THE EFFECT OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS ON ~~INTERLEUKIN-1~~ IL-1 PRODUCTION ~~FROM MURINE PERITONEAL MACROPHAGES~~

A Thesis submitted by

ASHWANI KUMAR BAHL

for the degree of Doctor of Philosophy

in the university of London

1992

Department of Pharmacology

University College London

Gower Street

London WC1E 6BT

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ABSTRACT

The effect of non-steroidal anti-inflammatory drugs(NSAIDs) has been investigated on interleukin-1 (IL-1) production from resident murine macrophages . By the use of a more sensitive and selective bioassay than has previously been used, it was found that a variety of NSAIDs potentiated the secretion of IL-1 from lipopolysaccharide (LPS)-stimulated macrophages. However, high concentrations of NSAID were required (10^{-5}M or greater) and this effect could be dissociated from cyclo-oxygenase inhibition. The effect of NSAIDs on cell-associated IL-1 was also investigated. NSAIDs dose-dependently increased the accumulation of IL-1 by LPS-stimulated macrophages. This effect was found to parallel cyclo-oxygenase inhibition and could dose-dependently be reversed by prostaglandin E_2 . Two selective 5-lipoxygenase inhibitors were without effect on IL-1 production from LPS-stimulated macrophages in the presence or absence of a NSAID.

The ability of NSAIDs to affect IL-1 production by a range of receptor and post-receptor stimuli was tested. Contrary to published reports it was found that: 1). certain neuropeptides were unable to stimulate IL-1 production 2). tumour necrosis factor (TNF) and calcium ionophores only stimulated cell-associated IL-1 3). a phorbol ester was unable to stimulate IL-1 production. NSAIDs were unable to affect IL-1 production from TNF- or ionophore- stimulated macrophages, thus suggesting a mechanism specific to LPS was being affected. The ability of indomethacin to inhibit diglyceride metabolism was investigated as a possible mechanism contributing to enhanced IL-1 production. Indomethacin, unlike R59022 (a diacylglycerol kinase inhibitor) was without affect on dioctanoyl 1,2 (sn) glycerol-stimulated IL-1 production however, at 10^{-5}M both drugs potentiated IL-1 activation of EL4 NOB1s (a T cell line). This study supports a rôle for PGE_2 as a downregulator of IL-1 production and shows that NSAIDs can potentiate IL-1 production and action by mechanisms unrelated to cyclo-oxygenase inhibition.

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I would like to dedicate this thesis to Mum and Dad for their love and support which have served as the pillars of my life.

ACKNOWLEDGEMENTS

Many thanks to Dr.John Foreman for his patience and guidance during the production of this thesis and for the years he put up with me working next-door. To Dr.Maureen Dale for laying the "seeds" for this project and her enthusiastic interest. Thanks also to Nick Hayes for his proof-reading of this thesis and his continued friendship.

I have been privileged throughout my years at UCL to meet people who have given me inspiration not only through their friendship but also their infectious knowledge. Thus, to Breda Twomey, Stuart Hirst, Bob Muid , Marie Duncan, Adrienne Penfield, Nick Tilford, Richard Burt and Mark Duffy , to mention but a few, many thanks. Special thanks must go to Jude Oben who started as my mentor in the lab and has continued as a valued friend. Lastly thanks to Dr. Benny Chain for his endless supply of infection-free cell-lines and advice.

I am indebted to Viven Grant not only for helping type this thesis, but also for the hours of her time which provided humour, information, counselling and the cultivation of my scottish accent. To Alice Grant for her help in emergency typing. Although abstract from the university, I am also deeply indebted to the Mussetti family for providing me with comfort, a home, and food in my times of need.

This project was financed by a grant from the Emyily le Rossignol Fund and I am grateful to the MRC for their studentship award.

PUBLICATIONS

The following work has been submitted for publication during the course of this thesis:

The effect of non-steroidal anti-inflammatory drugs on IL-1 secretion from murine macrophages. (1989)
Br.J.Pharmac.,**98**:670P

The effect of prostaglandin E_2 and non-steroidal anti-inflammatory drugs on cell-associated interleukin-1
(1990) *Advances in Prostaglandin,Thromboxane and Leukotriene Research* 21:513-515

ABBREVIATIONS USED

AG	Antigen
AB	Antibody
AA	Arachidonic Acid
ASP	Aspirin
cGRP	Calcitonin gene related peptide
cAMP	adenosine 3′, 5′-cyclic monophosphate
CTLL	Cytotoxic T-cell line
DAG	Diacylglycerol
DMSO	Dimethylsulphoxide
dic_8	1,2 -dioctanoyl-sn-glycerol
DNA	Deoxyribonucleic acid
DK	Diacylglycerol kinase
DL	Diacylglycerol lipase
GM-CSF	Granulocyte Macrophage - colony stimulating factor
HEPES	4-(2-hydroxethyl)-1-piperazineethanesulfonic acid
HIPETE	Hydroperoxyeicosatetraenoic acid
HETE	Hydroxyeicosatranoic acid
HIFCS	Heat inactivated foetal calf serum
IL-(n)	Interleukin -1/2/3 etc.....
IND	Indomethacin
IFN	Interferon
KDO	3-deoxy-D-manno-2-octulosonic acid
LAF	Lymphocyte Activating Factor
5-LO	5-Lipoxygenase
LTs	Leukotrienes
LPS	Lipopolysaccharide

2-ME 2-mercaptoethanol

MTT 3-[4,5-dimethylthiazol-2-??]-2,5-diphenyl tetrazolium bromide

mRNA Messenger ribonucleic acid

NSAID Nonsteroidal anti-inflammatory drug

NK Neurokinin

NaM Sodium meclofenamate

OAG 1-oleoyl, 2-acetylglycerol

OA Osteoarthritis

PG Prostaglandin

PBS Phosphate buffered saline

PKC Protein Kinase C

PLA_2 Phospholipase A_2

PDBU Phorbol dibutyrate

PIR Piroxicam

PMN Polymorphonuclear leucocyte

PAF Platelet activating factor

PEC Peritoneal exudate cells

PLC Phospholipase C

PHA Phytohaemaglutinin

RA Rheumatoid arthritis

rh recombinant human

RPMI Roswell Park Memorial Institute

RIA Radio Immuno assay

SDS Sodium Dodecyl Sulphate

SP Substance P

TNF Tumour necrosis factor

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CHAPTER ONE
INTRODUCTION

1.1 Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) *eg.* aspirin, indomethacin *etc.* are widely used to treat the symptoms of inflammation that occur in a variety of musculo-skeletal disorders and injuries. As a class of drugs the NSAIDs are among the most widely prescribed agents in clinical practice. This is reflected in the sales figures for the top twelve selling drugs in the UK in 1988.

Table 1.1 Sales of pharmaceuticals (* is an NSAID)

Leading "ethical" pharmaceuticals			
PRODUCT	1988 SALES(£m)	MAIN THERAPEUTIC USE	COUNTRY DISCOVERED
Zantac	1,250	Peptic ulcer	UK
Adalat/Procardia	750	Angina	Germany
Capoten	680	Hypertension	USA
Tagamet	600	Peptic ulcer	UK
Kefral/Ceclor	570	Antibiotic	USA
Tenormin	560	Hypertension	UK
Renitic	550	Hypertension	USA
Cardizem	450	Angina	Japan
Voltaten*	440	Arthritis	Switzerland
Naprosyn*	430	Arthritis	USA
Feldene*	350	Arthritis	USA

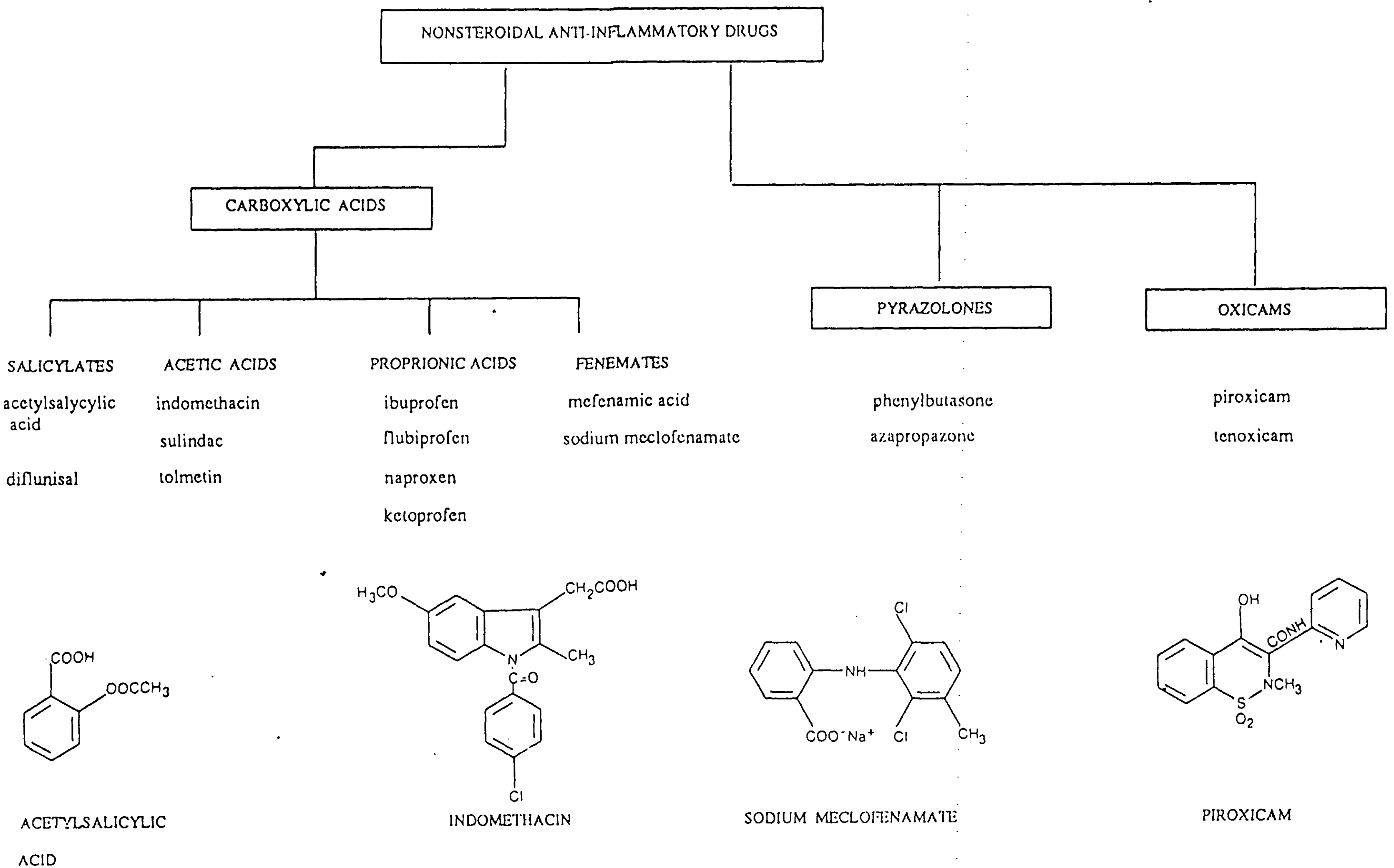


Figure 1.1 The chemistry of the non-steroidal anti-inflammatory drugs

In worldwide sales, three NSAIDs rank amongst the top ten leading products (Caven 1989). Looking through the British National Formulary (BNF) for 1990 some 24 different NSAIDs are available for prescription. The chemistry of the NSAIDs is summarised in fig.1.1

The NSAIDs are by no means a new class of anti-inflammatory drug. The events that have lead to the development of the "modern" NSAIDs are summarised by a list of historical points of interest :

- Hippocrates used certain plant extracts now known to contain NSAID-like compounds, to treat febrile illnesses.

- Dodens makes a reference to the analgesic properties of willow bark *(contains the natural glycoside salicin from which salicylic acid can be extracted)*. " The leaves and rind of Withy boiled in wine, do appease the paine of the sinuwes, and do restore againe their strength, if they be nourished with the fermentation of natural heat thereof " (from Ronald & Benedeck, 1970).

- In 1763 The Reverend Mr. Stone writes to the Royal Society commending the use of white willow bark to treat fever and joint pains.

- In 1829 H. Leroux, a French pharmacist isolates salicin in a pure state.

- In 1874 two German chemists, H. Kolbe and F. Lautemann devise a practical method for the commercial production of salicylic acid.

