of cells.

Adherent peritoneal (AP) cells were isolated from Dark Agouti (DA) rats and C3H/HeJ mice as previously described (Haynes et al 1988b, Chapters 2 and 3), resuspended in RPMI-1640 medium (Flow Laboratories) supplemented with 10% foetal calf serum (FCS), penicillin (50 IU/ml) and streptomycin (50 u/ml) and adjusted to a concentration of 1×10^6 /ml. 100 ul of the cell suspension (1×10^5 cells) was placed in wells of a flat-bottomed 96-well plastic culture tray . After incubation at 37°C in 5% CO₂ for 1 hour, cells not adhering to the bottom of the plastic wells were removed by washing 3 times with HBS. As described in Chapters 2 and 3, greater than 96% of these adherent cells stained positively for non-specific esterase indicating that they were nearly all macrophages.

Human peripheral blood mononuclear (PBM) cells from healthy volunteers were isolated using Ficoll-Hypaque as previously described (Haynes et al 1988a).

Rodent AP and human PBM cells were incubated in the presence of the test compounds in a final volume of 250ul. In the assays for cell spreading and cytokine production, 20 uM Piroxicam was included to inhibit endogenous PGE₂ production by the MNP (Haynes et al 1988a) so only the effects of the added PG's would be assessed. 5ug/ml Lipopolysaccharide (E. coli 0111:B4, Sigma) was used to stimulate cytokine production in the experiments measuring IL-1 and TNF production. After 24 hours the supernatants were collected and stored at -70°C until assayed for cytokines.

Biological assays for cytokines.

The lymphocyte activating factor assay was carried out as described (Chapter 2).

A relatively specific (cytotoxic) IL-1 assay was conducted using the IL-1 sensitive A375 cell line as described (Nakai et al 1988).

TNF was assayed similarly using the TNF-sensitive cell line L929 as described (Matthews and Neale 1987). In our hands this cell line was sensitive to both recombinant human TNF α and β .

To assay IL-6, 7TD1 hybridoma cells (with the kind permission of Dr. J. Van Snick, Ludwig Institute, Brussels, Belgium) were used as previously described (Bartold and Haynes 1991).

In these cytokine bioassays 1 unit/ml of activity was determined to be that present in a dilution of test sample which gave 50% of maximal activity. The appropriate recombinant cytokine was included in every assay to monitor variations between assays carried out at different times.

Other biological assays.

The assessment of macrophage spreading on glass cover slips was carried out as previously described (Chapter 3).

Immunoassay for IFN γ .

Human PBM cells were isolated as described above. 2×10^5 of these cells were incubated in a volume of 250ul of RPMI with various concentrations of the PG's and 5ug/ml PHA. After 24 hours the supernatant was sampled and assayed for human IFN γ with a commercial enzyme immunoassay kit (Commonwealth Serum Laboratories, Australia).

PGE₂ assay

The competitive immunoassay for PGE₂ was carried out as described in Chapter 2.

Extraction of RNA following prostanoid stimulation.

Foreskin fibroblasts (FSF), a gift from the Department of Virology, IMVS, were grown to confluence in DMEN media (Flow Laboratories) supplemented with 10% foetal calf serum (FCS), penicillin (50 IU/ml) and streptomycin (50 u/ml) in 13mm wells of a 24 well tray (approx 5×10^5 cells/well). These cells were washed and incubated in media with various PG's in the presence of 20 uM Piroxicam. After 3 hours, total RNA was extracted using the acid-guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi 1987).

Detection of IL-6 RNA.

The extracted RNA was adjusted to a concentration of 200 ug/ml (measured by OD at 260nm) and 10ul loaded into wells on an agarose gel. The gel was run for 3 hours then the RNA transferred onto a nitrocellulose membrane (Northern transfer) as described (Sambrook et al 1989). Hybridisation with a digoxigenin labelled IL-6 probe (antisense) was carried out using the method described in the Boehringer Mannheim product information bulletin (Nucleic Acid Detection Kit cat. no. 1175 041). The IL-6 probe was made from a cDNA for human IL-6 (Hirano et al 1986), a gift from Prof. Toshio Hirano, Institute for Molecular and Cellular Biology, Osaka University, Japan. The IL-6 probe was labelled with digoxigenin-UTP by in vitro transcription with T7 RNA polymerase using the method described the Boehringer Mannheim product information (RNA Labelling Kit (SP6/T7) cat. no. 1175 025).

Radio labelled ³⁵S-IL-6 probe was produced with ³⁵S-UTP (uridine 5'-[α-thio]triphosphate, [³⁵S]- Bresatec Adelaide , South Australia, Cat no. SRU-2) in a similar way to that described above, using the Message Maker in vitro

Transcription Kit (Bresatec Ltd., Adelaide, South Australia, cat. no. MMk-1). 2ul of the above RNA extracts were blotted directly onto nitrocellulose membrane and hybridised with the ^{35}S IL-6 probe as described above. Each of the dot blots were carefully cut out and the binding of probe determined by detection of ^{35}S associated with each blot using a scintillation counter.

Inhibition of ^3H -PGE₂ binding assay.

Human PBM and rat AP cells were isolated as described above. 10×10^6 cells were incubated in wells with 5nM ^3H -PGE₂ (Amersham, code TRK 431) and various PGs at a concentration of 50 nM in 500 ul RPMI media. After 30 minutes incubation at 37°C in 5% CO₂ the cells were washed 3 times in cold HBS. The cells were then lysed in Opti Phase "Hi Safe 3" scintillation fluid (LKB, England) to determine their ^3H content in a scintillation counter. Background binding was determined to be the ^3H bound in the presence of 2000-fold excess of non-radioactive PGE₂ (10uM). Maximum binding (100%) was determined to be the ^3H bound in the absence of competing PG.

Induction of intracellular cAMP.

Induction and measurement of intracellular cAMP was carried out as described (Finney et al 1990) using a cAMP assay kit (Amersham, code TRK 432).

Statistics.

The level of significance was determined to be $p < 0.05$ as calculated using an unpaired, two tail student T-test (Snedecor and Cochran 1989).

RESULTS

The effect of PGE's and cyclic nucleotides on IL-1 action

The effect of prostanoids on the lymphoproliferative action of human recombinant IL-1 was assessed using the LAF assay. PGE_{1,2,3} and Misoprostol were all

effective inhibitors of the IL-1 induced proliferation of mouse thymocytes (Fig. 4.1). PGE₁ and 2 were the most effective, inhibiting 50% of the activity (ID₅₀) of 10 units/ml of IL-1b at 4.4nM and 8.9nM respectively; PGE₃ and Misoprostol were less effective with ID₅₀'s of 17 nM and 87nM respectively. Bt₂cAMP also inhibited IL-1 activity (ID₅₀= 1900nM) whereas bt₂cGMP had no effect at the concentrations tested (10²-10⁶ nM).

The effect of Misoprostol on cytokine production

Figure 4.2 shows the effect of Misoprostol on the TNF, IL-1 and IL-6 activity and the concentration of IFN γ produced by i) human PBM cells, ii) murine AP cells and iii) rat AP cells . Misoprostol greatly inhibited the production of TNF (Fig. 4.2a) by human PBM cells and rat AP cells . AP cells of the mouse strain used (C3H/HeJ) produced no detectable TNF activity. Figure 4.2b shows the effect of Misoprostol on IL-1 production. We consistently found a slight increase in IL-1 activity produced by rat and mouse macrophages (AP cells) with the lowest concentrations of Misoprostol (1 -10 nM). This effect was also noted when the other PGE's were added to rodent AP cells but not observed in experiments with human PBM cells. Misoprostol stimulated IL-6 activity in the 3 cell types (Fig. 4.2c). Misoprostol was most effective at stimulating rat and mouse peritoneal macrophages. Misoprostol also inhibited the levels of IFN γ produced by human PBM (Fig 4.2d).

The effect of PGE's and cyclic nucleotides on cytokine production

Table 4.1 compares the inhibition (ID₅₀) of the PGE's and btcAMP upon the activity of TNF, IL-1 and concentration of IFN γ in the 3 cell types. The biological activity of TNF produced by stimulating these cells with LPS was inhibited by these 4 prostanoids and bt₂cAMP. IL-1 activity produced by human PBM cells was not significantly affected by exogenous PG's. These results were confirmed by immunoassays (Genzyme kit) for IL-1 (data not shown). However production of IL-1 by mouse, and to even a greater extent, by rat macrophages was reduced by

Figure 4.1. PG's and btcAMP inhibit the mitogenic effect of IL-1. The lymphoproliferative response of mouse thymocytes, was measured by the uptake of 3H-thymidine in the presence of 10 units/ml of recombinant human IL-1, suboptimal levels of PHA (1ug/ml) and PG's or cyclic nucleotides. Each point represents the mean of 4 experiments.

Figure 4.1

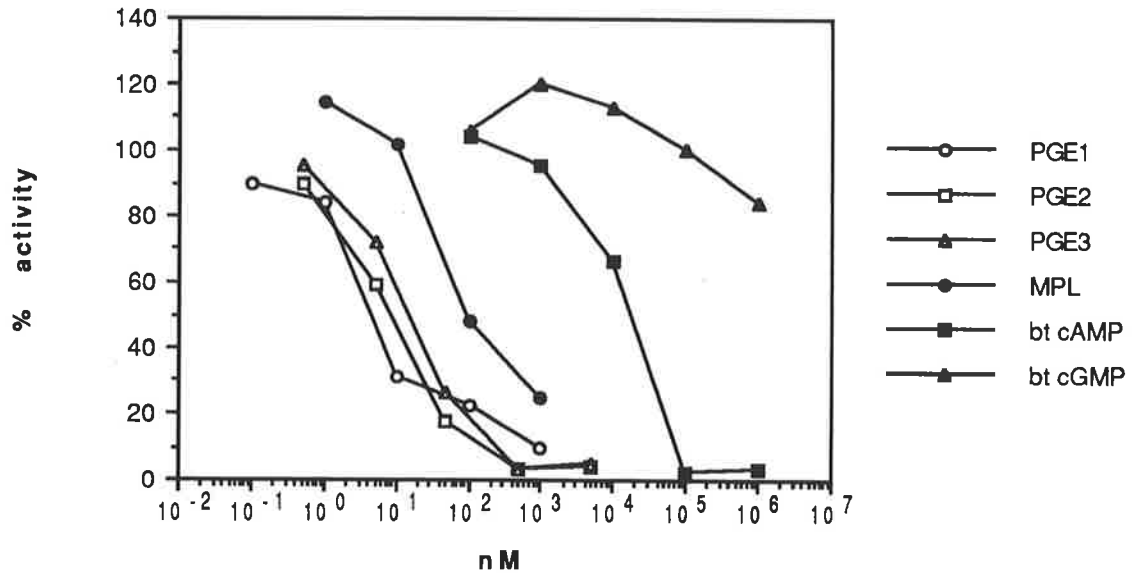


Figure 4.2. Misoprostol inhibits the production of a)TNF, b)IL-1 (in rat and mouse cells) and c) IFN γ but stimulates IL-6 production. Human PB mononuclear and rodent adherent peritoneal cells were incubated with 5 ug/ml LPS (except for IL-6 assays) and Misoprostol at various concentrations. After 24 hours the supernatants were sampled and their cytokine content measured as described in the methods. Each point represents the mean \pm the standard error for at least 4 human donors (squares) and at least 3 experiments for mice (circles) and rats (triangles).

Figure 4.2

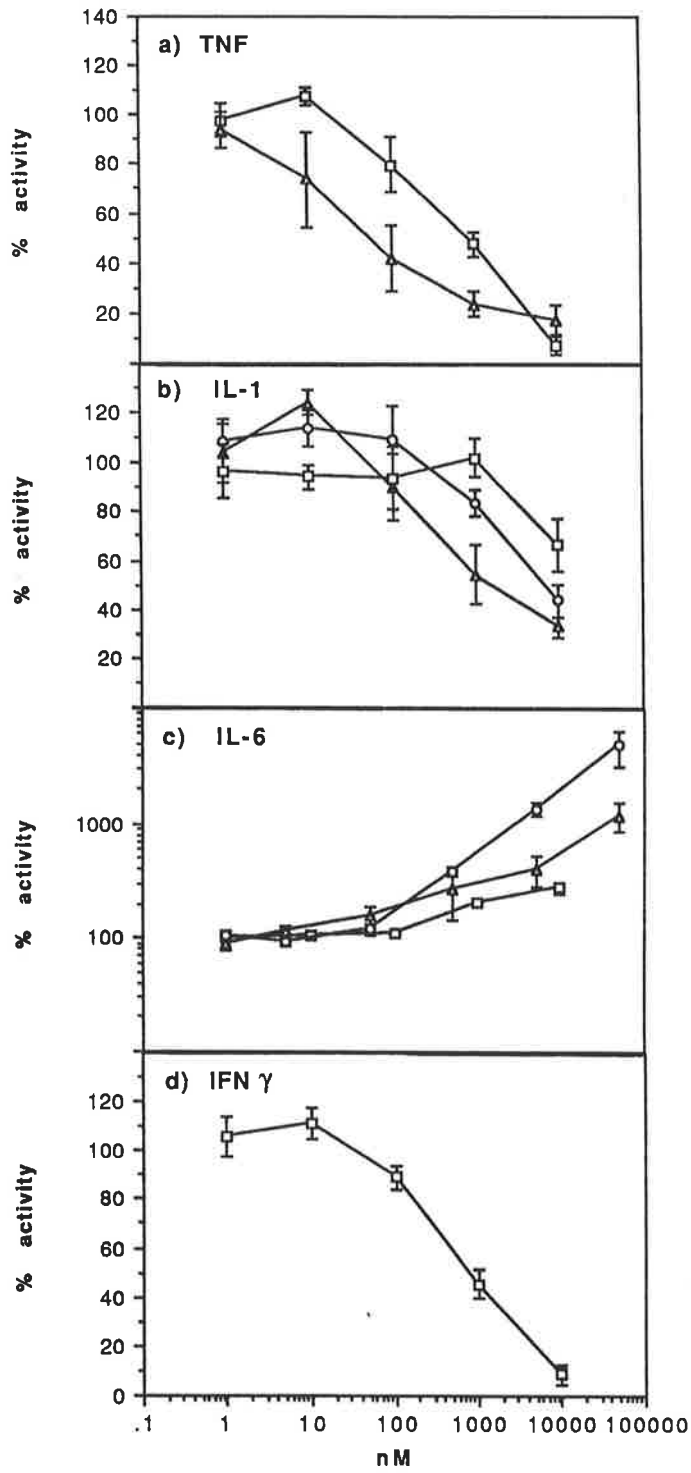


Table 4.1

Inhibitory effect of E-prostaglandins on the production¹ of, a) TNF, b) IL-1 and c) IFN γ by three types of mononuclear phagocytes.