- In 1877 Germain Seé announces that salicylates relieve chronic rheumatoid arthritis.

- Felix Hoffmann, a chemist at Bayer, finds an improved method for the synthesis of acetylsalicylic acid *(Hoffmanns father had rheumatoid arthritis and was intolerant of available salicylates*). Hoffmanns director, Dreser, coins the term ASPIRIN® in 1898 (A for acetyl, SPIR from *Spirea ulmaria* the flowers of which were used to extract salicylic acid, IN suffix).

- In 1949 the advent of cortisone demonstrates that corticosteroids have anti-inflammatory properties and the term NSAID is to be coined 3 years later.

- In 1963 indomethacin is formulated.

Inter-twined with the history of NSAIDs are the mechanisms that have been proposed for their anti-inflammatory action. **Table 1.2** summarises the main explanations that have been put forward. Arguments for rejecting many of the theories that have been proposed to explain the anti-inflammatory mechanisms of NSAIDs have been addressed elsewhere (Ferriera & Vane (1974)).

It was Vane (1971) who proposed the now widely accepted mechanism that accounts for the anti-inflammatory, analgesic and antipyretic action of NSAIDs. Vane discovered that NSAIDs selectively inhibit the synthesis of prostaglandins and subsequently showed that all NSAIDs inhibit the enzyme cyclo-oxygenase and that their anti-inflammatory activity *in vitro* parallels their ability to inhibit this enzyme.

Cyclo-oxygenase catalyses the addition of molecular oxygen to arachidonic acid and cyclizes it to form the cyclic endoperoxide PGG_2 which can be converted by other enzymes either to the " classical " prostaglandins (PGE_2, PGF_2, PGD_2 etc.) or to prostacyclin (PGI_2) or to thromboxane A_2 (TXA_2). The molecular structure of cyclo-oxygenase still remains to be investigated by x-ray crystallography therefore, all of the

currently available information regarding the molecular nature of the active site of the enzyme is based on structure-activity relationships with various inhibitors of the enzyme (Appelton & Brown 1979). The mechanisms by which NSAIDs inhibit cyclo-oxygenase fall into three broad categories:

(i) reversible competitive inhibition at the substrate site of the enzyme.

(ii) Irreversible competitive inhibition of the enzyme by covalent interaction.

(iii) Reversible non-competitive inhibition by scavenging fatty acid peroxides responsible for prostaglandin synthesis.

Ibuprofen inhibits prostaglandin synthesis by competitive inhibition at the substrate site. Aspirin and indomethacin are irreversible competitive inhibitors. Aspirin forms a covalent acetylated derivative with the enzyme (Roth & Majeus, 1975), indomethacin covalently attaches to the active site via its carboxylic and halogen moieties (Lands, 1985). Mefenamic acid acts as a competitive inhibitor and also as a non-competitive scavenger of the peroxide substrates required for prostaglandin synthesis. Sodium meclofenamate possesses all of the three inhibitory mechanisms on cyclo-oxygenase mentioned above. Thus, although NSAIDs are all inhibitors of the enzyme cyclo-oxygenase, they do not act via a single mechanism. Other subsequent explanations have been put forward, in addition to the inhibition of prostaglandin synthesis, which extend the proposed anti-inflammatory mechanisms of NSAIDs. These include:

1. **Inhibition of the 5-lipoxygenase pathway:**
Products of the lipoxygenase pathway include the hydroperoxyeicosatetranoic acids (HPETEs), hydroxyeicosatetraenoic acids (HETEs), lipoxins and

leukotrienes. The enzyme 5-lipoxygenase is composed of a single 75-80 KDa polypeptide chain that is anionic at neutral pH, and is responsible for the synthesis of leukotrienes. The leukotrienes exert a number of pro-inflammatory effects (see Samuelson et al., 1987 and Salmon & Higgs, 1987, for reviews). Briefly, the leukotrienes are potent bronchoconstrictors, increase permeability in post-capillary venules and stimulate mucus secretion. The dihydroxy derivative, LTB_4 causes adhesion and chemotactic movement of leucocytes and stimulates aggregation, enzyme release and generation of superoxide in neutrophils. There have been conflicting reports regarding the ability of NSAIDs to affect the 5-lipoxygenase pathway. Hamberg and Samuelson (1974) reported that aspirin and indomethacin were unable to inhibit platelet lipoxygenase. Higgs et al. (1982) reported that low doses of indomethacin (0.1 to 2 $mgKg^{-1}$) caused an enhancement of leukocyte accumulation in rabbit skin, whereas high-doses (5 to 50 $mgKg^{-1}$) caused dose-dependent inhibition of leukocyte migration. The authors proposed that the enhancement of leukocyte accumulation at low doses could be explained by the inhibition of cyclo-oxygenase and the diversion of arachidonic acid substrate towards the production of chemotactic lipoxygenase products, while the observed inhibition of leukocyte migration at high indomethacin doses may be due to the dual inhibition of lipoxygenase and cyclo-oxygenase. Siegel et al. (1980) reported that a variety of NSAIDs including aspirin inhibited the formation of lipoxygenase products as well as prostaglandins in rat neutrophils. Paajanen et al (1982) showed that aspirin inhibited both lipoxygenase and cyclo-oxygenase pathways in isolated hamster lungs. *In vitro* studies have shown that benoxaprofen causes a marked inhibition of leukotriene synthesis whilst also having a mild inhibitory effect on prostaglandin synthesis (Dawson et al., 1982). Therefore, the efficacy of some NSAIDs may well be dependent upon their ability to inhibit the 5-lipoxygenase and cyclo-oxygenase pathways.

2. **Antagonism of prostaglandin receptors:**
The fenamates such as sodium meclofenamate and mefenamic acid have been shown to inhibit myometrial PGE_2 receptors with an ED_{50} of 20μM and 200μM respectively (Rees et al.,1989). This may provide an explanation for the effectiveness of the fenemates in the treatment of primary dysmenorrhoea and menorrhagia compared with other NSAIDs.

3. **Inhibition of free-radical production:**
Twomey (1990) has studied the effect of NSAIDs on the receptor and post-receptor-mediated respiratory burst in human neutrophils. The study revealed that NSAIDs, could be divided into three categories:

(a) those that increased superoxide production e.g. benoxaprofen, indomethacin, sodium meclofenamate and mefenamic acid.

b) those that have no effect on superoxide production e.g. diclofenac, ketoprofen, sulinidac, ibuprofen, naproxen and aspirin.

c) those that decrease superoxide production e.g. phenylbutazone and piroxicam.

The mechanism by which certain NSAIDs inhibited the respiratory burst remained unresolved by Twomey. The ability of the NSAIDs to potentiate the respiratory burst will be dealt with later in this introduction.

4) **Inhibition of cytokine production:**
Schenkellars et al., (1990) observed that diclofenac 0.5mg Kg-1 day-1, administered to rats in which adjuvant arthritis had been induced, inhibited the early increase in IL-1 production from LPS-stimulated peritoneal and blood macrophages isolated from such animals. However, the above is an isolated report amongst a number of increasing reports suggesting that NSAIDs can potentiate cytokine production.

5) **Inhibition of phospholipases:**

In general, the inhibition of PLA_2 by the NSAIDs indomethacin and diclofenac is less sensitive than cyclo-oxygenase inhibition, requiring concentrations of >10μM to produce significant inhibition. Indomethacin, at micromolar concentrations ranges has been shown to inhibit phospholipase C (PLC) in human neutrophils (Shakir et al., 1989) and aspirin has been shown to induce a PLC inhibitory protein in human monocytes (Bomalaski et al., 1986). Thus, it is possible that certain NSAIDs may prevent the liberation of AA from lipid pools in plasma membranes. Indeed, O'Niel & Lewis (1989) have shown that indomethacin and diclofenac at micromolar concentrations inhibited radiolabelled AA release from IL-1α-stimulated synovial cells. Concomitant with this inhibition was the increased radioactivity associated with phosphatidylethanolamine (PE) and triglycerides (TG) in the plasma membrane. The authors suggest that the NSAIDs may increase the acylation of AA into PE and TG. Thus, by inhibiting various phospholipases and altering the cycling of lipids within the plasma membrane NSAIDs may inhibit the generation of various lipid-based inflammatory mediators.

6. **Impairment of transduction mechanisms:**

NSAIDs have been shown to inhibit the pertussis toxin-dependent adenosine phosphate-ribosylation of the G protein in neutrophils (Abramson et al., 1988). The authors propose that NSAIDs, by inhibiting the transduction via such G proteins, could affect the activation of such cells by a variety of stimuli that employ such pathways.

Table 1.2 Putatative mechanisms of NSAID action

MECHANISM OF ACTION	REFERENCE
Uncoupling of oxidative phosphorylation .	Adams & Cobb (1958).
Inhibition of generation / action of kinins.	Collier (1969).
Displacement of an endogenous anti-inflammatory peptide from plasma proteins	Aylward & Maddok (1974). McArthur *et al* (1971). Smith *et al* (1971).
Inhibition of lysosomal enzyme release.	Arrigioni-Martelli & Restelli (1972)
Hyper-polarization of neuronal membranes.	Levitan & Barker (1972).
Inhibition of Complement activation.	Harrity & Goldust (1974).

The discovery that the NSAIDs, by inhibiting prostaglandin synthesis, exert a range of anti-inflammatory activities *in vitro* and *in vivo,* has provided strong evidence for the role of prostaglandins as pro-inflammatory mediators. Indeed the role of prostaglandins belonging to the E series, in particular PGE_2, has been well established in oedema, fever, increased vascular permeability and sensitization of pain receptors. However, research has also shown that prostaglandins, in particular PGE_2, can exert profound anti-inflammatory effects *in vitro* and *in vivo*.

PGE_2 is synthesized by a number of different cell-types and tissues. Macrophages/monocytes are particularly efficient secretors of PGE_2 in response to a variety of inflammatory stimuli. The PGE_2 produced by macrophages and other cells at the site of inflammation can, by means of local interactions, regulate the immunological response of the macrophage itself and also of other cell-types involved in the inflammatory process. Table 1.3 summarises the anti-inflammatory effects that have been attributed to PGE_2. The inhibitory effects of PGE_2 summarised in table 1.3 are thought to be mainly mediated by elevation in intracellular adenosine 3', 5'-cyclic monophosphate (cAMP) caused by the interaction of PGE_2 with its cell-surface receptor.

In addition to the inhibitory effects of PGE_2 on isolated and mixed cell populations outlined above, Zurier & Quagliata (1971) have shown that administration of PGE_2 can ameliorate experimental adjuvant-induced arthritis and have shown that PGE_2 can inhibit immune-complex-induced nephritis *in vivo*. This data raises the question: can the inhibition of prostaglandin synthesis lead to a potentiation of an inflammatory reaction ?

Table 1.3 Immunomodulatory effects of PGE_2

CELL TYPE	ANTI-INFLAMMATORY ACTION	REFERENCE
T-CELLS	● inhibition of T cell function	Morley (1974)
	● inhibition of IL-2 production.	Lewis & Barret (1986)
	● down regulation of high affinity IL-2 receptors.	Chouaib (1987)
	● down regulation of transferrin receptors.	Neckers & Cossman (1983)
	● generation of a suppressor peptide from glass-adherent lymphocytes.	Goodwin & Webb (1980)
	● induction of suppressor T-cell populations and enhanced suppressor activity.	Chouabi (1984)
B-CELLS	● inhibition of proliferation ● inhibition of antibody production in mixed lymphocyte culture	(Staite & Panayi 1982)
MØ	Inhibition of:	
	● IL-1	Kunkel 1986a
	● TNF	Kunkel 1986b
	● IL-6	Callery 1990
	● GM-CSF	Garnett 1982
	● MHC II expression	Tripp 1986
NK/LAK CELLS	● inhibition of LAK cell differentiation	Becker 1987
	● suppression of NK activity	Henny 1972

1.2 PRO-INFLAMMATORY EFFECTS OF NSAIDs

In contrast to the above account, a number of studies have shown that NSAIDs may possess pro-inflammatory effects both *in vitro* and *in vivo*. Prostaglandin-dependent and-independent mechanisms have been proposed for the pro-inflammatory activities of NSAIDs. Summarized below are some of the observed pro-inflammatory activities of NSAIDs.