ID₅₀ (nmoles/L)²

Cells	Human PBM	Mouse AP	Rat AP
a) TNF			
PGE ₁	43.1 ± 16.1 ³	N.D. ⁴	17.3 ± 8.2
PGE ₂	44.3 ± 20.2	N.D.	34.5 ± 11.0
PGE ₃	126.6 ± 60.3	N.D.	274.6 ± 95.7
MPL	750.7 ± 256.6	N.D.	232.5 ± 69.1
bt ₂ cAMP	18,300 ± 6,300	N.D.	71,300 ± 9,670
b) IL-1			
PGE ₁	>1,000	811 ± 261	24.1 ± 9.5
PGE ₂	>1,000	953 ± 348	20.2 ± 0.3
PGE ₃	>1,000	1,123 ± 313	170 ± 28.9
MPL	>10,000	7,100 ± 1740	346 ± 76.6
bt ₂ cAMP	692,00 ± 186,000	456,000 ± 49,500	60,000 ± 2890
c) IFNγ			
PGE ₁	29.8 ± 11.3	.5	-
PGE ₂	33.1 ± 10.2	-	-
PGE ₃	39.3 ± 13.3	-	-
MPL	591 ± 168	-	-
bt ₂ cAMP	29,700 ± 5,070	-	-

¹ As measured in, a) L929 cytotoxicity assay, b) A375 cytotoxicity assay and c) IFN γ immuno assay.

² Concentration (nanomolar) which inhibited by 50% the activity of cytokine produced after stimulation with 5ug/ml LPS (for IL-1 and TNF) or 5ug/ml Con A (for IFN γ). Values were derived from dose-response curves based on data derived from the means of duplicate experiments, using at least 5 donors for human PBM cells and 3 separate experiments using animal AP cells. Endogenous PG production was suppressed with 20 uM Piroxicam (see Methods).

³ Standard error.

⁴ Not detectable following LPS stimulation of C3H/HeJ mouse AP cells.

⁵ Not tested.

these PGE's. Dibutyryl cAMP at concentrations greater than 670uM reduced IL-1 activity produced by human PBM cells by more than 50%, IFN γ production could only be measured in experiments with human cells since the immunoassay used was specific for human IFN γ . Like TNF production, IFN γ production was inhibited by all 4 PGE's and btcAMP. Summarising the results given in table 4.1, a) PGE₁ and 2 were just as effective at inhibiting TNF, IL-1 and IFN γ production by the cell types tested; b) PGE₃ was slightly less effective; and c) Misoprostol was always markedly (often 10-40 times) less effective than PGE₁ and PGE₂.

In contrast to other cytokines, the (mitogenic) activity of IL-6 present in the supernatants from all 3 cell types was enhanced after treating the cells with the prostanoids or bt₂cAMP (Table 4.2). This stimulation of IL-6 production was observed without LPS stimulation. The relative (stimulant) potencies of the PGE's was very similar to that noted for their inhibitory effects on the production of other cytokines (IL-1, TNF and IFN γ).

The IL-6 response of human peripheral blood (PB) cells to PG's

Table 4.3 show the response of adherent PB cells from healthy donors to various PG's and cyclic nucleotides. The numbers of adherent cells were calculated with the aid of a graticule as described in chapter 3. Cells from 5 different donors all responded to the three PG's at concentrations of 1 uM, and to Misoprostol at 10 uM, by increasing IL-6 production by about 2-3 fold. 1mM cAMP strongly stimulated IL-6 production while 1 mM cGMP only slightly stimulated IL-6 production. cAMP and cGMP together were less potent than cAMP alone indicating that the effects of the two cyclic nucleotides were not additive. The last 3 columns of table 4.4 shows the combined data for 3 types of PB cell populations. The PG's and nucleotides had similar effects on adherent and non-adherent populations; however the nonadherent population produced about 5 fold less IL-6 per cell than the adherent population.

Table 4.2

Stimulatory effect of E-prostaglandins on the production of IL-6 activity¹ by three types of mononuclear phagocytes.

ED₂ (nmoles/L) ²

Cells.	Human PBM	Mouse AP	Rat AP
Treatment			
PGE ₁	180 ± 45.2 ³	23.0 ± 8.3	24.2 ± 9.5
PGE ₂	417 ± 160	7.1 ± 3.0	20.2 ± 0.3
PGE ₃	463 ± 121	180 ± 12.6	170 ± 28.9
MPL	966 ± 225	140 ± 38.3	346 ± 76.7
bt ₂ cAMP	437,000 ± 186,000	42,600 ± 9,650	60,000 ± 2,900

¹ As measured in a mitogenic assay using 7TD1 cell proliferation .

² Concentration (nanomolar) which stimulated the activity of IL-6 produced two fold (ED₂) above control levels. Each value derived from the dose-response curves based on means of duplicate experiments, using at least 5 donors for humans PBM cells and 3 experiments for animal AP cells. Experiments were carried out in the presence of 20 uM Piroxicam (see Methods).

³ Standard error.

Table 4.3

The effect of cyclic nucleotides and PG's on IL-6 production by adherent, non-adherent and total peripheral blood mononuclear cells (PBM).

Stimulus	Individual human PBM responses.					Averaged PBM responses		
	D.H.	S.G.	D.R.H.	L.B.	S.R.	adherent	non-adherent	total
control	217 ± 64 ¹	53 ± 7	243 ± 28	297 ± 109	190 ± 19	200 ± 41	7.6 ± 0.9	237 ± 37
1mM btcAMP	3767 ± 338	253 ± 32	1037 ± 63	1433 ± 240	1033 ± 120	1505 ± 597	36.5 ± 13.6	1800 ± 306
" btcGMP	940 ± 227	163 ± 13	397 ± 67	370 ± 51	400 ± 3	454 ± 129	15.2 ± 4.9	533 ± 100
1mM btcA + GMP	2733 ± 584	173 ± 23	663 ± 47	1367 ± 167	630 ± 144	1113 ± 448	15.9 ± 4.5	1060 ± 70
1uM PGE ₁	1183 ± 309	133 ± 27	353 ± 18	655 ± 25	407 ± 3	546 ± 180	12.5 ± 0.4	543 ± 109
1uM PGE ₂	597 ± 117	83 ± 15	347 ± 32	589 ± 89	527 ± 58	405 ± 94	25.6 ± 7.5	517 ± 242
1uM PGE ₃	393 ± 49	138 ± 28	327 ± 17	789 ± 108	573 ± 27	444 ± 111	17.9 ± 3.0	623 ± 62
10uM Misoprostol	663 ± 42	107 ± 24	790 ± 240	691 ± 95	823 ± 78	615 ± 130	21.9 ± 2.6	733 ± 233

¹ units/ 10⁴ cells ± standard error of at least 3 experiments for individuals and 3 donors for the pooled data.

Stimulation of IL-6 by PG's and cAMP occurs at the level of mRNA production

Figure 4.3 shows that all three PG's at 1 μ M and Misoprostol at 10 μ M stimulated IL-6 mRNA above basal levels in FSF. All these PG's stimulated IL-6 mRNA at similar levels when detected either visually by non-radioactive digoxigenin or by scintillation counting using ^{35}S labelled probes. While this radioactive probe detected mRNA in control (untreated) FSF, the digoxigenin probe failed to detect any mRNA by northern blot analysis. Both methods of detection indicated IL-1 was a strong stimulator of IL-6 mRNA in these cells (stronger than any of the PG's tested).

Competition of PGE's with $^3\text{H-PGE}_2$ for cell binding

Table 4.4 shows that the binding of $^3\text{H-PGE}_2$ (5nM) to human PBM and rat AP cells can be markedly inhibited by a 10 fold excess of cold PG's (50nM). PGE's $_{1,2}$ and $_3$ all inhibited by approximately 80% $^3\text{H-PGE}_2$ binding to human PBM cells. Misoprostol was significantly ($P < 0.005$) less effective, inhibiting 47% of $^3\text{H-PGE}_2$ binding. Inhibition of $^3\text{H-PGE}_2$ binding to rat cells was more affected by all the PGE's tested. As noted with human cells Misoprostol was significantly ($P < 0.05$) less effective than the other PG's.

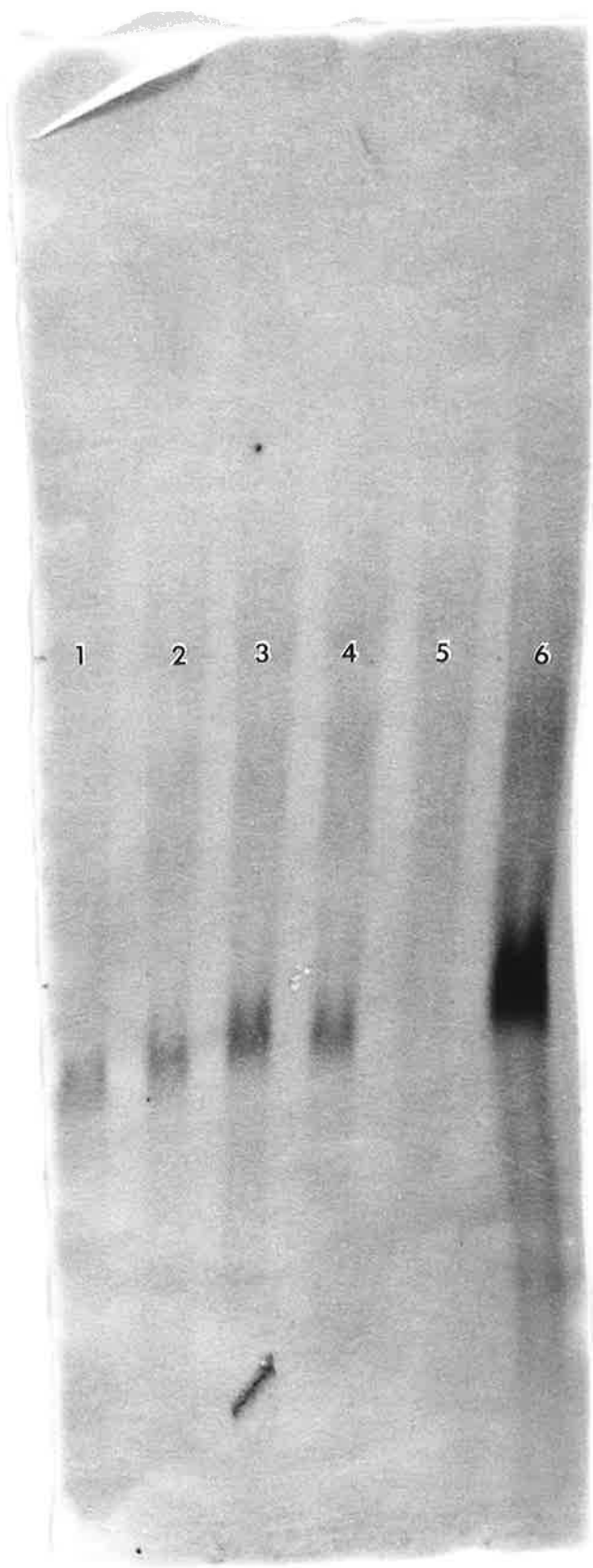
Stimulation of intracellular cAMP by PGE's.

The effect of the PGE's on intracellular levels cAMP in human PBM and rat AP cells is also shown in table 4.4. All PGE's markedly increased the levels of cAMP. In human PBM cells PGE's $_{1,2}$ and $_3$ increased the levels of cAMP approximately 10-fold whilst Misoprostol increased cAMP levels approximately 5-fold. All the PGE's had a similar effects on rat peritoneal cells; but the levels of cAMP detected were approximately 100-fold less than those detected in a similar number of human cells.