1.2.1 *IN VITRO*

- Lewis & Barret (1986) showed that indomethacin potentiated the proliferation of T-lymphocytes sub-optimally stimulated with phytohaemaglutinin (PHA). The effect was shown to be the result of enhanced production of IL-2 or IL-2-like activity from PHA-activated blood mononuclear cells. The authors also showed that PGE_2 added exogenously could inhibit the indomethacin-potentiated proliferation of T-lymphocytes.

- Sandborg et al (1986), Oppenheim et al (1980), Knusden et al (1986), Kunkel et al (1986a) and Brandwein (1986) have all reported that IL-1 production is enhanced by NSAIDs and decreased by PGE_2. Kunkel et al (1986b) have also shown that NSAIDs can potentiate tumour necrosis factor (TNF) production from macrophages. The effect of NSAIDs on cytokine production will be dealt with in greater depth in chapter 4.

- Raud et al (1987) have shown that indomethacin potentiates plasma extravasation from ovalbumin-challenged hamster cheek pouch. Exogenous PGE_2 completely reversed the indomethacin-induced potentiation of plasma leakage.

- Dale & Penfield (1987) have shown that indomethacin can dose-dependently potentiate the production of superoxide from human neutrophils *in vitro*. The ability of indomethacin to potentiate superoxide production appeared to be unrelated to its ability to inhibit PGE_2 synthesis. Dale & Penfield proposed that indomethacin may be working by inhibiting the breakdown of diacyl-glycerol (DAG), an endogenous activator of protein kinase-C (PKC). Their proposal was based on comparative studies with various inhibitors of DAG metabolism.

1.2.2 *IN VIVO*

- Newman & Ling (1985) observed a correlation between NSAID consumption and acetabular bone destruction in primary osteoarthritis (OA) of the hip. Rashad et al (1989) compared the effects of indomethacin and azapropazone (a weak cyclo-oxygenase inhibitor) on the progression of OA of the hip using the need for arthroplasty as the end-point of the destruction process in OA. Their findings suggested that OA of the hip progressed more rapidly in patients treated with a strong inhibitor of prostaglandin synthesis than in those treated with a weak inhibitor. Rashad et al proposed the importance of blood perfusion in maintaining the repair of joint structures of osteoarthritic joints. Thus by inhibiting the production of vasodilatory prostaglandins, the progression of OA would be accelerated.

Kalbhen (1982) observed that single or repeated intra-articular injection of NSAIDs (sodium salicylate, indomethacin and ibuprofen) into the knee joints of hens and rats induced progressive joint degeneration similar to OA. Maier & Wilhelmi (1983) using a mouse strain that develops OA spontaneously, observed differences between NSAIDs in their effect on the progression of OA. Progression of OA was found to increase when the animals were fed naproxen, ibuprofen, aspirin and indomethacin but not when fed piriprofen, piroxicam or diclofenac. Dingle &

Shield (1990) have reported that NSAIDs can potentiate cartilage breakdown caused by intra-articular injections of IL-1 and that the effect of NSAIDs is inhibited by the intra-articular administration of the stable prostaglandin E_1 analogue misoprostol®. Pettipher et al (1989) using an antigen-induced model of rheumatoid arthritis (RA) in the rabbit showed that intra-articular injections of indomethacin increased the loss of proteoglycan from articular cartilage and increased the accumulation of lymphocytes in the inflamed synovial lining. The authors put forward the proposal that indomethacin, by inhibiting PGE_2 synthesis within the joint, leads directly to greater local production of cytokines such as IL-1 which are known to mediate bone destruction in RA (to be discussed in detail later in the chapter). Alternatively, the inhibition of PGE_2 synthesis may contribute to the increase in lymphocyte numbers since PGE_2 inhibits lymphocyte proliferation. Thus, more lymphocytes are available to interact with an antigen and antigen-presenting cells, thereby resulting in an indirect increase in cytokine production.

- The NSAIDs are known to cause gastric ulceration, but the mechanism by which this occurs is still not understood fully. The gastrointestinal irritant effects of the NSAIDs may lead to life-threatening gastric complications in cases of chronic consumption by arthritic patients (Drugs Therapeutic Bulletin 1987). Ligumsky et al (1983) have demonstrated that gastric prostaglandin synthesis in rats could be inhibited by up to 95% without development of haemorrhagic erosions and suggest that inhibition of prostaglandin synthesis is unlikely to be the sole mechanism responsible for gastrointestinal ulceration. Indeed, a recent study by Wallace et al (1990a) in which rats were made neutropenic by prior treatment with a specific anti-neutrophil antibody, it was shown that the rats became significantly more resistant to the gastric-damaging

actions of indomethacin and naproxen than were control rats. The authors proposed that NSAIDs were contributing to the activation of neutrophils and thus predisposing the animals to gastric ulceration.

1.3 INTERLEUKIN-1 AN OVERVIEW

1.3.1 Interleukin-1 a cytokine

Cytokine is a term encompassing several polypeptide products of diverse cellular origin including interleukins, lymphokines and monokines. Cytokines participate in a variety of cellular responses, including the regulation of the immune system and the pathophysiology of a range of diseases. They are released in response to a number of stimuli, but in contrast to the chemical composition of antibodies produced by immune cells, their chemical composition is not dependent upon the stimulating agent. Once released, cytokines can act on other cells in the immediate vicinity (a paracrine effect) or on the same cell that produces them (autocrine effect). Cytokines that are produced in large amounts, and gain access to the circulation, can act in a hormonal fashion and have profound systemic effects (endocrine effect). Cytokines are extremely potent molecules generally acting at picomolar concentrations, with high affinity receptors which are expressed in relatively low numbers (10-10000 per cell). The response of a cell to a given cytokine is dependent on the local concentration of the cytokine and of other cell regulators to which it is concomitantly exposed.

Several cytokines have been named according to their biological effect. However, since most cytokines possess more than one biological activity, descriptive names can be misleading. Therefore, a nomenclature has been proposed that employs the term " interleukin " followed by a number. At the Sixth International Congress of Immunology in 1986, it was agreed that a new cytokine would first be named according to its biological properties, but once the amino acid sequence of the human form was elucidated then an interleukin number would

be assigned. The term interleukin is a misnomer for some interleukins such as IL-1, IL-2, and IL-6, since these molecules are not merely inter-leucocyte-kinins but are released by and affect a number of cell-types.

1.3.2 History of interleukin-1

IL-1 has been independently discovered by a number of researchers as a proteinaceous mediator possessing inflammatory effects. Because of its wide ranging biological actions, a host of names had been assigned to IL-1 before the interleukin nomenclature became widely accepted. Table 1.4 summarises the biological activities which have led to the various synonyms for IL-1.

Table 1.4 Biological activities leading to synonyms for IL-1

BIOLOGICAL ACTIVITY / EVENT	SYNONYM	REFERENCE
Peritoneal cells release substance producing fever	Granulocyte pyrogen	Beeson (1948)
Injection of endotoxin into rabbits causes production of circulating protein able to produce fever	Endogenous pyrogen (EP)	Atkins et al (1967)
Material from leucocytes mediates acute phase response	Leucocyte endogenous mediator (LEM)	Kampschmidt et al (1972)
Macrophages produce factor which stimulates thymocyte proliferation	Lymphocyte activating factor (LAF)	Gery et al (1972)
Factor from PBMC stimulates adherent synovial cells to release PGE_2 and collagenase	Mononuclear cell factor (MCF)	Dayer et al (1976)
International Lymphokine Workshop	Interleukin-1 (IL-1)	(1979)
Stimulation of cartilage matrix breakdown	Catabolin	Saklatvala & Dingle (1980)
Stimulation of plasminogen activator and PGE_2 synthesis from chondrocytes by factor from human synovial lining cultures	synovial factor (SF)	Meats et al (1980)

The above table was adapted from "The Synovial Lining" by Henderson & Edwards (1987).

1.3.3 The structure of interleukin-1

Complementary DNA cloning, protein purification and sequencing studies have lead to the isolation of two types of IL-1 polypeptide: IL-1α and IL-1ß. IL-1ß was cloned from human blood monocytes (Auron et al 1984) and IL-1α was cloned from the mouse macrophage line, P388D (Lomedico et al 1984). To date, the cloning of both IL-1 molecules has been reported in 5 species: man, mouse, rat, rabbit and cow. Between various species the primary amino acid sequences of IL-1ß is conserved in the range of 75-78%, whereas with IL-1α the sequence is conserved in the range of 60-70%. IL-1α and IL-1ß molecules within a species share only 22-26% amino acid homology and approximately 45% nucleotide homology (Dinarello 1989). Both IL-1α and IL-1ß are synthesized as propeptides of molecular weight approximately 31kDa, which are subsequently cleaved at the carboxy-terminal portions to yield the mature IL-1 forms. In humans, the mature IL-1α molecule consists of 159 amino acids and has a molecular weight (Mw) of 17.5kDa and an approximate pI of 5, whereas the IL-1ß molecule has 153 amino acids and a Mw of 17.5kDa with a pI of 7. Although the specific amino acid sequence constituting the IL-1 molecule varies between species, the approximate Mws and pIs remain the same. Indeed, the biological activities of the IL-1 molecules are not dependent on the species of animal nor are they IL-1α- or ß- restricted. Potency differences have been observed between IL-1α and IL-1ß for various biological actions. For example, recombinant murine IL-1ß was found to be 250-1250 fold less potent than recombinant human IL-1ß at inducing endothelial cell adherence of human monocytes (Thieme et al 1987). However, Bird et al (1987) have shown that the concentration of porcine IL-1ß required to elicit half maximal IL-2 production from EL4 NOB1 cells (see chapter 2) was 100 fold greater than for porcine IL-1α. Human IL-1ß has been crystallized (Priestle et al 1989) and the crystal structure has revealed 12ß strands forming a complex of hydrogen bonds resulting in a distorted tetrahedral structure. The interior of the IL-1ß molecule is strongly hydrophobic with no charged

amino acids. Computerised molecular modelling has revealed the likely structure of IL-1α to be similar to that of IL-1ß (Dinarello et al 1989).

1.4 INTERLEUKIN-1 SYNTHESIS AND SECRETION

1.4.1 How is Interleukin-1 synthesized?

Cells that synthesize IL-1 possess little or undetectable levels of mRNA encoding for IL-1α or IL-1ß production prior to stimulation (Burchett et al 1988, Schindler & Dinarello 1988, Warner et al 1987.). However, upon appropriate stimulation such cells rapidly transcribe mRNA for the IL-1 pro-peptides. Maximum levels of mRNA for IL-1 are reached 2-6 hr. after LPS-stimulation of human peripheral blood monocytes (Burchett et al 1988, Matsushima et al 1986, Hazuda et al 1988) and gradually decrease over 24 hrs. The decrease in mRNA accumulation is thought to be due to the induction of transcriptional repressors which suppress further transcription of mRNA and/or increase mRNA degradation (Shapiro et al 1987). Fenton et al., (1988), showed that cycloheximide treatment of LPS-stimulated macrophages (this would result in inhibition of protein synthesis) resulted in a 2-fold increase in IL-1ß mRNA levels compared with in untreated LPS-stimulated macrophages. This suggests that a protein is synthesized together with the mRNA which limits IL-1 accumulation. Studies that have investigated the production of IL-1 from cultured monocytes/macrophages have shown that cells cultured *in vitro* for more than 24hr. exhibit a 95% reduction in IL-1 secretion. Burchett et al (1988), have shown that the reduction in the secretory capacity for IL-1 in cultured monocytes/macrophages is the result of a decrease in the transcription of the mRNA for IL-1ß but not for IL-1α. The authors also showed that the stability, as measured by accumulation of mRNA, was markedly decreased for both IL-1α and IL-1ß mRNA. The ability of the mRNAs for IL-1 to be differentially regulated has also been highlighted by Warner et al (1987), who showed that recombinant IL-1α and IL-1ß

stimulate the rapid accumulation of IL-1ß mRNA in endothelial cells (EC) in a dose-dependent and time dependent manner. However, the mRNA for IL-1α was only observed when EC were concomitantly treated with cycloheximide, thus suggesting that the mRNA for IL-1α is under tighter repressor control. Table 1.5 illustrates the differences that have been observed in the expression of mRNAs for IL-1 in a variety of cell-types:

Table 1.5. Expression of IL-1 mRNA

CELL TYPE	mRNA EXPRESSED	IL-1 ACTIVITY
HUMAN MONOCYTES	ß>>α	ß>>α
HUMAN KERATINOCYTES	α>ß	α>ß
MURINE MACROPHAGES	α>ß	α>ß
BRAIN ASTROCYTES	ß	ß

Adapted from Koybayashi et al.,1989

1.4.2 Translation of interleukin-1 mRNAs

The induction of mRNA synthesis for IL-1 does not necessarily result in the synthesis of the IL-1 peptides. For instance adherence of macrophages/monocytes to plastic or glass surfaces, in the absence of LPS contamination, induces mRNA transcription for IL-1ß without inducing IL-1 peptide synthesis. Small amounts of LPS can rapidly induce translation of this mRNA to the pro-peptide (Schindler & Dinarello 1989). Since most commercially available media and sera contain LPS concentrations in the range of 10-150 $pg.ml^{-1}$, it is likely that most cultures of macrophages/monocytes translate the mRNA induced upon adherence. However, the pro-peptide may not necessarily be secreted.