Preliminary experiments with 4 other PG's (PGA_2 , $\text{PGF}_1\alpha$, $\text{PGF}_2\alpha$ and PGD_2) showed that they were poor mimics of PGE's in regulating cytokines, as

Figure 4.3. PG's and IL-1 stimulate the production of IL-6 mRNA in human foreskin fibroblasts. RNA was extracted from human foreskin fibroblasts incubated with PG's and human recombinant IL-1 β for 3 hours and the levels of IL-6 mRNA detected using a digoxigenin labelled ribo-probe as described in the methods. Each track is loaded as described below and the results of the dot blot analysis (see text) of the same RNA extracts using a ^{35}S labelled IL-6 probe are given in the right hand column.

Track	Treatment	DPM
1.	PGE ₁ (1 μM)	7,517
2.	PGE ₂ (1 μM)	8,370
3.	PGE ₃ (1 μM)	8,432
4.	Misoprostol (10 μM)	7,549
5.	none	3,679
6.	IL-1 β (10 ⁹ units/ml)	10,801



1

2

3

4

5

6

Table 4.4**Inhibition of ^3H -PGE₂ binding and stimulation of intracellular cyclic AMP production by E-prostaglandins and Misoprostol.**

Cells	%Inhibition ¹		nM cAMP ²	
	Human PBM	Rat AP	Human PBM	Rat AP
PG				
untreated	0	0	1.43 ± 0.35	0.01 ± 0.00
PGE ₁	79.9 ± 12.3	86.4 ± 5.4	13.8 ± 5.12	0.19 ± 0.05
PGE ₂	85.6 ± 9.4	88.0 ± 3.5	14.6 ± 2.08	0.12 ± 0.11
PGE ₃	88.5 ± 7.6	90.4 ± 6.4	14.5 ± 3.11	0.15 ± 0.08
MPL	47.2 ± 6.9	73.6 ± 8.8	7.00 ± 0.72	0.08 ± 0.07

¹ Percent inhibition of binding of ^3H -PGE₂ by a 10 fold excess (50nM) of PG. Mean ± standard error of 3 experiments.

² Picomoles cAMP produced per 2×10^5 cells. Mean ± standard error of 3 experiments.

determined in the above assays. Only PGA_2 had any significant effect at concentrations below 1000nM (data not shown).

Stimulation of PGE production by cyclic nucleotides

Figure 4.4a shows that *bt*cAMP at levels of 100 μM or greater strongly stimulated PGE production by human PB monocytes and rat peritoneal macrophages. However, concentrations of *c*AMP up to 10,000 μM did not affect PGE production by C3H/HeJ mouse peritoneal macrophages. *Btc*GMP, while not as potent as *bt*cAMP, also stimulated PGE production in human PB monocytes and mouse peritoneal macrophages. Dark Agouti rat peritoneal macrophages (AP cells) did not respond to *bt*cGMP and PGE production may have even been inhibited at the highest concentrations (10,000 μM). When this experiment was repeated with Porton rat peritoneal macrophages, *c*GMP stimulated PGE production in a similar way to its effect on human PB monocytes. The normal levels of PGE produced per 10^4 cells were, 3,100pM for the Porton rat, 1,150pM for the Dark Agouti rat, 2,750pM for the C3H/HeJ mouse and 1,700pM for human cells.

The effect of Misoprostol on PGE production

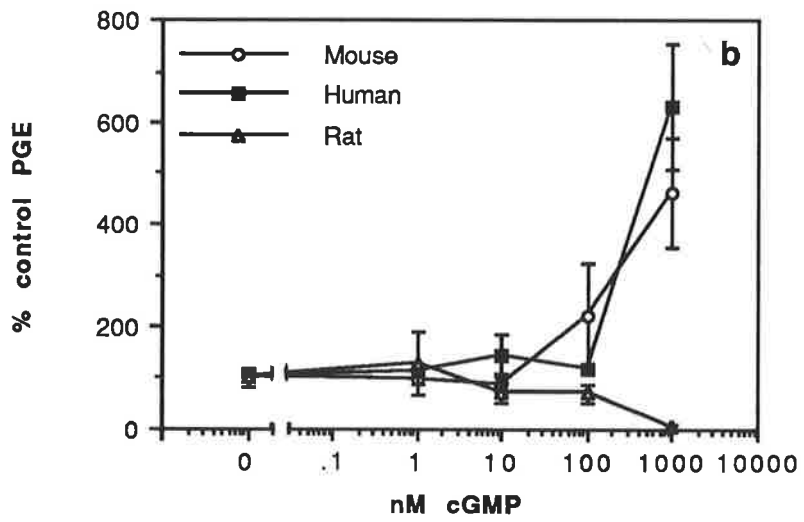
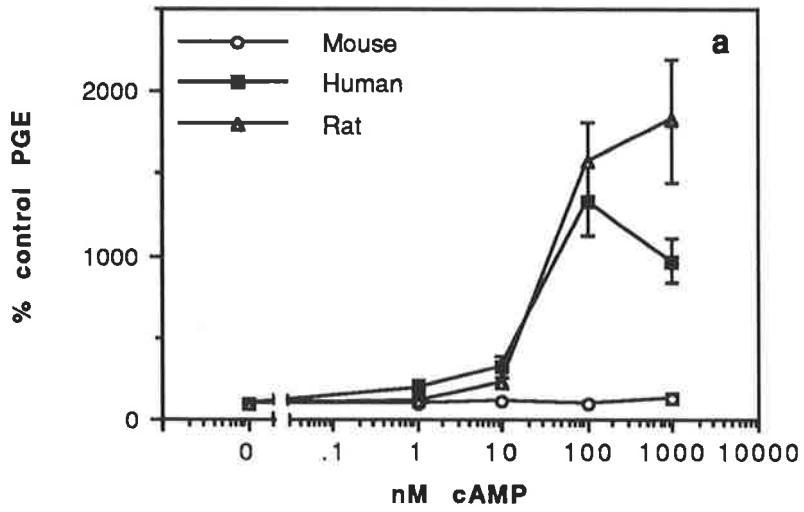
Since Misoprostol and the other PG's could stimulate intracellular *c*AMP (table 4.4), this in turn might stimulate PGE production. However, Misoprostol at concentrations up to 100nM had no measurable effect on PGE production. Higher concentrations could not be tested as Misoprostol itself, like PGE's 1,2 and 3 were all detected with approximately equal efficiency in this immunoassay. Any endogenous PGE produced would be swamped by an excess of exogenous PG's added as stimulant.

DISCUSSION

Inhibition of cytokine action

Figure 4.4. Bt_2cAMP stimulates prostaglandin release by human PB mononuclear cells, and rat AP cells but not C3H/HeJ mouse AP cells in vitro (a). Bt_2cGMP stimulates prostaglandin release by human PB mononuclear cells and C3H/HeJ mouse AP cells but inhibits its release by rat AP cells (b).

Figure 4.4



PGE₂ may inhibit lymphocyte proliferation induced by a variety of stimulants in vitro and in vivo (Chapter 2). This is largely due to its inhibition of IL-2 production by lymphocytes (Chapter 2). Both PGE₁ and PGE₃ (which may be formed from dietary fatty acids) and the PGE₁ analogue Misoprostol, suppressed lymphocyte proliferation in vitro, indicating their potential use as suppressants of lymphoid functions in vivo.

Inhibition of cytokine production

Production of TNF, IL-1 and IL-6 was assessed by biological assays in this study. The biological activity expressed represents the sum of the actions of a) the cytokine produced and b) any inhibitors also present in the test sample. Therefore we selected assays considered to be relatively specific and not likely to be affected by extraneous PG's present in the test samples. However, we cannot rule out the effects of other, as yet, unidentified factors that might also be regulated by any PG's present in the test supernatants. It is, however, the total biological activity produced (cytokine plus inhibitor) which will determine the final effects of PG's on the cytokine mediated immune responses.

The inhibitory effect of PGE₂ upon TNF β production has been well documented (Endres et al 1989). This inhibition occurs predominantly, if not totally, at the level of mRNA production (Scales et al 1989). PGE₁, PGE₃ and Misoprostol were also inhibitors of the activity of TNF produced by human mononuclear cells and rat peritoneal macrophages.

There has been some confusion in the literature concerning the ability of PGE₂ to regulate IL-1 production. It was initially reported that IL-1 production was inhibited by PGE₂ (Kunkel and Chensue 1985). Our experiments using human PBM indicate that PGE's_{1,2,3}, and Misoprostol did not significantly affect IL-1 production detected using the A375 cell assay. However, when we used other, less specific assays, to detect IL-1 (eg LAF assay or the EL4-nob1/ CTLL cell assay

(Ghering et al 1987), the apparent IL-1 activity was markedly reduced following treatment with PGE's. This was probably due to the effects of the PG's on the reporter assays used (Otterness et al 1988, fig 4.2). Dialysis of the supernatants only partially altered this inhibition (unpublished data) possibly indicating that PGE's were only incompletely removed. Recent reports suggest that PGE₂ does not affect the levels of IL-1 mRNA produced by human monocytes (Knudsen et al 1986) or mouse macrophages (Scales et al 1989).

In contrast to the results obtained with human cells, activity of IL-1 produced by rodent macrophages was reduced by PGE's as measured in our bioassay. However the concentrations of prostanoids required were much higher than those affecting the other cytokines (tables 4.1 and 4.2). Whilst high levels of TNF might also be detected in this IL-1 bioassay, it is unlikely that we measured only TNF suppression. Firstly we noted this effect using peritoneal macrophages from C3H/HeJ mice which did not produce detectable TNF (Table 4.1, 21). Secondly, the levels of TNF found in the rat macrophage supernatants (<20 units/ml) did not affect the action of IL-1 on A375 cells. At very low concentrations of PGE's we consistently observed a slight increase in IL-1 activity indicating that PGE's may possibly both stimulate (Kassis et al 1989) and suppress the formation of this cytokine depending on its concentration. The effects of PGE's on IL-1 production may therefore vary depending upon the species and concentrations used.

Unlike the other cytokines, IFN γ production was measured in an immuno-assay. The inhibitory effect of PGE₂ on IFN γ production has been described previously and its effects on immune responses have been discussed (Cesario et al 1989). IFN γ has been extensively described as a stimulator of many monocyte/macrophage functions, including inflammatory cytokine production. Therefore, suppression of IFN γ may markedly affect progression of inflammatory diseases.

Stimulation of IL-6 production

All the prostanoids stimulated IL-6 production, in marked contrast to their effects on the other cytokines investigated. This was noted at nanomolar concentrations with both rodent AP and human PBM cells. It is consistent with reports that PGE₁ and other stimulators of intracellular cAMP enhance IL-6 production in human foreskin fibroblasts (Zang et al 1988).

PG receptors

Since PGE_{1,2,3} and Misoprostol all had similar effects on the range of biological activities investigated, they probably have the same mechanism of action and bind to the same cell surface receptor(s). The relative abilities of the PGE's to compete with radiolabelled PGE₂ for cell binding was similar to their biological activities. This may indicate that observed differences in potency reflect their different affinities for such a receptor, ie PGE₁=PGE₂= or >PGE₃>Misoprostol. Of the other PG's tested only PGA₂ had some effect (PGF₁α, PGF₂α and PGD₂ had no effect) probably indicating they bind to different receptor types as has been reported (Kennedy et al 1982).

Regulation of intracellular cAMP

Cells generally respond to prostanoids by increasing the intracellular levels of cAMP, a 'second messenger' involved in the transduction of signals from membrane receptors to other sites within the cell (Berridge 1985). We found that bt₂cAMP had the same effects as these prostanoids on macrophages/monocytes. Also, the PGE's were able to increase the intracellular levels of cAMP (in human and rat cells) with similar relative potencies to their cell binding and their effects on cytokines. This suggests that the effects of all 4 of the E-prostanoids are mediated by their ability to increase intracellular cAMP.

The stimulation IL-6 production by PG's was preceded by increased levels of IL-6 mRNA (Fig 4.4) as has been reported for PGE₁ (Zang et al 1988). This indicates that stimulation by all the 3 PGE's (1,2 and 3) and Misoprostol was due to

transcriptional rather than post-transcriptional effects. Some cytoplasmic proteins are known to be phosphorylated by cAMP-dependent enzymes (Hunt et al 1984). Two of these in particular, CREB (cyclic AMP response element binding protein) and AP-1, recognise and bind to specific sequences of DNA. If these proteins bind to sites in the promoter region of genes, they can regulate the transcription and eventually expression of these gene products. Sequences corresponding to CRE sites have been identified in the promoter sequence of IL-6 (Tanabe et al 1988) and many other genes. Therefore the 3 PGE's (1,2 and 3) and Misoprostol's may not only induce CREB to promote IL-6 transcription but (at the same time) regulate transcription of other cytokines. For example, the promoter region of TNF, which is inhibited by PG's and cAMP, has sequences which closely resemble CRE and AP-1 (Economou et al 1989).

Generally, we found that human PBM cells were less sensitive to the PGE's than rodent peritoneal cells. However, rather than reflecting a species difference, this may indicate that mature peritoneal macrophages are more sensitive than immature peripheral blood monocytes and lymphocytes. This is supported by the observation that rat AP cells produce much less intracellular cAMP, either normally or following PGE treatment, than human PBM cells. More studies would be needed to determine if this is due to increased sensitivity of the rodent to intracellular cAMP, or to other factors, such as differences in the numbers of PGE receptors.