That the mRNAs for IL-1 are translated into pro-peptide forms prior to secretion was first shown for IL-1α synthesized by

the P388D1 murine macrophage cell-line (Giri et al., 1985). Since Giri's observation, several groups have reported the existence of intracellular pro-peptide forms for both IL-1α and IL-1ß that have Mws in the range of 31-33 KDa (Matsushima et al 1986, Bayne et al 1986, Hazuda et al 1988, Koybayashi et al 1988). Mature peptide forms of IL-1 (17.5 KDa) are not formed intracellularly (Hazuda et al 1988). Antibody staining and RIA techniques coupled with electron microscopy have revealed that the IL-1ß pro-peptide is primarily located in the cytosol of macrophages/monocytes and is lacking from the endoplasmic reticulum, golgi and plasma membrane fractions (Singer et al 1988, Sission & Dinarello 1988). Studies on IL-1α have revealed a similar profile of intracellular localisation, with the notable exception of the pro-peptide form of IL-1α which has been detected in the plasma membrane fraction by some researchers (Beuscher et al 1987). Koybayashi et al.,(1988) and Beuscher et al.,(1988) have shown that the IL-1α pro-peptide exhibits a 10-fold greater phosphorylation than the IL-1ß pro-peptide. Koybayashi et al., (1988) have suggested that the greater phosphorylation of the IL-1α pro-peptide may facilitate its interaction with components of the plasma-membrane, and this may be important in facilitating its processing to the mature peptide form. Hazuda et al.(1988) have determined the half-life for the intracellular-residence of the IL-1 pro-peptides in LPS-stimulated monocytes. The IL-1α pro-peptide was found to reside in the cytoplasm for 10-15hr. compared to 3-4hr. for the IL-1ß pro-peptide. This difference in the intracellular half-lives reflects a difference the rate at which the two IL-1 forms are secreted and not the rates of intracellular degradation. Maximal rates of IL-1ß secretion occur 2hr. after LPS stimulation whilst the IL-1α secretion rate was maximal 13hr. after LPS stimulation. Both pro-peptides were shown to be rapidly translated from their respective mRNAs, thus the time delay in secretion reflects a lag between translation and secretion.

1.4.3 Processing and secretion of interleukin-1

IL-1α and IL-1ß are unusual peptides in that both lack a hydrophobic "signal" peptide component either at the N-terminus or internally, as would be expected for a peptide that is actively secreted by a cell. In this respect, IL-1 peptides are similar to ß fibroblast growth factor (FGF), platelet derived-endothelial cell growth factor (PD-ECGF), ADF a thyroid-like molecule released by leukaemic T cells, blood coagulation factor XIIIa and the yeast mating pheromone a-factor (Abraham et al 1986, Ishikawa et al 1989, Tagaya et al 1989, Grundmann et al 1986 and Kuchler et al 1989). The "signal" sequence allows the co-translational, vectorial transfer of the peptide across the membrane of the endoplasmic reticulum and it is often cleaved before completion of the transmembrane transport of the peptide (Milstein et al 1972). The lack of a signal sequence raises the possibility that IL-1α and IL-1ß are not actively secreted from cells but are released as a result of cell-damage. However, several lines of evidence exclude this possibility:

i. A difference in the kinetics of release has been observed for IL-1α and IL-1ß

ii. Viability studies performed in conjunction with IL-1 secretion studies have shown that secretion of IL-1 can occur without a compromise in cell-viability.

iii. Certain drugs can differentially regulate the secretion of IL-1α and IL-1ß.

In cultured macrophages and macrophage-like cell-lines, processing of IL-1 pro-peptides into their mature forms is not required for the secretion of IL-1 peptides (Hazuda et al 1988, Hazuda et al 1987, Mizel 1988, and Beusher et al 1990). Indeed the pro-peptides and the mature forms of IL-1ß are secreted with identical kinetics suggesting that the

secreted with identical kinetics suggesting that the two peptides may be secreted by the same mechanism (Hazuda et al 1988). As previously mentioned, IL-1 is not located in the endoplasmic reticulum or Golgi apparatus and does not possess a signal sequence. Therefore, how is IL-1 secreted ? Rubatelli et al (1990), have reported that IL-1ß and, to a lesser extent IL-1α, are localised in distinct vesicles within the cytoplasm of monocytes. The IL-1 in such vesicles was found to be resistant to protease digestion whereas non-vesicular IL-1 was degraded by proteases. The ability of the vesicles to protect IL-1 from protease digestion was eliminated by treatment with a detergent. Cells that translate IL-1ß but lack the ability to secrete it also lack the vesicular compartmentalisation of IL-1 in the cytosol. Furthermore, methylamine, a compound known to inhibit exocytosis (Maxfield et al.,1979), inhibited the secretion of IL-1ß from LPS-stimulated monocytes by 90%. The authors suggest that such vesicles, when exocytosed, lead to IL-1ß secretion. However, the study offered no explanation for the ability of IL-1 not found in such vesicles to be secreted, and neither did it provide direct evidence for the existence of such vesicles eg. by using electron microscopy techniques. A role for heat shock protein 70 (hsp 70) in the post-translational translocation of IL-1 into such cytoplasmic vesicles and directly to the plasma membrane has also been conjectured by Rubartelli et al.(1990).

The IL-1α pro-peptide is as biologically active as the mature IL-1α peptide (Mosley et al 1987) whereas the IL-1ß pro-peptide is biologically inactive and processing to the 17.5 KDa form is required for biological activity (Mosley et al 1987, Black et al 1988, Hazuda et al 1988 and Hazuda et al 1990). The location at which IL-1 is processed into the mature peptide forms ie. intracellular or extracellular, has been the subject of a number of studies:

- Hazuda et al., (1988) showed that ^{35}S-labelled recombinant IL-1ß was not processed to the 17.5 KDa biologically active form when incubated in the cell-free culture supernatants from LPS-stimulated monocytes. However, the cell-lysates of such monocytes were capable of processing the IL-1ß pro-peptide into the biologically active form.

- Black et al., (1988) showed that incubation of the IL-1ß pro-peptide with membranes from the human monocyte or myeloid cell-lines HG-60,and KG-1, resulted in a 500-fold increase in the biological activity present in the culture fluid. This was shown to be the result of processing of the IL-1ß pro-peptide to biologically active peptides that ranged in Mw from 17.5-19 KDa.

- Black et al (1989) showed that the supernatants from PHA-stimulated monocytes contain an enzyme capable of processing recombinant IL-1ß pro-peptide into a biologically active form. The enzyme involved was completely inhibited by metal ion chelators but not by inhibitors of serine, cysteine, or aspartate proteases. The enzymatic activity was dependent on both Ca^{2+} and Zn^{2+}. The enzyme was not inhibited by the substrate-based metalloprotease inhibitors: phosphoramidon, benzyloxycarbonyl-Gly-Leu-NH_2 and N-(2-Carboxy-3-phenylpropionyl)-Leu. N-terminal sequence analysis of the processed IL-1ß pro-peptide and the cleavage sequence of synthetic peptides revealed that the enzyme present in the culture medium was specific for pre-aspartate cleavage. It should be noted that the authors concentrated the cell-free supernatants of the PHA-activated monocytes by 200-fold and no mention was made of the purity of the cell-population under study. Thus, the actual source of the enzymatic activity remains in doubt.

● Kostura et al (1989) compared the ability of a number of cell-types to process recombinant IL-1ß pro-peptide to a biologically active form. Monocytes and THP-1 cells (a human monocyte cell-line) were capable of processing IL-1ß pro-peptide to a biologically active 17.5 KDa peptide. Amino-acid sequencing revealed the processed peptide was identical to the 17.5 KDa IL-1ß present in the supernatants of stimulated macrophages/monocytes. Lymphoid cells, fibroblast cell-lines and epithelial cell-lines were unable to process the IL-1ß pro-peptide. The authors traced the processing activity to the membrane /cytosolic fraction of the monocytes and THP-1 cells and referred to it as monocyte "IL-1ß convertase".

● Beuscher et al (1990) reported that IL-1ß is secreted exclusively as its pro-peptide form from murine peritoneal macrophages stimulated with 0.5-1 $\mu g.ml^{-1}$ LPS. However, when the LPS concentration was increased to 10-20 $\mu g.ml^{-1}$ a biologically active 20 KDa form was detectable in the supernatant. The authors also showed that the cell-lysates and cell-free supernatants from macrophages stimulated with 10-20 $\mu g.ml^{-1}$ of LPS were able to process recombinant IL-1ß pro-peptide into a biologically active form. In contrast, the supernatants from cells stimulated with 0.5-1 $\mu g.ml^{-1}$ LPS were devoid of processing activity, whilst the cell-lysates of such-cells could still process the IL-1ß pro-peptide. This suggests that appropriately stimulated macrophages can release a processing enzyme into the extracellular milieu.

● Hazuda et al (1990) have looked at the ability of proteases found in the inflammatory exudates of patients suffering from sarcoidosis, RA and emphysema, to process recombinant IL-1ß pro-peptide. The authors showed that purified collagenase, elastase and cathepsin G all processed IL-1ß pro-peptide, at distinct cleavage sites, into biologically active peptides *in vitro*. Synovial fluid collected from patients suffering from inflammatory polyarthritis, and bronchiolar-lavage fluid from patients with sarcoidosis and emphysema also possessed the

ability to process the IL-1ß pro-peptide. Control fluids from patients who had no symptoms of inflammatory disease did not exhibit processing activity. Since several studies have demonstrated that, *in vivo*, IL-1 can induce neutrophil accumulation, the subsequent degranulation of neutrophils at inflammatory sites and the ability of IL-1 to induce collagenase from synovial cells may play a role in the proportion of biologically active IL-1ß released from macrophages *in vivo* . Trypsin and plasmin have also been implicated in the processing of IL-1 *in vivo* (Matsushima et al 1987).

From the above studies, it can be concluded that both cellular and extracellular enzyme activity is responsible for the processing of IL-1ß into a biologically active form. The exact nature and mechanism of processing by the enzyme found in monocyte/macrophage cells awaits further investigation.

1.5 IS THERE A BIOLOGICALLY ACTIVE, MEMBRANE-ASSOCIATED, INTERLEUKIN-1 ?

Kurt-Jones et al (1985a) were the first to describe the existence of an IL-1-like biological activity associated with paraformaldehyde-fixed macrophages and isolated membranes from stimulated macrophages. The fixed macrophages and membranes were able to replace IL-1 in T cell bioassays for IL-1 (Kurt-Jones et al 1985b, Kurt-Jones et al 1986). The authors also showed that an anti-IL-1α antibody was able to inhibit the IL-1 activity associated with macrophage membranes and cells. Further evidence for implicating IL-1α as a membrane associated peptide was provided by Conlon et al (1987) who used fluorescent anti-IL-1α antibody to show that IL-1α was localised at the surface of monocytes . Beuscher et al (1988) reported a 33 KDa membrane peptide that reacted with a specific anti-IL-1α antibody.

Membrane IL-1 activity has also be reported on fibroblasts, epithelial cells, B cells and T cells (Le et al 1987, Kurt-Jones et al 1987, Kurt-Jones et al 1985b, Acres et al 1987.). A role for membrane-associated IL-1 in activation of T cells during antigen presentation has been proposed by Unanue et al (1987), whilst the secreted form of IL-1 is thought to be involved in systemic inflammation. However, there is a growing body of evidence which suggests that IL-1 does not exist as an integral membrane-associated peptide:

- Bayne et al.(1986) could not detect IL-1 on the surface of stimulated monocytes by immunocytochemical techniques.

- Singer et al.,(1987) failed to detect human IL-1 in plasma membranes using immunoelectron microscopy.

- The lack of a hydrophobic "signal" region in the IL-1 peptides suggests that IL-1 does not resemble other existing membrane-associated and secreted proteins.

More recently Minnich-Carruth et al.,(1989) and Suttles et al.(1990) have provided evidence against the existence of an integral membrane-associated IL-1. Their evidence is summarised below:

1. After initial trypsinisation was used to remove any IL-1 associated with the membrane, short-term fixation of stimulated macrophages with 4% paraformaldehyde (15min) still allowed IL-1α to leak from the cytoplasm of the fixed cells into the extracellular medium.

2. Employing longer fixation times with 4% paraformaldehyde (2hr) after trypsinisation, abrogated the appearance of IL-1 activity on the membrane and in supernatants of the fixed cells.

I have looked at secreted and total cell associated IL-1 throughout the present study. This was achieved as outlined in Materials & Methods. Freeze-thawing and sonication of the macrophage monolayers allows the determination of IL-1 activity associated with the membrane and cytosol. It should be noted that the use of the NOB1 bioassay does not enable selective quantitation of the two IL-1 forms. Furthermore, although IL-1ß exists as its biologically inactive pro-peptide form intracellularly disrupting the cells and exposing the pro-peptide to membrane associated enzymes such as "IL-1ß convertase" causes a rapid conversion to its biologically active form as would be expected (Hazuda et al.,(1988), Black et al.,(1988), Kostura et al.,(1989) and Beuscher et al.,(1990)).

Fig. 1.2 summarises the events leading to the synthesis and secretion of IL-1 as outlined above.

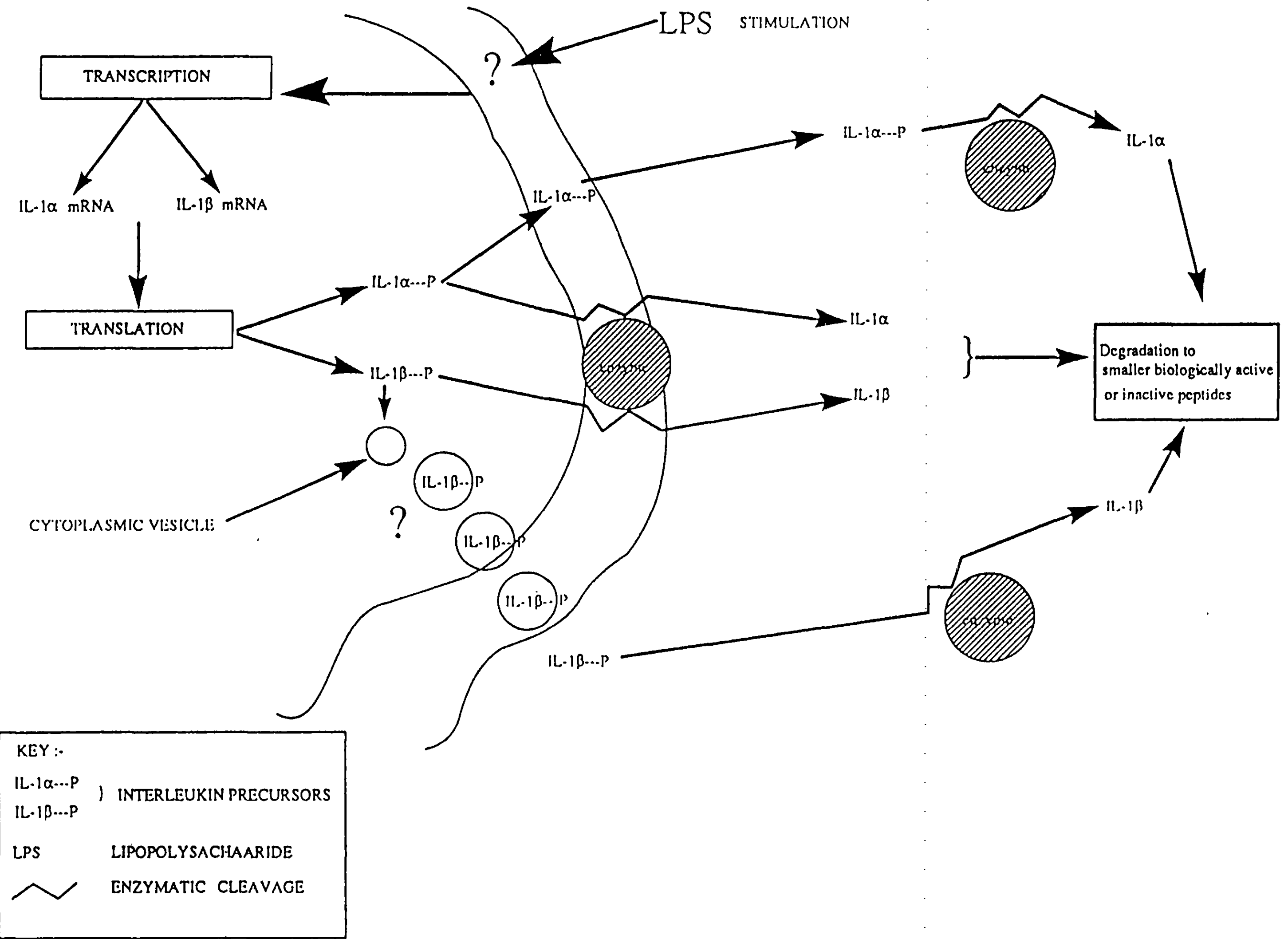


Figure 1.2 **synthesis and secretion of IL-1.** IL-1 is synthesised on demand and requires transcription and translation of mRNA. The precursor forms of IL-1 are localised in the cytoplasm. The mechanisms for their secretion and processing remain poorly understood. See text for detailed discussion of the above figure.

1.6 THE INTERLEUKIN-1 RECEPTOR

Both forms of IL-1 act at the same receptor site to trigger biological events. Unlike the IL-2 receptor, the IL-1 receptor is expressed on unstimulated IL-1-responsive cells but can, like the IL-2 receptor, be up-or down-regulated (Dower & Urdal 1987., Savage et al 1988). Dower et al (1986ab) showed that binding of radiolabelled IL-1ß to murine T-lymphoma cells can be blocked completely by unlabelled IL-1α and vice versa. Furthermore, [^{125} I] murine IL-1 binding to murine T-lymphoma cells can be blocked by both human IL-1α and IL-1ß (Kilian et al.,1986). Binding studies with the propeptide forms of IL-1 have shown that the IL-1ß propeptide is incapable of binding to the IL-1 receptor and is biologically inactive, whereas the IL-1α propeptide can bind to the receptor and is biologically active (Mosley et al 1987ab). Although the IL-1 receptor recognises both forms of IL-1, the affinity between the ligand and its receptor may differ for the two forms of IL-1 (Chin et al 1988). In a study by Chin & Horuk (1990) IL-1α was found to be 23-fold more potent than IL-1ß at stimulating PGE_2 synthesis from rabbit articular chondrocytes. The authors showed that less than 1% receptor occupancy was required for 50% maximal PGE_2 production with IL-1α.

Table 1.6 summarises the cellular distribution of IL-1 receptors in a number of cell lines. The cellular distribution of receptors was found to be a good indicator of biological responsiveness in the cells studied.

The molecular nature of the IL-1 receptor is currently being investigated by a number of research groups. Studies have revealed an 80 kDa IL-1 specific binding protein on a number of cell lines, and for the murine form the cDNA has been cloned and corresponds to a 65 kDa protein (Simms et al 1988). It recognises both IL-1α and IL-1ß. Glycosylation sites of the receptor may play a part in regulating the affinity of the

Table 1.6 cellular distribution of IL-1 receptors

SPECIES	CELL TYPE	RECEPTORS NUMBERS FOR IL-1
murine	thymocytes	< 10
murine	spleen cells	< 10
human	PBM	27
human	T cell line	100
murine	EL4	185 ± 81
murine	EL4 6.1 C10	$1.1 \pm 0.45 \times 10^{-4}$
murine	fibroblast(3T3)	5×10^{-3}
human	T lymphoma (jurkat FHCRC)	< 10
human	monocyte(U937)	< 10

(Adapted from Dower & Urdal 1987.)

receptor and could also account for the various molecular weights that have been reported for the IL-1 binding protein. The cloned 80 kDa receptor is a member of the immunoglobulin superfamily. The extracellular segment contains the characteristic tertiary structure of two ß-pleated sheets connected by disulphide bonds. The receptor has three distinct domains :

i) a 319 amino acid extracellular portion
ii) a 21 amino acid hydrophobic transmembrane segment
iii) a 217 amino acid piece thought to be located in the cytoplasm.

The sequence of the cytoplasmic segment does not show any similarities to that of protein tyrosine kinase (a kinase involved in cell activation by a number of cytokines). Evidence that the receptor is phosphorylated is lacking, although a Lys-Lys-Ser-Arg-Arg sequence in the receptor may

represent a PKC phosphorylation site. A second type of IL-1 receptor is thought to occur on B lymphocytes, pre-B-cells and B-cell lines (Matsushima et al 1986, Bomsztyk et al 1989, Horuk & McCubrey 1989.). It is smaller than the characterized 80kDa receptor and has a higher affinity for IL-1.

Giri et al (1990) have reported the identification and characterization of a soluble IL-1 receptor from Raji Human B-Lymphoma cells that resembles the membrane IL-1 receptor found on the Raji cells. An analogy can be drawn between this soluble IL-1 receptor and the p55 (p represents the *in situ* molecular weight in KDa determined by electrophoretic movment) component of the IL-2 receptor the so called "soluble IL-2 receptor, or TAC antigen" (Robb et al 1986.). Indeed, extending the analogy with the IL-2 receptor, some researchers have proposed that like the low affinity p70 chain of the IL-2 receptor, the IL-1 receptor also possesses a low affinity chain and, depending upon the presence of a second smaller chain, the affinity of the IL-1 receptor can vary (Bird et al 1987).

1.7 CELLS PRODUCING IL-1

A wide variety of cells are capable of producing IL-1 in response to a number of different stimuli. The macrophage / monocyte remains the most active cell-type at synthesising and processing IL-1 for secretion: 100 $fg.cell^{-1}.24h^{-1}$ of IL-1ß has been quoted (Dinarello 1988). Table 1.7 summarises the cell types known to produce IL-1 (adapted from di Giovine & Duff 1990). Although all of the cell types listed synthesize IL-1, the proportion of IL-1α to IL-1ß can vary depending upon the stimulus used and the source of cells in question. Also, cells such as keratinocyte, whilst synthesising large amounts of IL-1 propeptide, process only very small amounts of the propeptide for secretion into the extracellular environment.

Table 1.7 CELL TYPES PRODUCING IL-1

CELLULAR SOURCES OF IL-1	
MONOCYTES	
	Blood
	Placental
TISSUE MACROPHAGES	
	Alveolar macrophages
	Kupffer cells
	Synovial cells
	Peritoneal macrophages
LYMPHOCYTES	
	Helper T-cells
	B cells
	NK cells
VASCULAR CELLS	
	Smooth muscle cells
	Endothelial cells
BRAIN CELLS	
	Astrocytes
	Microglia
	Glioma cell
SKIN CELLS	
	Keratinocytes
	Langerhans
MISCELLANEOUS	
Dendritic, Fibroblast, Neutrophils, Corneal epithelial Chondrocytes, Thymic epithelial, Noradrenergic neural.	

1.8 BIOLOGICAL EFFECTS OF IL-1

IL-1 has pleiotropic effects on many cells, most of the effects being associated with an inflammatory response. IL-1 can also induce the transcription and synthesis of other cytokines such as IL-2, interferons, IL-3, IL-6 (Van Damme & Billiau 1987), IL-1 (Dinarello et al 1987., Warner et al 1987.) TNF (Kunkel et al 1987b) and IL-8 (Matsushima & Oppenhime 1989). The responses that can be delineated from studying the *in vitro* and *in vivo* effects of recombinant IL-1 are bringing to light how and to what extent the observed biological effects of IL-1 are mediated via the induction of other cytokines and other immunological regulators. For example IL-1 has a poor chemotactic effect on neutrophils *in vitro* in a Boyden chamber but *in vivo* IL-1 is strongly chemotactic for neutrophils and this has been shown to be due to the ability of IL-1 to induce the production of IL-8 *in vivo* (Matsushima & Oppenhime 1989).