Regulation of PGE production by cyclic nucleotides

BtcAMP was found to stimulate PGE synthesis in human PB monocytes and rat (Dark Agouti and Porton strains) peritoneal macrophages. Surprisingly btcGMP also stimulated PGE release but at higher concentrations than cAMP. The Dark Agouti rat was different from the Porton strain in that cGMP inhibited PGE release at the highest dilution. Most of these effects were noted at high

concentrations of the dibutylated cyclic nucleotides and it is difficult to determine if the levels of intracellular cyclic nucleotide attained are similar to those found under physiological conditions. This stimulation of PGE synthesis by cyclic nucleotides contrasts to a report that they inhibit prostanoid synthesis in platelets (Alvarez et al 1989).

Since Misoprostol and the other PGE's will stimulate higher intracellular cAMP levels, it was of interest to see if they also can stimulate PGE release. However, Misoprostol did not stimulate PGE release at concentrations up to 100nM. Unfortunately higher levels could not be tested since the polyclonal antibody used not only detected PGE in the immunoassay but also the exogenous Misoprostol. This may indicate either, i) higher concentrations of Misoprostol are needed to induce the required levels of cAMP for stimulation of PGE release, or ii) although high enough levels of cAMP are attained, binding to the PGE receptor induces other signals which repress PGE release. If PGE's do stimulate their own release this would be a potent mechanism by which PGE's can amplify acute inflammation by a positive feedback effect.

PG regulation of cytokine mediated inflammation

The ability of E-prostaglandins to regulate the production of inflammatory cytokines and other MNP functions described here, may be examples of natural feedback mechanisms for controlling inflammation. Suppression of MNP spreading, IL-1 action, IFN γ , TNF and, occasionally, IL-1 production, whilst stimulating the comparatively less acutely cytotoxic cytokine IL-6, may lead to an overall reduction of inflammation. IL-6 is also a potent stimulator of acute phase protein production by the liver (reviewed Segal et al 1989, Le and Vilcel 1989), and a common function of these acute phase proteins may be to help restore the homeostatic balance of the inflamed tissue (Koj 1985).

These regulatory effects may be particularly relevant during the acute phase of the inflammatory process, such as active arthritis, where high levels of PGE₂ have been detected in synovial fluids (Robinson and Levine 1974). These concentrations (1-100nM) are similar to those at which we observed significant effects *in vitro*. Since MNP's not only produce PGE's, but also are affected by them, PGE's effects need only be short-ranged. Therefore, much higher effective concentrations in this microenvironment may be attained. In addition, in this study we have often assessed the PGE's suppression of responses to strong stimuli (10u/ml IL-1 and 5ug/ml LPS), but these PGE's may be even more effective at suppressing responses to weaker stimuli.

Such a regulatory mechanism has two major implications for current and prospective therapies. Firstly, the commonly used NSAIDs which strongly inhibit PG production, may be effective at relieving some symptoms of inflammation (eg pain, vasodilatation etc) but might not reduce the long-term progression of the disease sustained or mediated by inflammatory cytokines (eg IL-1, TNF etc). Secondly, dietary control of inflammation by fish oils etc, may be more effective in the long term if allowed to augment the natural regulation by PGE₂ by producing additional PGE₁ or 3. Conversely, if dietary treatment were to result in lower levels of total PGE (ie PGE₂ + 1 or 3), as has been reported (Broughton 1991), the suppressive effects of PGE's may be reduced. However, suppression of cytokine production by modifying the production of leukotriene B₄ (Endres et al 1989, Rola-Pleszczynski and Lemaire) or other products of lipoxygenase (Sirko et al 1991) may balance/replace the loss of PGE₂ suppression.

Misoprostol was developed to reduce the damaging effects of NSAIDs and other gastrotoxins on the stomach. However, recent findings suggest it may have other beneficial effects such as prolonging the survival of transplants. This may reflect the immunosuppressive (Wiederkehr et al 1990) and antiinflammatory (Whitehouse et al 1990a) effects of the drug. Although the data shown in this

report was generated with the ester form of Misoprostol, the acid form showed identical activity in all the assays used (data not shown). This probably indicated that cells used in these assays contain sufficient esterase activity to rapidly generate the Misoprostol free acid. In all the experiments, Misoprostol closely resembled the naturally occurring prostanoids, supporting the suggestion that stable synthetic PG's might be useful in therapy where such immune regulation is beneficial (Nicholson 1990). Its use in conjunction with other therapies reducing PG production, eg NSAID or dietary controls as described above, may be of particular value.

The results of this study indicate that PG's can modify the cytokine-dependent immune responses. However the potential immunoregulant action of PGE₁ and PGE₃ indicate that diet enrichment with their precursor fatty acid might also affect certain immunopathies by modifying the proportions of cyclooxygenase products (PG's).

Chapter 5

Cyclosporin prevents experimental arthritis in rats by regulating leucocyte subpopulations and inflammatory mediators.

INTRODUCTION

The fungal cyclic peptide, cyclosporin (CsA), has been used extensively to promote the survival of organ grafts (Morris 1981, Cohen et al 1984). CsA is very effective in preventing allograft rejection and elicits less side effects in the graft recipients than many other forms of immunosuppressive therapy (eg. Nitrogen mustards, antimetabolites). However, despite its wide usage, the exact mode of action of CsA remains unclear.

Extensive *in vitro* studies have shown that CsA will selectively inhibit a number of immune functions, and in most cases this is attributable to its suppression of T lymphocyte activity. This inhibition may cause not only selective depletion of T-helper cells (Clerici and Shearer 1990) but also suppress accessory cell (Varey et al 1986, Esa et al 1988) and B lymphocyte functions (Klaus 1988). These observations, and the success of CsA in transplantation, led to the evaluation of CsA for treating a variety of autoimmune disorders including rheumatoid arthritis (Dougados et al 1988, Sany 1990).

CsA has been shown to be effective in the treatment of experimentally induced arthritis in rats and mice (del-Pozo et al 1990, Cannon et al 1989, Rofe et al 1990, Rofe et al 1992). Not only does CsA treatment reduce the severity of disease, but treatment during the induction period also prevents development of adjuvant arthritis and renders the animals resistant to further challenge with the same arthritogen (Whitehouse and Vernon-Roberts 1989). Surprisingly the effect of CsA

on lymph nodes draining the site of adjuvant injection and adjuvant disease (paws) indicated that CsA modified, rather than suppressed, immune responses to the arthritogenic adjuvant, probably by selective suppression of some T lymphocyte cytokines.

MATERIALS AND METHODS

Adjuvant disease

Adjuvant arthritis was induced as previously described (Haynes et al 1988b). Briefly, on Day 0, groups of male Dark Agouti (~250g) rats were injected subdermally near the tail base with 50ul of finely ground, heat-killed, delipidated mixed human strains of Mycobacterium tuberculosis dispersed in squalane at a concentration of 10mg/ml. Co-administration of CsA with the adjuvant was achieved by grinding the CsA (100 mg/ml) in with the adjuvant. In some experiments, CsA was injected subcutaneously into the hind flank of the animal. On Day 14 after adjuvant injection, the polyarthritis was assessed by scoring each paw for disease severity as previously described (Whitehouse et al 1990b).

Detection of CsA in the blood

Blood levels of CsA were measured with an immunoassay ("Cyclotrac" SP RIA - Incstar Corp. Minnesota, USA) by Dr. Ray Morris at the Queen Elizabeth Hospital, Woodville, S.A.

Preparation of rat paws for histology

Rat rear paws were amputated from animals sacrificed on days 3, 7 and 14 after adjuvant injection. The skin was carefully removed and the ^{remaining} tissue fixed in a 10% formaldehyde solution for 1 week before transfer to a solution of "Decal" reagent (Omega Chemical Corp. New York, USA) for decalcification for 36 hours at 37°C. The paws were cut sagittally, embedded in paraffin blocks and sectioned when required. Sections were taken from the central part of the paw.

Cell isolation

Peripheral blood mononuclear (PBM) cells were isolated as previously described (Haynes et al 1988a). Popliteal lymph nodes (PLN) and inguinal lymph nodes (ILN) were removed immediately after the rats were killed by cervical dislocation and kept in cold Hank's buffered salt solution (HBS). The lymph nodes were passed through a fine wire mesh and the single cell suspensions washed in HBS. Adherent peritoneal (AP) cells were isolated as described in previous chapters.

³H-thymidine uptake

Triplicate 100ul cultures of the PBM, PLN and ILN cells in RPMI-1640 medium containing 2×10^5 cells were incubated with 50ul of 0.5 uCi ³H-thymidine in 96-well microtitre trays, 3 hours later the cells were harvested on glass filters and ³H-thymidine incorporation measured as described (Haynes et al 1988a).

Cytokine production (ex vivo)

2×10^5 PBM, PLN and ILN cells were incubated in a final volume of 250ul RPMI medium with 10% FCS. These cells were unstimulated or stimulated with either 5 ug/ml LPS or 5 ug/ml Con A. After 24 hours the supernatants were sampled and assayed for the various cytokines. The in vitro effect of CsA on PBM, PLN and AP cells isolated from normal Dark Agouti rats was carried out in the same manner with the addition of CsA at various concentrations. Samples of supernatants were assayed immediately or stored at -70°C for up to one month before assay.

Assays for cytokines

The following assays were carried out as described in Chapter 4. IL-1 was assayed using the IL-1 sensitive A375 cell line (Nakai et al 1988). Although these cells are also sensitive to high levels of TNF, the levels of TNF in the supernatants were always too low to be detected at the dilutions used. TNF was assayed using the TNF-sensitive cell line L929 (Matthews and Neale 1987). 7TD1 hybridoma cells

were used to assay IL-6 (Bartold and Haynes 1991). IL-2 was assayed using the CTLL-2 cell line (Gillis et al 1978).

To assay IFN γ , an enzyme-linked immunoassay kit was used (Holland Biotechnology, Netherlands) specific for rat IFN γ .

Assay for PGE₂

A competitive radioimmunoassay was used as described in Chapter 2.

Monoclonal antibodies (mAbs)

Mark 1 mAb's (Bazin et al 1984) were gift from Prof. H. Bazin, Brussels, Belgium. R73 mAb's (Hunig et al 1989) were a gift from Dr. T Hunig, Wurtzburg, Germany. OX6 , OX 19 , W3/13, OX8, and W3/25 mAb's have all been previously described (Fukumoto et al 1982, Dallman et al 1982, Mason et al 1980).

Immunofluorescence assays

Isolated rat lymph node cells were stained with mAb in an immunofluorescence assay essentially as described (Gadd and Ashman 1983). Briefly, 50ul of lymph node cells (10^7 /ml) (isolated as described above) were mixed with an equal volume of mAb in HBS and incubated for 30 min on ice, followed by two washes in phosphate-buffered saline containing 0.1% sodium azide and 1% bovine serum albumin. The cells were resuspended in 50 ul of 1/50 dilution of fluorescein-conjugated sheep anti-mouse immunoglobulins (Silenius Laboratories, Australia) and incubated for 30 min on ice. The cells were then washed three times with the PBS-azide and analysed by flow cytometry on a FACScan (Becton Dickinson). To block non-specific binding of mAb, 10% normal rat serum was included in all incubations.

Staining of nodes with mAb

Lymph nodes required for staining with mAb were immediately embedded in OTC compound (Miles Scientific, Il., USA) and stored at -70°C before 5 µM sections were cut on a cryostat. The staining of sections was carried out within 24 hours on serial sections as described (Mayrhofer and Spargo 1990).

Statistical analysis

Statistical significance values were calculated using a Student's t test (Snedecor and Cochran 1989).

RESULTS

(summarised in table 5.7)

The effect of CsA on the expression of adjuvant-induced arthritis and associated weight loss

Table 5.1 shows that both the weight loss and disease onset were prevented when CsA was administered mixed with the arthritogenic adjuvant (given at day 0). Subcutaneous administration of 10 mg/kg CsA every second day from day -1 to day 13 or from day -1 to day 7 was equally effective at preventing disease and weight loss.

Histological examination (Fig 5.1) of the rear ankle joints and surrounding tissues from untreated polyarthritic animals (Fig 5.1.a) showed there was a large influx of polymorphonuclear cells, lymphocytes and macrophages. Occasional multinucleate giant cells (Fig 5.1.b) were present in the eroded pits of bone and there was a large amount of cartilage destruction associated with an ingrowth of thicker synovial membrane (Fig 5.1.c). Neither destruction of bone and tissue nor the presence of multinucleate giant cells and polymorphonuclear cells were seen in or around the ankle joint of CsA-treated animals (Fig 5.1.d), which appeared identical with those from normal animals (no adjuvant treatment).

Table 5.1

The effect of CsA treatments on the induction of arthritis by a mycobacterial adjuvant (Adj) in male Dark Agouti rats.