Tables 1.8 and 1.9 summarise the biological effects of IL-1 *in vitro* and *in vivo*.

Table 1.8 biological effects of IL-1 *in vivo*

SYSTEMIC EFFECTS OF RECOMBINANT IL-1	
CNS fever, Brain PGE_2 synthesis ↑ ACTH ↑ slow wave sleep ↓ REM sleep ↓ Appetite	**METABOLIC** Hypozincaemia, Hypoferraemia ↑ Acute phase protein ↑ Corticosteroid synthesis ↑ Proteoglycan release ↓ Albumin synthesis
HAEMATOLOGICAL Neutrophilia ↑ GM-CSF ↑ Bone marrow cell cycling Bone marrow stimulation Tumour necrosis	**VASCULAR WALL** Hypotension ↑ leucocyte adherence ↑ PGE_2 synthesis ↑ Heart rate ↑ Cardiac output Chemoattractant

Adapted from Dinarello 1989 Advances in Immunology vol.48 p 153 ↑ represents increase ↓ represents decrease.

Table 1.9 in vitro effects of recombinant IL-1

LOCAL EFFECTS OF IL-1
Histamine release from basophils (weak)
Eosinophil degranulation
Increased metalloproteinases from synoviocytes
Increased serine proteinases from synoviocytes
Bone resorption
Induction of fibroblast and endothelial-proliferation
CSF activity
PGE_2 synthesis from dermal and synovial fibroblasts
Induction of PLA_2
Cytotoxic for human melanoma cells and-ß-islet cells
Cytotoxic for thyrocytes
Keratinocyte proliferation
T cell activation
IL-2 production
Increased IL-2 receptor expression
B cell activation
Synergism with IL-6, TNF, IL-2, IL-4
Increased cytokine production :TNF, IL-6, IL-3, IL-1, GM-CSF, IFNγ, IL-8.
Activation of NK cells

(Adapted from Dinarello 1989)

From tables 1.8 and 1.9 it can be seen that many of the biological effects produced by IL-1 are particularly pertinent to the destructive inflammatory processes that occur in arthritic conditions. Fig 1.3 attempts to summarise how some of these biological actions could result in compromised joint integrity.

1.9 THE ROLE OF IL-1 IN DISEASE

IL-1 has been implicated as a contributing mediator in a number of pathological states such as: hypotensive shock, Crohn's disease, peridontitis, psoriasis, immune complex glomerulonephritis, kidney allograft rejection, dialysis complications, diabetes, Hodgkins disease, infertility in endometriosis, and arthritis (for references see di Giovine & Duff 1990).

The following discussion will concentrate on the evidence implicating IL-1 as a pivotal mediator of the arthritic process.

1.9.1 Interleukin-1 in body fluids

Studies that have used bioassays to quantitate IL-1 in synovial fluid have yielded variable results probably as a result of the ubiquitous presence of IL-1 and IL-2 inhibitors in such fluids (for review see Hopkins et al 1988). However, a number of studies have shown increased levels of IL-1 in the joint fluid of patients suffering from RA, OA, traumatic arthritis and psoriatic arthritis (Nouri et al 1984,. Fonta et al 1987,. Wood et al 1983., di Giovine et al 1988). In a study by Symons et al (1989) in which a radioimmunoassay (RIA) was used to quantitate IL-1α and IL-1β levels in the range of 500 $pg.ml^{-1}$ were found in RA patients whilst levels of approximately 90 $pg.ml^{-1}$ of IL-1β and 300 $pg.ml^{-1}$ of IL-1α were found in the controls. Wood et al (unpublished observation quoted in IL-1, infection, immunity and disease 1989) using concurrent, *in situ* hybridisation and cellular immunophenotyping on the same section of tissues obtained from arthritic patients, have shown that the predominant IL-1 producing cell is the $CD14^{+}$ tissue macrophage. These cells occurred in the synovial lining layer, in cellular aggregates and in intermediate stromal areas of joints. Levels of circulating IL-1β have been shown to correlate positively with disease activity in patients suffering from RA. It is possible that circulating IL-1 levels may serve as a diagnostic marker of disease activity (Eastgate et al 1988).

1.9.2 Production of Interleukin-1 *ex vivo*

Rheumatoid synovial tissue obtained at arthroscopy synthesizes large amounts of both IL-1α and IL-1ß upon stimulation *in vitro* (Miyasaka et al 1988). This increased capability of IL-1 synthesis was not observed in non-rheumatic synovial tissue from age-matched controls. Circulating monocytes from RA patients with recently active disease, spontaneously secrete IL-1 *in vitro* (Shore et al 1986). Danis et al (1987) have also shown spontaneous production of IL-1 from blood monocytes, synovial fluid and tissue macrophages from patients with RA and ankylozing spondylitis but not from normal individuals or patients suffering from OA.

1.9.3 Interleukin-1 mRNA

High levels of IL-1α and IL-1ß mRNAs have been detected in the synovial membranes from arthritic patients (Buchan et al 1988,. Firestein et al 1989.). Buchan et al. have also shown that synovial cells possessing high levels of mRNA for IL-1 show enhanced secretory capabilities for IL-1 upon stimulation *in vitro*.

1.9.4 Interleukin-1 in experimental synovitis *in vivo*

Observations in experimental animal models of arthritis support the hypothesis that IL-1 is an important mediator of tissue destruction. For instance, intra-articular injection of highly purified or recombinant IL-1 into the knee joints of rabbits induced a transient synovitis with leucocytic infiltration into the synovial lining. In addition, intra-articular injections of recombinant IL-1 induced a dose-dependent loss of proteoglycan from articular cartilage (Henderson & Pettipher 1988). The loss of proteoglycan from the mid-zone of the articular cartilage in rabbits has been shown to be unrelated to the number of infiltrating leucocytes attracted by IL-1 (Pettipher et al 1988). Repeated injections of IL-1 into the ankles of normal rats resulted in a chronic synovitis that was characterized by the presence of mononuclear cells and fibrosis, but failed to produce

cartilage and bone destruction (Stimpson et al 1988). However, in joints that had been previously injected with a streptococcal cell-wall peptidoglycan-polysaccharide complex, subsequent injections of IL-1 markedly attenuated the acute inflammatory response but produced pannus formation and cartilage destruction (Stimpson et al 1988). Figure 1.3 summarises the destructive effects of IL-1 on bone and cartilage

1.10 BIOASSAYS FOR INTERLEUKIN-1

A number of the biological effects of IL-1 have been utilised to develop bioassays to quantitate IL-1. Table 1.9 summarizes the various bioassays that have been developed. A problem that is encountered with all bioassays is the effect of inhibitory substances in biological fluids that interfere with the biological response. Where the inhibitory substance is of relatively low Mw (eg. PGE_2) these can be removed by dialysis. Alternatively, drugs can be used to supress certain inhibitory mediators (eg. NSAIDs with PGE_2). However, the complication of the effect of the drug on the assay system and/or IL-1 production arises. High Mw protein inhibitors of IL-1 have been shown to be produced by peripheral blood monocytes (PBM) (Berman et al 1986), macrophage cell lines (Carter et al 1990) and are also found in the urine of febrile patients (Seckinger et al 1987). For such inhibitors, physico-chemical purification procedures are not satisfactory unless large volumes of fairly active material are being investigated. Gearing & Thorpe (1989) have shown that such proteinaceous inhibitors are best removed by dilution of the original supernatants containing the IL-1 by a factor 1:50 or 1:100. This necessitates the use of a sensitive bioassay since the supernatants of activated macrophages normally contain IL-1 in the range of 0.1-10 $ng.ml^{-1}$ so the bioassays that requiring IL-1 concentrations of $ng\text{-}pg.ml^{-1}$ are unsuitable for such procedures.

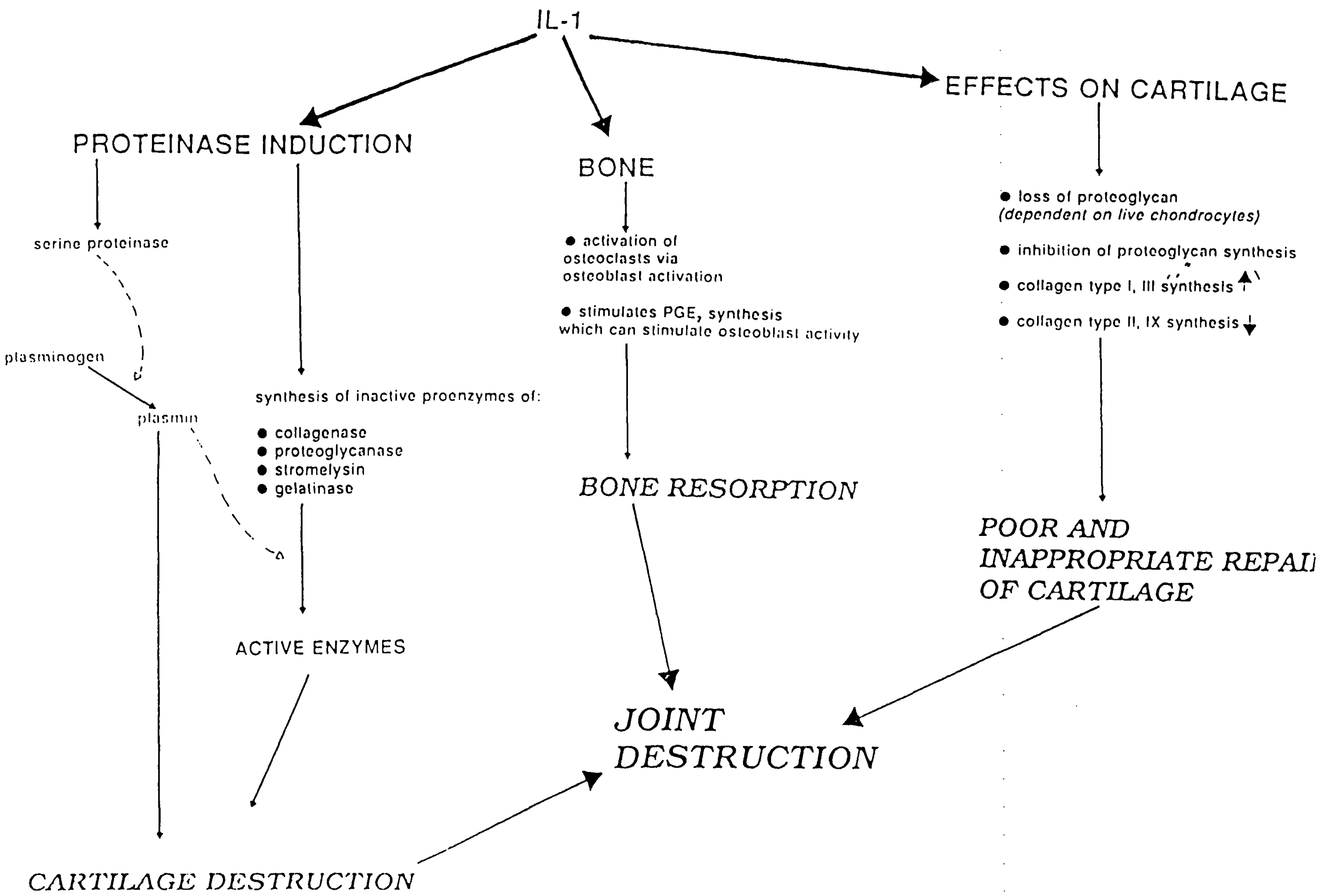


Figure 1.3 A schematic representation of the destructive effects of IL-1 on bone and cartilage

Table 1.10 bioassay for IL-1

BIOLOGICAL EFFECT	ASSAY	SENSITIVITY / SPECIFICITY	
Induction of fever	Rabbit/mouse pyrogen	ng-μg	very poor
Acute phase response	Rabbit/mouse acute phase protein synthesis	ng-μg	very poor
Tissue remodelling	Cartilage breakdown & proteoglycan release.	ng	poor
	Bone resorption.	ng	very poor
Endothelial /fibroblast	PG production	ng	very poor
	Proliferation	ng	very poor
T cell	Thymocyte proliferation(LAF)	pg	very poor
	IL-2 production		
	LBRM33.	100fg	good
	EL4 6.1 + CTLL	100fg	good
	EL4 NOB1 + CTLL	100fg	good
	IL-2 receptor Production EL4 6.1	pg	good
	Proliferation of D10 cells	fg	good
Cytotoxicity	Tumour cell killing	100pg	good

The lack of specificity which many of the bioassays show has come to light with the characterization of cytokines that possess overlapping biological activities with IL-1 eg. IL-6, granulocyte macrophage colony stimulating factor (GM-CSF), IL-8 and TNFα. It is outside the scope of this report to compare critically, in detail the specificity and sensitivity

of all the bioassays used for IL-1 in an attempt to justify the use of the bioassay employed in the present study (Table 1.10 presents a basic overview). However, since much of the work published to date, which parallels the study undertaken in this report, has relied on the use of the lymphocyte activating factor assay (LAF) to quantitate IL-1 in biological supernatants, a comparison of the bioassay used in this study (EL4 NOB1 + CTLL) with the LAF assay will be made. The specific details of the assay protocol employed in the following study will be discussed in detail in the materials & methods section (chapter 2).