Treatment	Number of rats	Severity of arthritis ¹	Weight change (gm) ²
None	20	0	+32.6 ± 4.2
Adj	31	7.85 ± 0.70	-21.5 ± 3.0
Adj + CsA ³	32	0	+25.4 ± 2.4
Adj + CsA s.c. (Days-1to7) ⁴	5	0	+32.2 ± 7.8
Adj + CsA s.c. (Days-1to13) ⁴	9	0	+17.3 ± 5.3

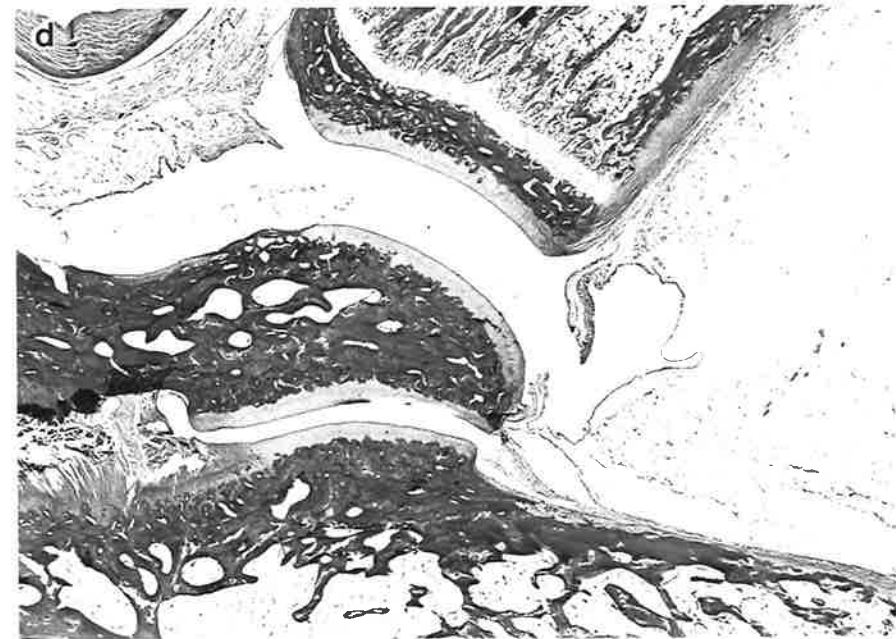
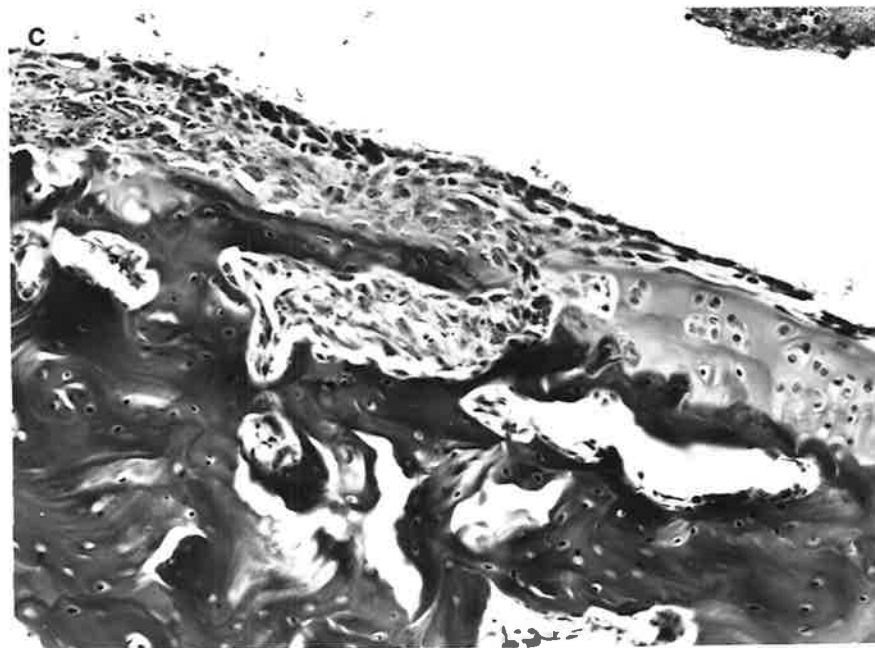
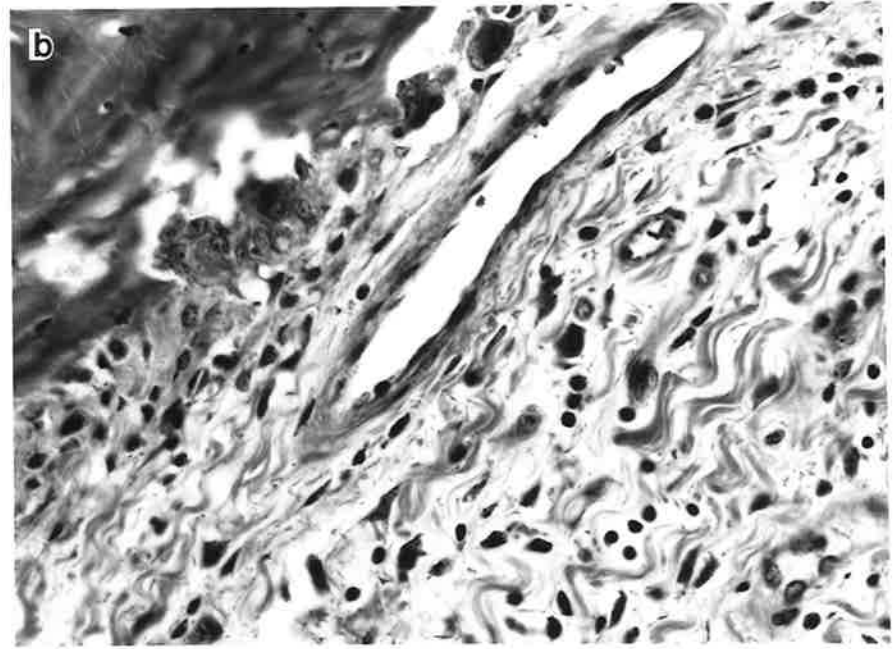
¹ Mean ± standard error scored 14 days after inoculating adjuvant in tail base. The total score per rat was calculated by tallying the scores for each rear paw (maximum 4 each) and front paw (maximum 3 each).

² The mean ± standard error of the weight change over 14 days.

³ 40mg/Kg CsA admixed with the Adjuvant injected in the tail (see methods).

⁴ 10mg/Kg CsA injected subcutaneously every second day.

Figure 5.1. The histology of the rear rat paw (ankle joint) shows the destruction of tissue and bone occurring during expression of arthritic disease (day 14) in rats injected with adjuvant alone (5.1a). The soft tissue is crowded with inflammatory cells, mainly polymorphonuclear, but many mononuclear cells are present. There is extensive erosion of the bone and multinucleate giant cells are often seen in the eroded pits (5.1b). Loss of cartilage is associated with an ingrowth and thickening of the synovial membrane (5.1c). In contrast none of these destructive and inflammatory processes are seen at day 14 in rats injected with CsA admixed with the adjuvant (5.1d). The histology was identical to that seen in normal healthy rats (not adjuvant injected or CsA treated).



Blood levels of CsA following administration of CsA admixed with the adjuvant are shown in Figure 5.2. High levels ($> 0.3 \mu\text{M}$) were detected for the first 7 days post injection, falling to less than $0.1 \mu\text{M}$ on day 14.

Analysis of lymphoid cell populations

Figure 5.3 shows the yields of lymphoid and blood cells isolated from Dark Agouti rats injected in the tail base with either adjuvant alone or a mixture of adjuvant and 40 mg/Kg CsA. ^3H -thymidine incorporation by these cells is also shown. Cells were isolated from 3 separate sites, namely mononuclear cells from the peripheral blood (PBM), total cells from the inguinal lymph node (ILN) and total cells from the popliteal lymph node (PLN). The yields of cells and the uptake of ^3H -thymidine by PBM did not vary significantly throughout the 14 day period of the experiment (Fig 5.3.a). However, there was a slight reduction in ^3H -thymidine labelling on days 3 and 7 in the CsA-treated group. The yields of cells isolated from the ILN increased markedly on days 3 to 14 in both groups of animals (Fig 5.3.b). ^3H -thymidine labelling rose dramatically only on day 3; however, the levels were significantly lower ($P < 0.05$) in CsA-treated animals. The yields of PLN cells (Fig 5.3.c) from both adjuvant and adjuvant/CsA-injected rats significantly increased after day 7. ^3H -Thymidine labelling was slightly greater on day 3 in both groups but lower in cells from CsA-treated animals than those from untreated animals. There was a marked increase in ^3H -thymidine uptake by PLN cells from both groups of animals on day 14.

PLN cells were isolated from normal, adjuvant-injected and adjuvant/CsA-injected rats on day 14. Similar results were obtained when CsA was administered subcutaneously. Subpopulations of cells were labelled with monoclonal antibodies and compared by flow cytometric analysis (Table 5.2). There was no significant difference between the three groups in the percentages of cells binding mAb W3/13 (CD43, pan T cell marker) or OX8 (CD8⁺ subset of T cells). The proportion of OX6 positive (MHC class II) cells was significantly higher ($P < 0.005$) in all the

Figure 5.2. Levels of CsA detected by a immunoassay in the blood of rats given a single dose co-administered with the adjuvant in the tail base. Each point represents the mean \pm standard error of 3 experiments.

Figure 5.2

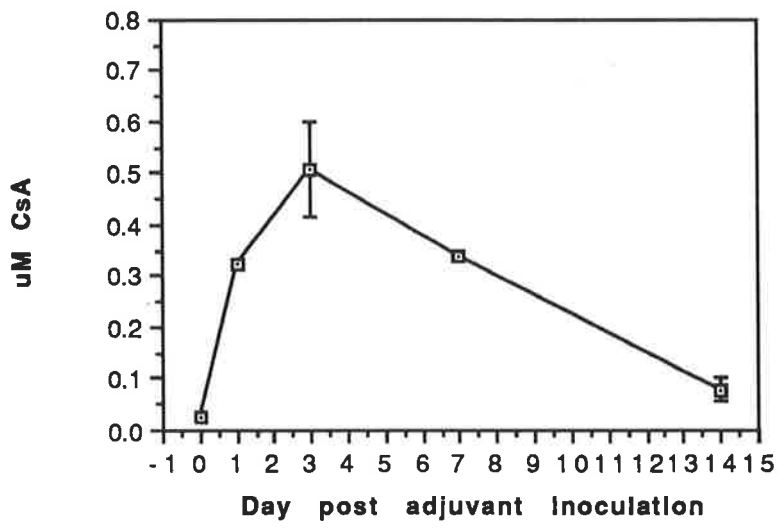


Figure 5.3. (i) The yield of and (ii) ^3H thymidine uptake by, (a) mononuclear cells isolated from the peripheral blood, (b) total cells from the inguinal lymph node and (c) total cells from the popliteal lymph node. Each point represents the mean \pm standard error of 6 experiments.

Figure 5.3

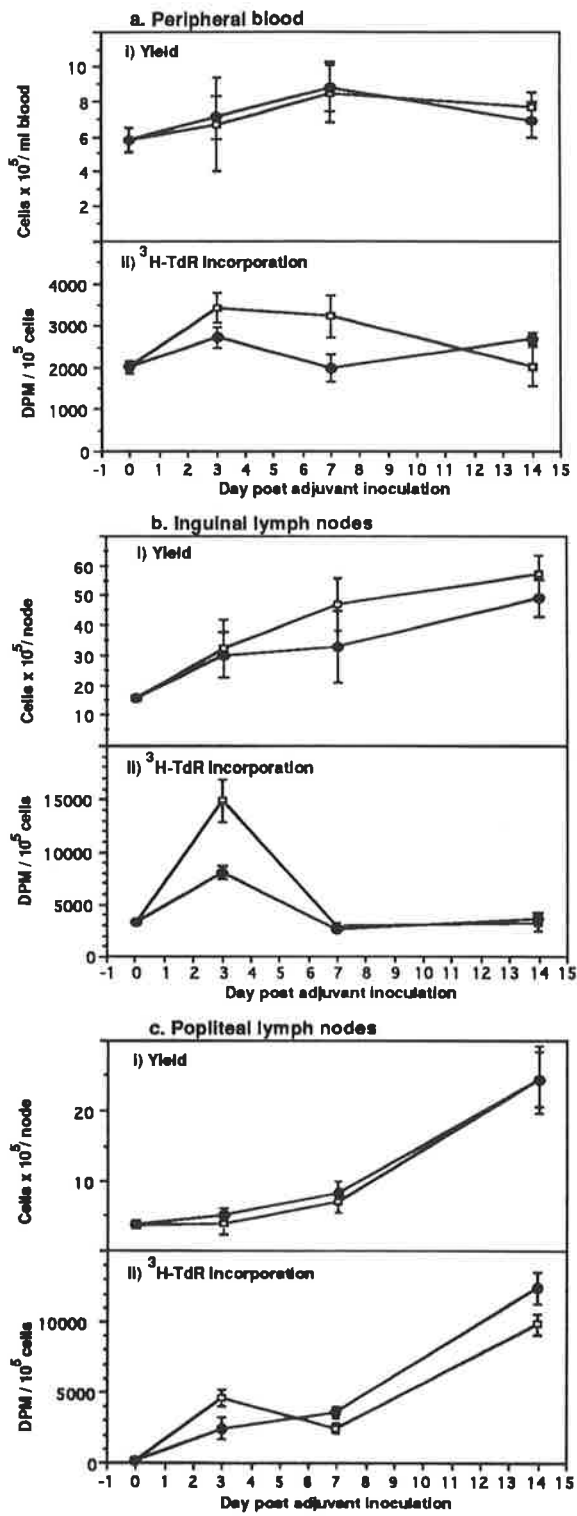


Table 5.2

Popliteal lymph node cell subpopulations 14 days after mycobacterial adjuvant injection (Adj) ± cyclosporin (CsA) in rats.