1.10.1 The LAF assay

The LAF assay has for many years been the assay of choice for the quantitation of IL-1. The assay relies on the ability of IL-1 to stimulate, dose-dependently, murine thymocyte proliferation in the presence of a sub-optimal concentration of a mitogenic lectin (eg. PHA (Gery et al 1972). Although thymocytes also proliferate in response to lipopolysaccharide (LPS) and IL-2, this can be overcome by using thymocytes from the LPS unresponsive mouse C3HEJ and carrying out the assay in saturating concentrations of IL-2 (Falk et al 1988). However, specificity with respect to other cytokines is poor since TNFα, IL-6, IL-4, IL-7 and GM-CSF can all cause murine thymocytes to proliferate and TNFα and IL-6 strongly synergize with IL-1 to stimulate thymocyte proliferation (Ranges et al 1988., Suda et al 1989., Helle et al 1988.).

The LAF assay is also relatively insensitive, requiring at least 10-20pg.ml^{-1} of recombinant IL-1 to produce a detectable response. Thus, when samples require dilution to eliminate the influence of endogenous inhibitors (as mentioned previously) this lack of sensitivity can completely obscure the presence of IL-1. For instance, PGE_2 is a potent inhibitor of thymocyte proliferation: 2ng.ml^{-1} being sufficient to inhibit IL-1-induced thymocyte proliferation by 50% (Le Moal et al 1988). Since levels of 100-20 ng.ml^{-1} of PGE_2 can be present in biological fluid in the joints of animals in which arthritis

has been induced (Petippher et al 1989) or from macrophages stimulated *in vitro* (Kunkel et al 1986., Otterness et al 1988.), dialysis of such supernatants is required before the IL-1 can be assayed. However, dialysis is not always appropriate to remove all inhibitory molecules, as mentioned earlier.

1.10.2 EL4 NOB1 + CTLL assay

This assay relies on the dose-dependent production of IL-2 from EL4 NOB1 cells (a sub-clone of the murine EL4 thymoma) by direct stimulation with IL-1 alone. The cytotoxic T cell line CTLL is used to quantitate the IL-2 thus produced (Gearing et al 1987). The assay can reliably detect as little as a few hundred $fg.ml^{-1}$ of recombinant IL-1 (see chapter 2). As the cell lines are grown in continuous culture their response is reliable and reproducible, unlike the thymocytes which have to be obtained fresh from mice each time an assay is performed. The assay responds weakly to TNFα requiring $10ng.ml^{-1}$ of rhTNFα and $1ng.ml^{-1}$ of rmTNFα . The assay does not respond to TNFß. Only 50% of the maximal stimulation achieved with IL-1 can be achieved with TNF. However, the relatively high dilutions of the original supernatant that are usually required is sufficient to reduce the TNF concentration to a negligible level. Low levels of TNFα that are not stimulatory to EL4 cells do not synergize with IL-1 to stimulate the EL4 cells (Gearing, personnel communication). IL-6 fails to stimulate EL4 NOB1 cells at concentrations up to $100ng.ml^{-1}$ and this concentration is unlikely to be found in diluted supernatants of stimulated cells. The major disadvantage of the assay is the need to maintain cell-lines, in particular the CTLL cell, which is time-consuming and requires a developed expertise.

1.10.3 Immunoassays

Immunoassay for IL-1 based on radioimmunoassay (RIA), immunoradiometric assay (IRMA) and enzyme linked immunosorbent assay (ELISA) have recently become commercially available.

The advantages of the immunoassays over bioassays is that they are quick to perform, do not require culturing of cell-lines and can be very specific for a desired antigenic cytokine. The immunoassays are also unaffected by the inhibitors mentioned above. However, immunoassays cannot distinguish between biologically active or inactive forms of the cytokine. The assays are usually species specific as well as specific for the IL-1α or IL-1β forms, thus necessitating the use of two assay systems if the total IL-1 content is required (although this can be an advantage in studying the independent production of the IL-1 forms). Lastly the commercially available immunoassays are prohibitively expensive to use on a routine basis to assay a large number of samples for IL-1.

1.11 AIM OF THE PROJECT

The NSAIDs are widely used in the symptomatic treatment of arthritic conditions. Recent studies suggest that this group of drugs possess pro-inflammatory activities either through prostaglandin-dependent or -independent mechanisms. This raises the question of whether NSAIDs could be detrimental to and/or contribute to the pathology of certain inflammatory diseases. The effect of NSAIDs on IL-1 production is of prime importance in this respect since IL-1 has been shown to be an important mediator of chronic inflammation. As macrophage-like cells have been implicated as the major local producers of IL-1 in arthritic conditions and are prolific secretors of IL-1 *in vitro*; the murine peritoneal macrophage was chosen as a cell to investigate the effect of NSAIDs on IL-1 production. Resident murine macrophages were chosen in this study because:

1. They are easy to isolate and purify.

2. Relatively inexpensively obtained.

3. Do not require the stringent facilities laid down by the COSHH report (1988) for their handling or culture as compared with the handling of human macrophages obtained from blood.

4. As an eliciting agent is not employed to increase macrophage accumulation in the peritoneum, the complex nature of priming and signal transduction pathways for the eliciting agent do not have to be taken into consideration when trying to examine the mechanism of action of NSAIDs

Although the effects of NSAIDs on IL-1 secretion have been investigated by a number of researchers (see chapter 4) conflicting observations have been made regarding the ability of NSAIDs to modulate IL-1 secretion from macrophages *in vitro*. The conflicts may well be due to the LAF assay that was employed by such researchers for the quantitation of IL-1.

Thus, we have used the EL4 NOB1 assay to re-evaluate the effect of NSAIDs on IL-1 secretion from macrophages and have attempted to investigate the mechanisms of such effects.

CHAPTER TWO

M A T E R I A L S   A N D   M E T H O D S

2.1 Materials

MATERIAL	OBTAINED FROM
ASPIRIN	Sigma Chemical Co, Poole UK
A23187	Sigma Chemical Co, Poole UK
BWA4C	Gift from Wellcome Res.Labs.
CALCITONIN GENE RELATED PEPTIDE	Gift Dr. Marshall (UCL)
DIMETHYL SULPHOXIDE	Sigma Chemical Co, Poole UK
diC_8	Sigma Chemical Co, Poole UK
FOETAL CALF SERUM	Gibco Ltd. Paisley UK
GENTAMICIN	Gibco Ltd. Paisley UK
GLUTAMINE	Gibco Ltd. Paisley UK
INDOMETHACIN	Sigma Chemical Co, Poole UK
INTERLEUKIN-1 α RECOMBINANT	Gift from Dr.Sedgwick (Roche)
IONOMYCIN	Calbiochem Notts. UK
L-651,392	Gift. Merk Sharp & Dohme UK
LIPOPOLYSACCHARIDE (LPS)	Sigma Chemical Co, Poole UK
2-MERCAPTOETHANOL	Gibco Ltd. Paisley UK
MTT	Sigma Chemical Co, Poole UK
NEUROKININ A & B	Gift Dr. Marshall(UCL)
PLATELET ACTIVATING FACTOR	Sigma Chemical Co, Poole UK
PHORBOL 12, 13- DIBUTYRATE	Sigma Chemical Co, Poole UK
PHOSPHATE BUFFERED SALINE	Gibco Ltd. Paisley UK
PIROXICAM	Sigma Chemical Co, Poole UK
PROSTAGLANDIN E_2	Sigma Chemical Co, Poole UK
RIA PGE_2	NEN research products UK
RHC 80267	Gift from Dr.C.A. Sutherland (Revlon Health Care), NY. USA
R59022	Janssen Pharmaceutica (Belg).
RPMI-1640	Gibco Ltd. Paisley UK

RPMI-1640 DUTCH MODIFICATION	Gibco Ltd. Paisley UK
SODIUM DODECYL SULPHATE	Sigma Chemical Co, Poole UK
SODIUM MECLOFENAMATE	Parke Davis & Co., Pontypool
SUBSTANCE P	Peninsular Labs.
TISSUE CULTURE WARE	Falcon Ltd. Oxford UK
TUMOUR NECROSIS FACTOR α	Gift from Xenova Ltd.
TRYPAN BLUE	Gibco Ltd. Paisley UK

BWA4C	N-(3-phenoxycinnamyl)-acetohydroxamic acid
diC8	1,2-dioctanoyl-sn-glycerol
L 651-392	4-bromo-2,7-dimethoxy-3H-phenothiazin-3-one.
MTT	3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide.
RHC80267	1,6-di(O-(carbamoyl)cyclohexanone oxime) hexane.
R59022	6-[2-[4-[(4-fluorophenyl) phenylmethylene-1-piperidinyl]-7-methyl-5H-thiazolo[3,2-a]pyrimidin-5-one

2.2 Dilution of Drugs, Neuropeptides and Cytokines

All compounds were finally diluted in supplemented RPMI-1640 before being placed onto the macrophages or cell-lines.
All NSAIDs, R59022, RHC80267, diC_8, BWA4C, L-651,392, ionomycin, A23187 and phorbol 12,13-dibutyrate (4ß-PDBu) were dissolved in DMSO as concentrated stock solutions (10^{-1} - 10^{-3} M). The solutions of NSAIDs, R59022, RHC80267, BWA4C and L651,392 were freshly made from their powder forms whilst the other compounds were diluted from aliquoted stock solutions kept at -20°C. The maximum concentration of DMSO never exceeded 0.1% v/v in the final solution to be placed onto the cells. Controls were carried out to test for any effects of the DMSO on the cells at concentrations of 0.1% and lower and they consistently showed no effect.

The neuropeptides, neurokinin A & B (NK-A, NK-B), calcitonin gene related peptide (cGRP) and substance-P (SP) were all diluted to their appropriate concentrations from aliquoted stock solutions (made up in phosphate buffered saline (PBS) containing 10% w/v human serum albumin). The minimum dilution of the neuropeptide stock into supplemented RPMI-1640 was never less than 1:1000.

PGE_2 and platelet activating factor (PAF) were diluted to the appropriate concentrations from stock solutions of 3 x 10^{-3}M in absolute alcohol and $2 mg.ml^{-1}$ in chloroform respectively, both stock solutions were kept at -20°C. The solvents were evaporated before diluting the PGE_2 and PAF; polypropylene containers and pipette tips were used in these dilution procedures to minimise binding of the compounds to plastic surfaces.

MTT was made up at $5 mg.ml^{-1}$ in PBS and filtered through a $0.22 \mu m$ sterile filter into a sterile container and kept in the dark at 4°C until required.

2.3 Recombinant human IL-1α (rhIL-1α) particulars of its activity and composition

ACTIVITY	3×10^6 Uml^{-1} (LAF)
PROTEIN CONCENTRATION	0.36 $mg.ml^{-1}$(by amino acid analysis)
SPECIFIC ACTIVITY	1×10^7 Uml^{-1}
ENDOTOXIN	~ 150 Uml^{-1} (LAL)*
BUFFER	30mM Tris-Cl, 0.4M NaCl (pH 7.8)

**LAL limulus amoebocyte lysate (assay for endotoxin)*

The original stock solution was diluted to 2000 U ml^{-1} with RPMI-1640 containing 1% heat inactivated foetal calf serum (HIFCS) and filtered with a low protein binding, 0.22μm filter (Millipore) into sterile bijoux bottles (sterilin) and stored at -20°C until required.