Antibody (indicating)	Normal (no Adj)	Adj	Adj + CsA in tail	Adj + CsA s.c. every 2nd day from day -1 to 7	Adj + CsA s.c. every 2nd day from day -1 to 13
OX6 (MHC Class II ⁺ cells)	20.6 ± 1.3 ¹ (14) ²	28.3 ± 1.9 (23) **	36.4 ± 2.1 (23) **	34.4 ± 1.3 (4) *	31.1 ± 2.3 (4)
W3/13 (mainly T cells)	79.2 ± 2.7 (12)	78.3 ± 2.4 (15)	76.4 ± 2.1 (22)	ND	ND
OX19 (CD5 ⁺ cells)	78.6 ± 2.2 (10)	80.9 ± 2.1 (14)	71.9 ± 2.7 (14) *	74.7 ± 4.1 (4)	74.9 ± 1.3 (4)
OX8 (CD8 ⁺ cells)	27.5 ± 2.8 (7)	24.4 ± 1.8 (14)	26.1 ± 1.1 (14)	25.3 ± 3.7 (4)	22.3 ± 0.4 (4)
W3/25 (CD4 ⁺ cells)	62.4 ± 0.8 (11)	56.9 ± 1.9 (15) *	46.3 ± 2.0 (14) **	49.2 ± 1.2 (4) *	49.5 ± 1.7 (4) *
Mark I (B cells)	15.4 ± 1.2 (7)	13.3 ± 1.6 (6)	24.8 ± 2.7 (8) **	22.6 ± 2.2 (4) **	22.6 ± 2.2 (4) **
R73 (αβ T cells)	72.7 ± 1.3 (7)	70.8 ± 1.1 (8)	59.6 ± 1.8 (8) *	66.2 ± 1.5 (4)	63.2 ± 1.8 (4) *

¹ Percentage of cells staining positive with the monoclonal antibody ± standard error as determined by fluorescent activated cell sorting analysis as described in methods.

² Number of animals per group.

* and ** significance is p<0.05 and p<0.005 respectively. For (i) Adj (no treatment) animals compared to normal animals and (ii) Adj + CsA treated animals compared to Adj (adjuvant alone injected) animals.

groups that had received the adjuvant compared with normal animals (no adjuvant). The proportion of cells staining with Mark I mAb (B cells) was unchanged in animals receiving adjuvant alone. However, the proportion of cells staining with OX6 and Mark I antibodies was significantly greater in rats receiving adjuvant plus CsA. The proportion of cells labelled by W3/25 mAb (CD4⁺, T cells and some macrophages) was reduced in all groups receiving adjuvant and lowest in the groups which also received CsA ($P < 0.005$ when compared to adjuvant alone). Similarly, the proportion of cells staining with R73 antibodies (α/β T cells) was also reduced significantly, particularly in those groups receiving adjuvant and CsA. The proportion of cells staining with OX19 (CD5⁺ T cells) was only reduced slightly by CsA treatment. CsA administered by subcutaneous routes (either on days -1 to 7 or days -1 to 13 to adjuvant treated rats) gave similar results to CsA admixed with the adjuvant.

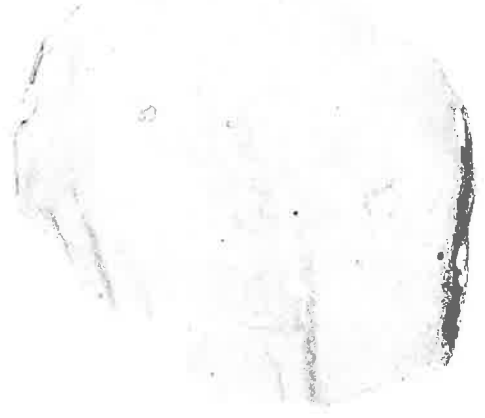
Immunohistology was performed on PLN isolated from animals at day 14. Figure 5.4 illustrates that CsA treatment (CsA admixed with the adjuvant) had little effect on the appearance of PLN following adjuvant injection. CsA treatment often resulted in larger nodes, as represented in Figures 5.4b., d., f. and g. However, this was not reflected in a significant increase in the yield of cells from these nodes (Fig 5.3). Lymph nodes isolated from both groups of animals had the features of reactive lymph nodes, with expansion of both B cell areas (follicles) of T dependent areas (paracortex). Staining with R73 antibodies indicated that large numbers of α/β positive T cells were evenly distributed throughout the paracortical and cortical region in nodes from animals given adjuvant alone (Fig 5.4.c). However, CsA treatment resulted in large circular areas in the paracortical region which were not α/β positive T cells (Fig 5.4.d). A serial section of this node stained with Mark 1 antibodies showed that these areas were probably B cells and indicated the presence of follicles in the paracortical region (Fig 5.4.f). This was not noted in nodes isolated from animals injected with adjuvant alone (Fig 5.4.e). Staining with OX6 mAbs indicated that the class II positive cells were mainly B

Figure 5.4. Immunohistology of PLN from rats injected with adjuvant (5.4a, c, e, g) and CsA admixed with adjuvant (5.4b, d, f, h) rats was carried out with mAb directed against various cells subset markers as described in the text. Control antibody (an IgG class 1 mAb directed against a giardia cell wall antigen) did not stain PLN from either group of animal indicating background staining is low (5.4a and b). Staining patterns obtained with the mAb's R73 directed against the α/β T cell receptor (5.4c and d), Mark I directed against cell surface immunoglobulin (5.4e and f) and OX6 directed against Class II antigens (5.4g and h) are shown. Magnification ~20x.

a



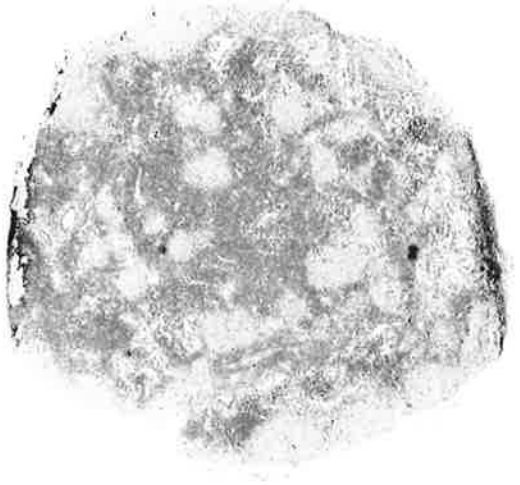
b



c



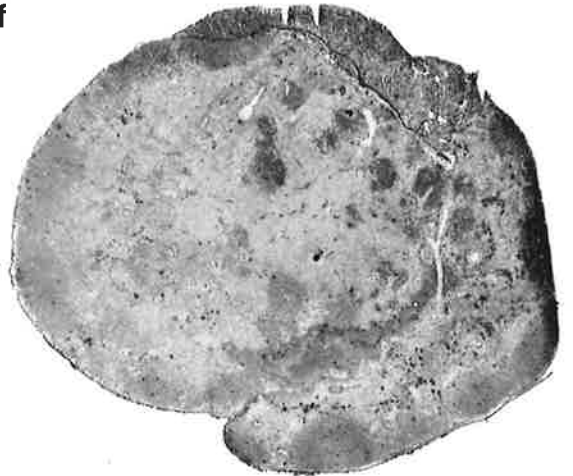
d



e



f



g



h



cells in nodes isolated from both groups of animals (Fig 5.4.g and h). The other T cell mAb's (W3/13, OX19, OX8 and W3/25), like the R73 mAb, stained the paracortical region of nodes from animals injected with adjuvant alone but large circular areas, consistent with B cell follicles, remained unstained in the paracortex of nodes from animals treated with CsA.

Cytokine production by cells ex vivo after CsA treatment in vivo

Table 5.3 shows the effect of CsA treatment on the ability of PLN cells to produce a number of cytokines. Although the results represented here were obtained with animals injected with CsA admixed with the adjuvant, similar results (for PLN, PBM and AP cells) were obtained when the CsA was administered subcutaneously. Cells were isolated from control animals and from animals during the initiation (days 1 and 3) or the establishment phases of the arthritic disease. Unstimulated cells produced levels of all cytokines (except IL-6) below the detectable limits of our assays. Con A stimulated secretion of IFN γ , IL-2, TNF and IL-6 but had little effect on IL-1 secretion, while LPS induced secretion of IL-1, TNF and IL-6. The secretion of all cytokines, except TNF, was increased in animals that had received the arthritic adjuvant. CsA treatment significantly suppressed ($p < 0.05$) IFN γ (Days 1 and 3), IL-2 (day 1), and IL-6 (day 14) production by Con A-activated lymph node cells from adjuvant stimulated animals. TNF production was also reduced by in vivo CsA treatment (measured on days 1, 3 or 14) but this was not significantly ($p < 0.05$) different from animals given adjuvant alone. IL-1 and IL-6 secretion was also significantly ($p < 0.05$) suppressed by CsA in LPS-stimulated lymph node cells on day 14.

CsA treatment had similar effects on cytokine secretion by PBM cells (table 5.4) but there was no effect on secretion of IL-6.

The effect of CsA treatment on cytokine secretion by adherent peritoneal (AP) cells stimulated with 5 μ g/ml LPS is shown in table 5.5. Although no IL-2 or IFN γ could

Table 5.3

Cytokine production by cells isolated from the popliteal nodes of DA rats with/without mycobacterial adjuvant (Adj) ± cyclosporin (CsA).

Group\Cytokine	IFN γ		IL-2		IL-1		TNF		IL-6	
	+Con A	+LPS	+Con A	+LPS	+Con A	+LPS	+Con A	+LPS	+Con A	+LPS
Normal	3.81 ± 1.13 ²	<1	35.5 ± 8.1 ³	<2	<2 ³	4.8 ± 2.3	92.5 ± 1.8 ³	2.1 ± 1.4	0.59 ± 0.10 ⁴	0.63 ± 0.22
Day 1¹										
Adj	24.3 ± 5.0	<1	86.0 ± 16.6	<2	<2	2.3 ± 1.3	51.0 ± 16.7	2.5 ± 2.5	156 ± 85.6	31.4 ± 22.0
Adj/CsA	6.75 ± 0.78*	<1	27.8 ± 10.4*	<2	<2	<2	27.0 ± 7.3	<2	253 ± 35.2	2.98 ± 1.38
Day 3										
Adj	38.7 ± 2.5	<1	55.0 ± 14.4	<2	2.8 ± 1.2	3.4 ± 2.0	25.0 ± 7.5	3.8 ± 1.3	15.9 ± 13.3	15.3 ± 8.5
Adj/ CsA	16.6 ± 6.1*	<1	75.5 ± 32.5	<2	2.6 ± 0.7	7.8 ± 1.0	8.5 ± 1.1	2.1 ± 0.7	42.8 ± 22.9	4.08 ± 0.67
Day 14										
Adj	5.58 ± 1.39	<1	50.0 ± 9.0	<2	<2	13.6 ± 0.86	10.9 ± 2.86	<2	14.5 ± 2.8	6.69 ± 0.71
Adj/CsA	3.60 ± 0.20	<1	42.0 ± 14.1	<2	4.53 ± 0.68	3.59 ± 1.23*	5.53 ± 2.75	<2	36.6 ± 4.5*	31.8 ± 7.27*

¹ Post-injection with adjuvant.

² ng/10⁵ cells of IFN γ measured in a immunoassay as described in methods. Mean ± standard error of at least 4 animals.

³ units/10⁵ cells of cytokine measured in biological assays as described in methods. Mean ± standard error of at least 4 animals.

⁴ units/10³ cells of cytokine measured in biological assays as described in methods. Mean ± standard error of at least 4 animals.

* p<0.05

Table 5.4

Cytokine production by peripheral blood mononuclear cells isolated from the peripheral blood of DA rats with/without mycobacterial adjuvant (Adj) ± cyclosporin (CsA).

Group\Cytokine	IFN γ		IL-2		IL-1		TNF		IL-6	
	+Con A	+LPS	+Con A	+LPS	+Con A	+LPS	+Con A	+LPS	+Con A	+LPS
Normal	0.73 ± 0.15 ²	<1	12.5 ± 3.1 ³	<2	<2 ³	3.2 ± 0.6	20.0 ± 7.1 ³	95.0 ± 3.5	0.81 ± 0.21 ⁴	6.7 ± 0.42
Day 1¹										
Adj	18.8 ± 11.5	<1	8.5 ± 3.7	<2	<2	14.3 ± 2.4	35.4 ± 31.4	45.5 ± 25.7	0.37 ± 0.07	14.3 ± 3.7
Adj/CsA	1.50 ± 1.34*	<1	14.3 ± 0.6	<2	<2	11.8 ± 2.0	36.8 ± 13.2	62.3 ± 22.8	0.41 ± 0.13	14.0 ± 6.5
Day 3										
Adj	2.13 ± 0.99	<1	24.3 ± 5.4	<2	<2	3.5 ± 1.7	29.5 ± 8.9	95.0 ± 32.0	0.38 ± 0.05	12.8 ± 8.8
Adj/ CsA	3.38 ± 2.1	<1	38.7 ± 23.9	<2	<2	7.8 ± 0.9	9.30 ± 3.90	119 ± 45.6	0.22 ± 0.05	12.6 ± 6.1
Day 14										
Adj	0.99 ± 0.16	<1	32.5 ± 6.4	<2	<2	5.9 ± 0.8	25.0 ± 11.0	66.3 ± 16.7	6.70 ± 1.19	16.0 ± 2.16
Adj/CsA	0.74 ± 0.11	<1	18.0 ± 3.3	<2	<2	2.9 ± 0.6*	28.5 ± 11.9	90.3 ± 3.6	1.01 ± 0.34*	4.78 ± 1.58*

¹ Post-injection with adjuvant.

² ng/10⁵ cells of IFN γ measured in a immunoassay as described in methods. Mean ± standard error of at least 4 animals.

³ units/10⁵ cells of cytokine measured in biological assays as described in methods. Mean ± standard error of at least 4 animals.

⁴ units/10³ cells of cytokine measured in biological assays as described in methods. Mean ± standard error of at least 4 animal

*p<0.05

Table 5.5

Cytokine production by lipopolysaccharide (5ug/ml) stimulated adherent peritoneal cavity cells during the stages of disease initiation and expression.

Cells isolated from DA rats with/without mycobacterial adjuvant (Adj) ± cyclosporin (CsA).

Group\Cytokine	IL-1	TNF	IL-6
Normal	15.2 ± 2.9	89.5 ± 12.1	34.4 ± 4.8
Day 3 ¹			
Adj	10.3 ± 4.8	37.8 ± 8.3	255 ± 111
Adj/CsA	18.5 ± 5.5	17.8 ± 5.0*	295 ± 103
Day 7			
Adj	53.8 ± 7.5	145 ± 32.4	502 ± 147
Adj/ CsA	38.5 ± 6.2*	32.4 ± 14.7*	82 ± 28*
Day 14			
Adj	25.2 ± 5.2	271 ± 55.5	163 ± 22
Adj/CsA	13.8 ± 2.9*	47.3 ± 23.3*	63 ± 21*

¹ Post-injection with adjuvant.

² units/10⁵ cells of cytokine measured in biological assays as described in methods. Mean ± standard error of at least 4 animals.

³ units/10³ cells of cytokine measured in biological assays as described in methods. Mean ± standard error of at least 4 animals.

* p<0.05

be measured, substantial amounts of IL-1, TNF and IL-6 were produced by these cells. During the early stages of disease initiation (Day 3) CsA treatment only affected TNF production, significantly reducing its levels. During the later stages, just before and during disease expression (Days 7 and 14), IL-1, TNF and IL-6 production was markedly reduced by CsA treatment. CsA tended to raise production of these cytokines to the levels produced by normal (untreated and non-adjuvant injected) animals.

Effects of CsA on secretion of cytokines and PGE₂ by cells in vitro (table 5.6)

Cytokine production was also assessed following stimulation of normal popliteal lymph node (PLN), PBM and AP cells in vitro with either Con A or LPS in the presence of graded concentrations of CsA. Table 4 shows the concentrations of CsA calculated to inhibit cytokine production by 50%. IFN γ and IL-2 production by Con A-stimulated PLN and PBM cells were particularly sensitive to CsA treatment with ID₅₀'s of about 10nM or less. Con A stimulated TNF production by PLN and PBM cells and this was also inhibited by CsA in vitro (ID₅₀'s < 100nM). By contrast, IL-6 production was only inhibited by relatively high concentrations of the drug (ID₅₀'s > 1 μ M). Production of IL-1 by LPS-stimulated PBM cells was inhibited at moderate concentrations of CsA (ID₅₀'s < 150nM), but TNF and IL-6 production were only affected by much higher concentrations of CsA (> 1 μ M). Cytokine production by AP cells was resistant to CsA (ID₅₀'s > 1000nM). IL-6 production was unaffected by CsA in vitro (<10 μ M). CsA was toxic to the cell types tested at concentrations greater than 10mM as assessed by trypan blue exclusion.

PGE₂ production by PBM and AP cells was inhibited by CsA treatment (Table 5.6). Con A-stimulated PBM cells were more sensitive to the effects of CsA (ID₅₀ of 9.9nM) than either LPS-stimulated PBM cells or LPS-stimulated AP cells (ID₅₀'s of 2.9 and >10mM respectively). However Con A-stimulated PBM cells

Table 5.6

The differential effects of CsA in vitro on the production of IL-1, IL-2, IL-6, TNF and PGE₂ by rat popliteal lymph node (PLN) cells, peripheral blood mononuclear (PBM) cells and adherent peritoneal (AP) cells stimulated with 5ug/ml Con A or LPS.

	Cell type (Stimulus)			
	PLN (+ Con A)	PBM (+Con A)	PBM (+LPS)	AP (+LPS)
Cytokine¹				
IL-2	5.8 ± 3.5 ²	8.1 ± 5.7	NM ³	NM
IL-1	NM	NM	147 ± 57	3,300 ± 900
IL-6	6,930 ± 520	1,590 ± 530	7,130 ± 133	1,030 ± 120
TNF	85 ± 12	19 ± 7.8	411 ± 117	1,350 ± 521
IFN γ	11.2 ± 2.2	5.9 ± 1.1	NM	NM
Prostanoid⁴				
PGE ₂	NM	9.9 ± 4.1	2,930 ± 2,050	>10,000

¹ Cytokine measured in supernatant after 24 hours culture of cells with CsA measured in appropriate biological assay as described in text.

² The concentration (ID₅₀) of CsA which reduced the activity of cytokine or PGE₂ present in supernatant after 24 hours by 50%. Each value represents the mean ± standard error from at least 4 experiments.

³ NM = the level produced was too low to calculate an ID₅₀ value accurately (ie levels less than 2 units/ml activity for the cytokines and 3.1ng/ml for PGE₂).

⁴ PGE₂ measured in radioimmunoassay.

Table 5.7

Summary of the effects of experimental polyarthritis and CsA treatment on disease parameters, PLN cell populations and ex vivo PLN cytokine production in the rat.

	Polyarthritic rats (compared to normal rats)	CsA treated rats (compared to polyarthritic rats)
Disease parameters :		
Disease score	+++ ¹	---
Weight change	-	+
Cell populations :		
Yield	+++	0
Proliferation	+++	0
Proportion of:		
MHC Class II+ve	+	++
CD4+ve	0	--
B cells	0	+++
Cytokine production :		
IL-1	+	-
IL-2	+	--
IL-6	+++	+++
TNF	-	---
IFN γ	++	---

¹ Relative increase +, decrease - or no effect 0.

approximately 4 fold less PGE₂ than LPS stimulated PBM cells and 10 fold less than LPS stimulated AP cells per cell.

DISCUSSION

CsA as an antiinflammatory drug

There is abundant evidence that CsA prevents graft rejection and expression of some autoimmune disorders by affecting the activation of T lymphocytes. Although the adjuvant-induced arthritis in rats and rheumatoid arthritis are not identical, they share some clinical and pathological features. Both diseases are examples of immunologically mediated chronic inflammation in which T lymphocytes (Yoshino et al 1990), particularly CD4⁺ cells (Sany 1990, Larsson et al 1985), play a central role in both establishing and sustaining chronic inflammation. The present study (results summarised in table 5.7) shows that appropriate CsA treatment can completely prevent the expression of this experimental arthritis in rats by suppressing some T lymphocyte activities. This anti-arthritic effect of CsA may be due to its ability to selectively inhibit the secretion of several cytokines.

The levels of CsA achieved in the blood of rats receiving 40 mg/kg CsA admixed with the arthritogenic adjuvant were similar (>100nM) to those reported in rheumatoid arthritis patients during continuous treatment (Madhok and Capell 1988). Similar levels (> 0.2-0.3 uM CsA) were also found to be required for about 60 days during the induction of tolerance to tissue allografts in animals (reviewed, Lim and White 1989). High levels of CsA were maintained following the single injection of CsA, probably because it was released slowly from the lipid phase of the adjuvant, and because the hepatic extraction of CsA from the blood is approximately 10 times higher in man than in rats (Kahan 1985, Wagner et al 1987). CsA injected subcutaneously every second day from days -1 to 7, or days -1 to 13, was as effective in preventing polyarthritis as CsA given in a single dose mixed in with the adjuvant. Both these therapies also prevented the cachexia

which usually accompanies the adjuvant administration (Rofe et al 1990), and had similar effects on lymph node enlargement and lymphocyte functions. This indicates that CsA, to be effective, need not be given in close juxtaposition to the arthritogen, and its action is therefore unlikely to involve interaction and modification of the mycobacterial antigen(s) (Cohen et al 1985) that may initiate the disease. It is thus more likely to be affecting the initial response to the arthritogen.

The effect of CsA therapy on lymphocyte proliferation

Cohen et al 1985 suggested that the pathogenesis of adjuvant arthritis primarily involves T cell recognition of mycobacterial epitopes that are cross-reactive with components of synovium. The suppressive effect of CsA on expression of the disease might therefore be due to inhibition of T cell activation by the processed mycobacterial antigen(s). However the enlargement of the regional lymph nodes draining the hind feet of rats with active disease was also seen in rats treated with CsA. Since the increase in cell recoveries was also associated with a marked increase in ^3H -thymidine uptake, it is likely that node cell proliferation, rather than lymphocyte recruitment, mainly contributed to the node enlargement in both CsA-treated and untreated rats. The failure of CsA to reduced lymph node cell proliferation during the stages of disease expression (Day 14) was surprising since previous reports have indicated CsA may inhibit certain events underlying lymphocyte proliferation (Hess et al 1982, Groenewegen et al 1985). It is clear that responses to antigens by these node cells were not entirely suppressed since node enlargement was always evident, even in the absence of clinical or histological arthritis, in the CsA-treated rats. This suggests that lymphocytes do responds to the arthritogen in CsA-treated animals, but without generating the type of response that induces arthritis.

CsA is reported to affect some T lymphocyte subpopulations more than others (Clerici and Shearer 1990, Klaus 1988). Therefore, it is of interest to compare the

lymphocyte subpopulations in the affected lymph nodes of arthritic rats and rats protected by CsA treatment. The results indicate small but significant changes in lymphocyte subsets. The proportion of T lymphocytes (CD43⁺, CD5⁺) and B lymphocytes (Ig⁺) were similar to that previously reported (Mason et al 1980).

Adjuvant treatment, although increasing cell yields dramatically, did not change the proportion of cells detected by the series of mAb except for a modest (8%) increase in the proportion of cells expressing MHC class II antigens. The proportion of these cells was further increased by CsA treatment. This contrasts with claims that CsA treatment reduces the expression of MHC class II determinants in dog epithelial cells (Groenewegen et al 1985).

The effect of CsA therapy on leucocyte subpopulations

CsA treatment almost doubled the proportion of cells expressing Ig, presumably B cells, while simultaneously decreasing the proportion of cells expressing the CD4 antigen by more than 10%. Macrophages, as well as T lymphocytes, can express the CD4 antigen (Barclay 1981), and may account for at least part of the reduction of CD4⁺ cells. The effective treatment of experimental arthritis by anti-CD4 mAb also indicates that this cell subpopulation is essential for expression of arthritis (Larsson et al 1985, Van den Broek et al 1992).

Immunohistology of the PLN was consistent with the findings using immunofluorescence carried out with the same mAb. While small differences (~10%) in the proportions of CD4⁺ cells and total T cells are not readily detectable using immunohistology, the large increase in the proportion of B cells following CsA treatment was noted. There was an increase in the numbers of B cell follicles and these extended into the paracortical region, possibly at the expense of some T cell populations normally present there. These B cells also expressed class II antigens and may have accounted for the increase in the proportion of cells detected with the OX6 mAb in the nodes of CsA treated animals.

The most striking effect of prior in vivo treatment with CsA was a reduction in the secretion of IL-2, TNF and IFN γ , in response to Con A, by lymph node cells harvested during the early phase of arthritis induction. This suggests that the node lymphocyte population in CsA-treated rats is enriched in T cells of the T helper (T_H) type 1 class (Mosman and Coffman 1989). Unfortunately, there is no complementary data relating to the production of those cytokines (IL-4, IL-5, IL-10) that characterise the CD4⁺ T cells (T_H2) that promote humoral immunity. However, in vitro studies with human PBM cells have shown CsA will inhibit IL-4 and IL-5 production (Andersson et al 1992). The secretion of IL-6 (a B cell growth factor) by lymph node cells harvested from CsA-treated rats, may explain the increase in B cells observed in the lymph nodes of these animals, suggesting that not all B cells responses are affected by CsA (Shindani et al 1983). These findings are also consistent with reports of enhanced B cell responses in CsA treated mice (Kunkl and Klaus 1980) and elevated IgG levels in CsA-immunosuppressed transplant recipients (White et al 1980).

The effect of CsA therapy on ex vivo cytokine production

The effects of CsA treatment on cytokine production by PLN and PBM cells and during the early phase of disease initiation (days 1 to 3) were consistent with a direct effect of CsA on these cells in vitro. The inhibition by CsA of IL-2, IFN γ and TNF α production by human peripheral blood mononuclear cells in vitro has also been observed (Andersson et al 1992). That report also indicated that TNF α and IL-6 production by LPS stimulated monocytes was unaffected by CsA in vitro, as found in the experiments described here using rat PBM and AP cells. Supplementing the previous findings of Anderson et al, the results presented here show that Con-A stimulated IL-6 production by PLN cells was relatively unaffected by CsA in vitro. Since these cells are mainly lymphocytes, and Con A predominantly stimulates this type of cell, it seems likely that the IL-6 is produced

by lymphocytes. This indicates that in contrast to IL-2, TNF and IFN γ production, IL-6 production by lymphocytes is relatively unaffected by CsA.

Of particular interest was the observation that the Con A stimulated production of IL-6 by PLN and PBM cells was relatively unaffected by CsA in vitro. Since these cell populations are mainly lymphocytes, and Con A is predominantly a stimulator of lymphocytes, it seems likely that IL-6 production by these node populations originates from lymphocytes and is unaffected by CsA. IL-6 may be one of the rare factors produced by lymphocytes which is not affected by CsA in contrast to other cytokines (IL-2, IL-4, IL-5 and TNF α) which are sensitive to CsA in vitro (Andersson et al 1992).

The in vitro effect of CsA on cytokine production

The inhibition of in vitro TNF production by CsA was variable. The method used for assaying TNF production will detect both TNF α (mainly produced by macrophages and monocytes) and TNF β (mainly produced by lymphocytes). Con A is primarily a stimulator of lymphocytes and therefore of TNF β production; whereas LPS, a stimulator of monocytes and macrophages, should preferentially stimulate TNF α production. Indeed the present study found that Con A-stimulated TNF production was much more sensitive to CsA than the LPS-stimulated TNF production. This probably indicates that CsA more effectively inhibits TNF β production by lymphocytes than TNF α production by monocytes and macrophages as has been reported in studies with human PBM cells in vitro (Andersson et al 1992)

While in vivo CsA treatment reduced the levels of IL-1, TNF and IL-6 produced by macrophages (table 5.5), in vitro treatment with CsA had little effect on the production of these cytokines by the same cells (table 5.6). This may indicate that CsA does not affect macrophage cytokine production directly but will inhibit their ability to produce cytokines indirectly by affecting other types of cell which

maintain or stimulate cytokine production by macrophages. Since IFN γ is a stimulator of many macrophage functions, including IL-1, TNF and IL-6 production (Hart et al 1989, Cheung et al 1990), inhibition of lymphocyte derived IFN γ by CsA (Reem et al 1983, table 5.6) may result in decreased responsiveness of macrophages *in vivo*. This indirect effect is consistent with the *in vivo* findings (table 5.5) which indicate that CsA treatment did not affect the production of IL-1 and IL-6 by macrophages (AP cells) early (day 3) but markedly reduced production later (days 7 and 14). The indirect inhibition of macrophage functions, particularly by reducing lymphocyte-derived IFN γ as observed on days 1 and 3, may be a major disease-preventing effect of CsA.

CsA and prostanoids

Prostanoids, such as PGE₂, can regulate cytokines in a similar way to CsA (ie. inhibiting IL-1, IL-2, TNF and IFN γ whilst stimulating IL-6 production) (see chapter 4). If CsA stimulates PGE₂ release (Esa et al 1988) then the regulation of cytokines and cells by CsA may involve PGE₂ as a mediator. However, the present data show that CsA can inhibit, rather than stimulate, PGE₂ release as others have reported (Churchill et al 1990). CsA was a strong inhibitor of PGE₂ production by Con A-stimulated PBM cells, but not LPS-stimulated PBM or AP cells, indicating that PGE₂ production by monocytes or macrophages is, like TNF, relatively unaffected by CsA. Prostaglandin-mimics, such as Misoprostol, are sometimes used in conjunction with CsA for organ transplantation to reduce undesired effects caused by CsA's inhibition of eicosanoid production. These prostaglandin-mimics may not only prevent some of CsA's side effects (eg kidney damage) but also enhance its regulation of immune functions (Iyengar et al 1991).

In conclusion, CsA prevents both the polyarthritis and the cachexia associated with the immune response against arthritogenic adjuvant in rats, but this is not due to complete suppression of the immune response to the initiating arthritogen(s). CsA may modify the activation of certain subsets of lymphocytes,

essential for development of polyarthritis, by suppressing the production of some T cell cytokines. The data suggest that the expression of polyarthritis may involve the preferential activation of the TH1 subset. These findings are consistent with observations that TH1 activation is associated with development of chronic inflammation such as experimental allergic encephalomyelitis in mice (Merril et al 1992) and systemic inflammation in humans (Brod et al 1991). Therapy directed against TH1 cells (eg with CsA), perhaps combined with a prostanoid agonist that regulates monokine and cytokine production and complement the action of CsA on lymphocytes (chapter 4, Betz and Fox 1991), may be particularly helpful in the management of rheumatoid arthritis.

Chapter 6

General conclusions and future directions

While each chapter contains its own detailed discussion, there are some overall general conclusions that can be drawn from the findings presented in this thesis.

Prostanoids as regulators of inflammatory mediators

Chapters 2,3 and 4 deal with NSAIDs and the role prostanoids (PG's) may play in the regulation of inflammatory mediators. The results indicate they may have a beneficial effect on chronic inflammation. PG's ability to regulate the production and action of important inflammatory cytokines, such as TNF and IL-1, indicate they may be important regulators of inflammation. The results also confirm the long held view that PG's can suppress the production and action of the lymphoproliferative cytokine IL-2. IL-2, produced by lymphocytes, is important for maintaining and expanding some lymphoid populations. T lymphocyte populations in particular depend upon IL-2 for proliferation, and these cells are considered central to the cell mediated immune responses seen in auto-immune diseases. The production of IFN γ , a lymphocyte derived activator of macrophages, is also suppressed. Taken together these results indicate that PG's may play an active role in the resolution and suppression of chronic inflammatory diseases.

However PG's are not, as originally thought, just suppressors of immune responses. They are also stimulators of some immune functions. This study shows that IL-6 production is greatly enhanced in several cell types. Others indicate that additional cytokines such as GM-CSF (Quill et al 1989) and IL-5 (Betz & Fox 1991) may be stimulated by PGE₂. A consistent pattern of response has emerged from the recent literature, indicating that PG's (and other agents that elevate cAMP) inhibit the production of cytokines which characterise the T helper (T_H) type 1

subset of lymphocytes. By contrast, cytokine production by T_H -2 cells seems relatively unaffected, or even enhanced. These effects, in particular stimulating IL-6 production, may enhance various humoral immune functions. This is consistent with the reported enhancement of IgG1 and IgE production by PGE (Phipps et al 1991).

Taken together, these findings indicate that PG's may 'switch' the immune system from T_H -1 and cell mediated responses that generate immune-mediated chronic inflammation to less inflammatory T_H -2 and humoral responses. PGE's may, therefore, be a natural mechanism for directing immune responses away from generating chronic inflammation towards healing and repair. The episodes of acute inflammation, dominated by PGE production, that commonly flare up during rheumatoid arthritis may represent failed attempts by the body to regulate the underlying chronic inflammation which sustains the disease.

Can PGE's be used for therapy?

Administration of PGE's systemically may not be effective because of their very short half life in the circulation. Even analogues, such as Misoprostol, which have a much longer half life in vivo, may not remain active for long enough to provide the necessary benefit at an inflammatory site. Low doses of PGE's co-administered with other drugs may, however, be effective (Whitehouse et al 1990a). Therapies which raise levels of PGE's at inflammatory sites may be more potent. One way to achieve this may be through dietary control through essential fatty acids derived from fish and plant oils. Increasing the quantities of substrates available for cyclooxygenase (see figure 1.1) may increase the levels of PGE's produced at an inflammatory site where PGE synthesis is enhanced (probably stimulated by cytokines like IL-1). The results of Chapter 4 indicate that precursors derived from fish or plant oils may form PGE's which are as effective as PGE₂.

While therapies which elevate PGE at an inflammatory site may reduce chronic inflammation, the additional levels of a PGE may stimulate aspects of acute inflammation (such as vasodilatation, pain and fever) and so its benefit may be questioned. Since the effect of PGE's is mediated by intracellular cAMP, other agents having similar effects may also be considered. While PGE's deleterious pro-inflammatory role in acute inflammation is well understood, its more helpful role as a suppressor of cytokines mediating chronic inflammation indicate it may be overall a potent beneficial agent in the treatment of chronic inflammation.

Similarities between the action of PGE's and Cyclosporin A (CsA)

Following *in vitro* treatment both CsA and PGE's seem to down-regulate the T_H-1 sub class of lymphocytes as well as stimulating IL-6 production. However, unlike PGE's, CsA's direct effects seem confined to lymphocytes. CsA did not affect the production of cytokines by monocytes/macrophages or the morphology of macrophages. CsA's affect on lymphocytes was not mediated by PGE since its production was inhibited by CsA in these cells. The effectiveness of CsA in preventing adjuvant arthritis indicates that the action of lymphocytes are essential for the induction of the disease. However, CsA was less effective in suppressing pre-established disease (Whitehouse et al 1990b), and this may indicate that, although T_H-1 lymphocytes are essential for establishing the disease, other cells, such as macrophages are important for maintaining the disease.

Taking into consideration the ability of both CsA and PGE's to suppress aspects of chronic inflammation, a combination of both CsA and PGE's might be worth considering for the treatment of arthritis. Misoprostol is currently used in combination with CsA to counteract the side effects of CsA treatment in transplantation recipients.

Other therapies directed against T lymphocytes

In recent years several new "CsA like" drugs have been developed which seem to be effective at preventing graft rejection. These drugs (Lobenzarit, FK506 and Rapamycin) may also be effective, like CsA, in the treatment of auto-immune mediated inflammation. Some of these new drugs may have fewer side effects than CsA, a major cause for withdrawing long term effective CsA treatment. Further investigation of these drugs as suppressants of immune-mediated chronic inflammation is warranted.

The therapeutic potential of monoclonal antibodies directed against T lymphocyte antigens has also been demonstrated (Chapter 5 discussion). Antibodies directed against T lymphocyte subsets, such as CD4 cells, or against activation antigens, such as IL-2 receptor, may suppress T cell functions in a similar way to CsA therapy. Most monoclonal antibodies are derived from rodents, and are recognised as foreign by the human immune system and can produce harmful side effects. Recombinant cDNA techniques are being used to "humanise" the monoclonal antibodies by replacing the Fc portion of the rodents monoclonal antibody with a human Fc fragment.

Conditional pharmacology

The concept that an antiinflammatory drug's effect is enhanced by inflammatory mechanisms has been proposed (Whitehouse and Vernon-Roberts 1991, Gadd et al 1992). The activity of aspirin and its metabolites, that may be preferentially formed at an inflammatory site, indicate that aspirin may be a pro-drug. These metabolites are shown to regulate the activity of some inflammatory cytokines (Chapter 2). Drugs which become active at the inflammatory site may have reduced side effects because activity is targeted to where the drug will give most benefit.

New molecular therapies

A number of newer approaches, still at the experimental level, utilise molecular biological techniques to produce active proteins. These proposed therapies often

involve antagonists of particular cytokines (eg soluble receptors or synthetic peptides which block receptors) and trials are underway with purified material. Monoclonal antibodies are also being developed which are directed against particular cytokines or block their binding to receptors. These specific molecules target a particular cytokine. Given the complex relationships of inflammatory cytokines the effects of blocking a single cytokine, may have unexpected consequences. Furthermore the overlapping activities of many inflammatory cytokines (see table 1.1) may mean that blocking the activity of one cytokine leads to another cytokine replacing its activity (Finkelman 1992).

A therapeutic strategy which regulates groups of cytokines may be more effective. Cytokines, such as IL-4 and IFN γ , may modify the overall direction of the inflammatory response in similar ways to that described for PGE's and "Cyclosporin-like" drugs. These natural proteins may have less side effects than exogenous drug therapies. In addition, "designer drugs", specifically made to block transcription or translation of particular genes and small molecules which interfere with the signal transduction process are being considered (Gibbons 1992). Therapies which mimic or regulate intracellular messengers (such as cAMP and cGMP investigated in this thesis) may also have profound effects upon the activities of inflammatory cells and the course of inflammation in general.

This thesis aimed to explore how inflammation may be influenced by the regulation of inflammatory mediators. While it has explored only a small portion of this broad and expanding field the findings have presented important insights into the actions of endogenous and exogenous regulators of inflammatory mediators. By better understanding the processes of chronic inflammation and defining the mode of action of effective therapy this type of research may help identify new approaches to treat chronic inflammatory diseases.

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B/code
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Erratum

Page No/ Fig/ Table	Para/ line	Error	Correction
6	6	fetomolar	femtomolar
13	15	prostanoids	prostaglandins
13	21	arachidonate	arachidonate
13	22	hydroxyeicosatetraenoic	hydroxyeicosa-tetraenoic
15	2	Alison	Allison
18	15	reference to Azapropazone	delete
Fig 1.1		Feldine	Feldene
		eicosatreinoic	eicosantrienoic
Fig 2.4	4	"with"	delete
Table 2.1		dicloro	dichloro
		PlasmaConc	Average Plasma Conc
		BW755c source ?	Burroughs Wellcome UK.
23	6	dicloro	dichloro
23,24		effective	potent
24	1	compared to	compared with
35		2nd last sentence	remove full stop
Fig.3.7		2000x	3000x
44/113		Stopen	Stopen
45	3rd para	Fc receptors (Becker..) Ia antigens (Fertsch...)	MHC receptors (Becker..) Fc receptors (Fertsch...)
61	29	Zang	Zhang
63/103		Vilcel	Vilcek
103		protien	protein
64	23	reference date missing	add 1985
72	18	lager	larger
76	25	responds	respond
99	7	myelopoisis	myelopoiesis
	5	morphogenesis	morphogenesis
105	8	Ceraml	Cerami
108	18	phorbal	phorbol
109	30	ref Cuturi et al	move to after Cohen et al
110	15	Eliment	Element
114	30	tranasformed	transformed
117	1	Yaskawa	Yasukawa