Recombinant human TNFα (rhTNFα). The peptide was supplied as a stock solution at 2$\mu g.ml^{-1}$ in PBS. A minimum dilution of 1:667 was employed from the original aliquots.

2.4 Media used for cell culture and assay procedures

a. Phosphate buffered saline

Phosphate buffered saline (PBS) low in Ca^{2+} and Mg^{2+} chilled to 4°C was used to rinse out the peritoneal cavities of mice in order to obtain peritoneal exudate cells. The PBS was also used to wash the peritoneal exudate cells before they were placed into supplemented culture medium.

b. Roswell Park Memorial Institute Medium 1640 (RPMI-1640)-glutamine free.

All cell cultures were maintained in supplemented RPMI-1640 with or without heat-inactivated foetal calf serum depending on the cell line. The supplements that were added to the RPMI-1640 were as follows :

1. Glutamine 2×10^{-3}M final
2. Gentamicin 50$\mu g.ml^{-1}$ final

A more detailed account of the individual media requirements for each cell-line will follow later. The use of glutamine-free RPMI-1640 was proffered since this could then be stored at 4°C, with an expiry period of approximately 3 years, prior to the addition of a concentrated glutamine stock solution kept at -20°C, whereas RPMI-1640 containing glutamine must be stored at -20°C and a proportion defrosted each time it is required. Gentamicin (an aminoglycoside originally isolated from *Micromonospora purpurea*) was preferred over the penicillin/steptomycin (PS) combination as the antibiotic ,in culture media, for a number of reasons:

i. **Broader spectrum of anti-microbial activity.** Gentamicin is active against strains of Proteus and Staphylococcus which can sometimes be resistant to PS combination. Gentamicin is also active against Pseudomonas strains unlike PS.

ii. **Anti-Mycoplasma activity.** Gentamicin is active against several but not all strains of mycoplasma, whereas PS has no anti-mycoplasma activity.

iii. **pH stability.** Gentamicin is stable for 15 days at 37°C over a pH range of 2-10 in tissue culture media. Streptomycin is rapidly destroyed at alkaline pHs and penicillin is unstable at both acid and alkaline pH.

iv. **Temperature stability.** Gentamicin is stable at all temperatures and can withstand autoclaving.

v. **Activity is unaffected by serum.** Penicillin loses 30% of its anti-bacterial activity when it is added to tissue culture media containing serum.

vi. **Toxicity.** Gentamicin is less toxic over a wider range of concentrations than PS.

c. Dutch modification (DM)-RPMI-1640

This is RPMI-1640 with 20mM HEPES buffer (4(-2-hydroxyethyl)-1-piperazine ethanesulfonic acid). This medium was used to wash all cell-lines, apart from the peritoneal exudate cells, because of its ability to maintain its pH without the use of a carbon dioxide incubator.

2.5 ISOLATION OF MURINE PERITONEAL MACROPHAGES

Murine peritoneal macrophages were used as the cell type for studying the synthesis and secretion of IL-1 for the reasons given in the introduction. The cells were isolated and purified under strict aseptic conditions and the drugs added to the macrophages were also aseptically prepared.

2.5.1 Mice.

BKW male mice were purchased from Bantin & Kingman Ltd. Hull and kept in a clean environment within the departmental animal house. The mice were fed on a standard laboratory diet and allowed water ad libitum. The mice were sacrificed between the ages of 9 and 14 weeks by asphyxiation with CO_2 in an enclosed chamber prior to the harvesting of their peritoneal exudate cells (PEC).

2.5.2 Harvesting peritoneal exudate cells

Once killed the mice were doused with 70% industrial methylated spirits (IMS) and taken into a lamina air flow cabinet designated for aseptic isolation of cells from animals. The peritoneum was exposed by pulling back the fur from an incision point on the abdomen. 5mls of PBS chilled to 4°C, plus 1ml of air to allow for agitation, were introduced into the peritoneal cavity of a mouse. The peritoneal surface was then massaged gently for a minute before a small incision was made into the peritoneum to allow the PBS to be sucked up with a pasteur pipette. The pooled PEC from 5 mice were washed three times in cold PBS by spinning at 300g for 8 minutes before being placed into supplemented RPMI-1640 to which 5% HIFCS had been added . The cells were counted using a haemocytometer, trypan blue exclusion was used as an indicator of cell viability. A greater than 95% viability was always obtained. The percentage of macrophages within the exudate cells varied from 50-60% as determined by morphology and staining for non-specific esterase.

2.5.3 Purifying the macrophages from PEC

The macrophages contained in the PEC were purified by placing 1ml of supplemented RPMI-1640 containing 1 x 10^6 PEC into each well of a 24 well tissue culture plate. The cells were then incubated at 37°C in a CO_2 incubator at 5% CO_2, 95% air and 100% humidity. After 2hr incubation the non-adherent cells were removed by gently rinsing with supplemented RPMI-1640 at room temperature. The resulting monolayer was found to consist of 90% macrophages as judged by morphology and non-specific esterase staining. The wells containing the macrophage monolayer were then overlaid with serum-free, supplemented RPMI-1640 containing stimulant and/or NSAID. The 24 well plate was incubated for 20hr at 37°C in a CO_2 incubator at 5% CO_2, 95% air and 100% humidity, after which the cell-free supernatants were removed and frozen at -75°C until assayed for IL-1. The macrophage monolayer left in the well was rinsed once with RPMI-1640 and then overlaid with 2ml of RPMI-1640 containing 1% HIFCS. The plate was freeze-thawed at -75°C to fracture the cells and then sonicated for 15 minutes to disrupt the plasma membrane remnants.The resultant mixture was incubated at 37°C for 30min before being transferred to a -75°C freezer. This allowed for the determination of IL-1 remaining within the cell and bound to the plasma membrane which will be collectively termed "cell-associated IL-1".

2.6 CELL LINES AND THEIR MAINTENANCE

All manipulations involving the use of cells, media, preparations of drugs for addition to cells, was performed in a laminar air flow cabinet, adhering to strict aseptic technique. As mentioned above, gentamicin was used to limit possible microbial contamination. On visual evidence of a microbial contamination or abnormal cell structure or function, the cells were destroyed and a new batch grown from a cryo-preserved stock kept under liquid nitrogen.

2.6.1 The EL4 NOB1 murine T cell thymoma cell line

The EL4 NOB1 (NOB1) cells are a subclone of the murine EL4 6.1 T cell thymoma. The NOB1 cells produce very low levels of IL-2 at " rest". However, upon stimulation with IL-1α or IL-1ß the NOB1 cells produce large quantities of IL-2 in a dose-dependent manner. Unlike the EL4 6.1 cells the NOB1 sub-clone does not require co-stimulation with a mitogenic-lectin or calcium ionophore in order to produce IL-2. Functional receptors for IL-1 have been solubilised from the plasma membranes of NOB1 cells and these have been shown to have a Mw of ~ 80 kDa (Bird et al 1988).

The NOB1 cells were grown in supplemented RPMI-1640 containing 5% HIFCS, at 1×10^5 cells ml^{-1} for two days and 5×10^4 cells ml^{-1} over the weekend ie. the cells were harvested on Mondays, Wednesdays and Fridays. The NOB1 cells can also be grown in Dutch modification RPMI-1640 (DM-RPMI-1640) however, this adversely affects the ability of the cells to respond to recombinant IL-1 thus suggesting that HEPES- containing media are unsuitable for maintaining these cells in culture. When the NOB1 cells are grown in flat-bottomed tissue culture flasks they show a tendency lightly to adhere to the bottom of the flasks, but are easily dislodged by tapping the bottom of the flask on a hard flat surface. Figures 2.1a,b and c show EL4 NOB1s under transmission electron microscopy. The pictures were taken by D. Hockley at the National Institute for Biological Standards and Control (NIBSC) Blance Lane Hertfordshire. A brief outline of the procedure used by D. Hockley is given below:-

i. cells spun into pellet

ii. fixed in 2.5% gluteraldehyde in isotonic calcium cacodylate buffer containing 1% osmium tetroxide and 0.5% uranylacetate.

iii.embedded into Araldite

iv. sections stained with uranylacetate and lead citrate.

Figure 2.1a EL4 NOB1 cells have a dense granular cytoplasm filled with polyribosomes and mitochondria; reflecting a high metabolic potential for the cell. The cells are also motile as shown by the central cell in the picture sending out a pseudopodium. The dark circular granules in the cytoplasm represent intracellular virus particles **(IV)** and these are also found outside the cell **(EV)**. Total magnification x 9250

Figure 2.1b Shows intracellular virus particles.
Total magnification x 84 000

Figure 2.1c Shows a virus budding from the cell next to which is a free virus particle. The virus is a typical type-c murine retrovirus (D.Hockley personal communication).
Total magnification x 180 000

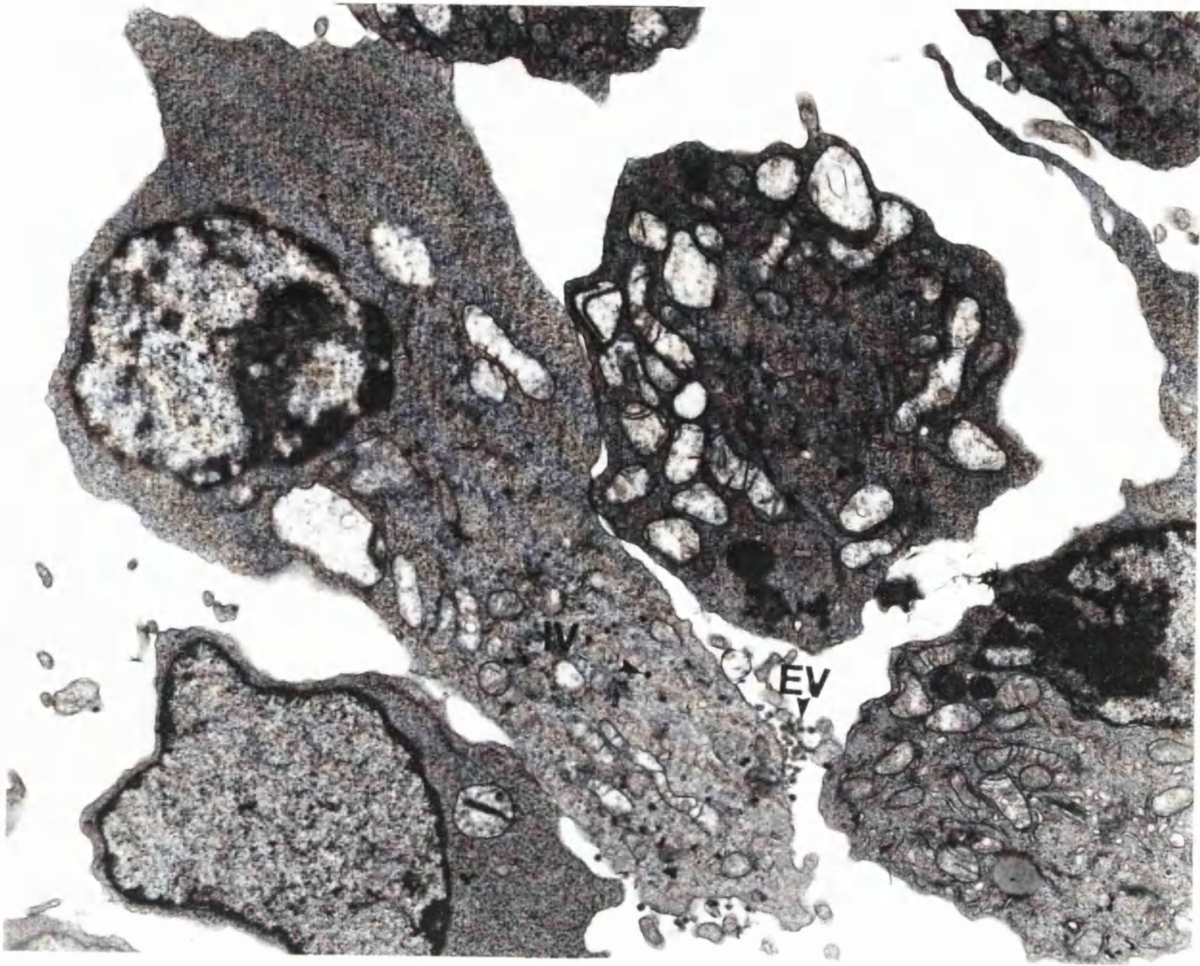
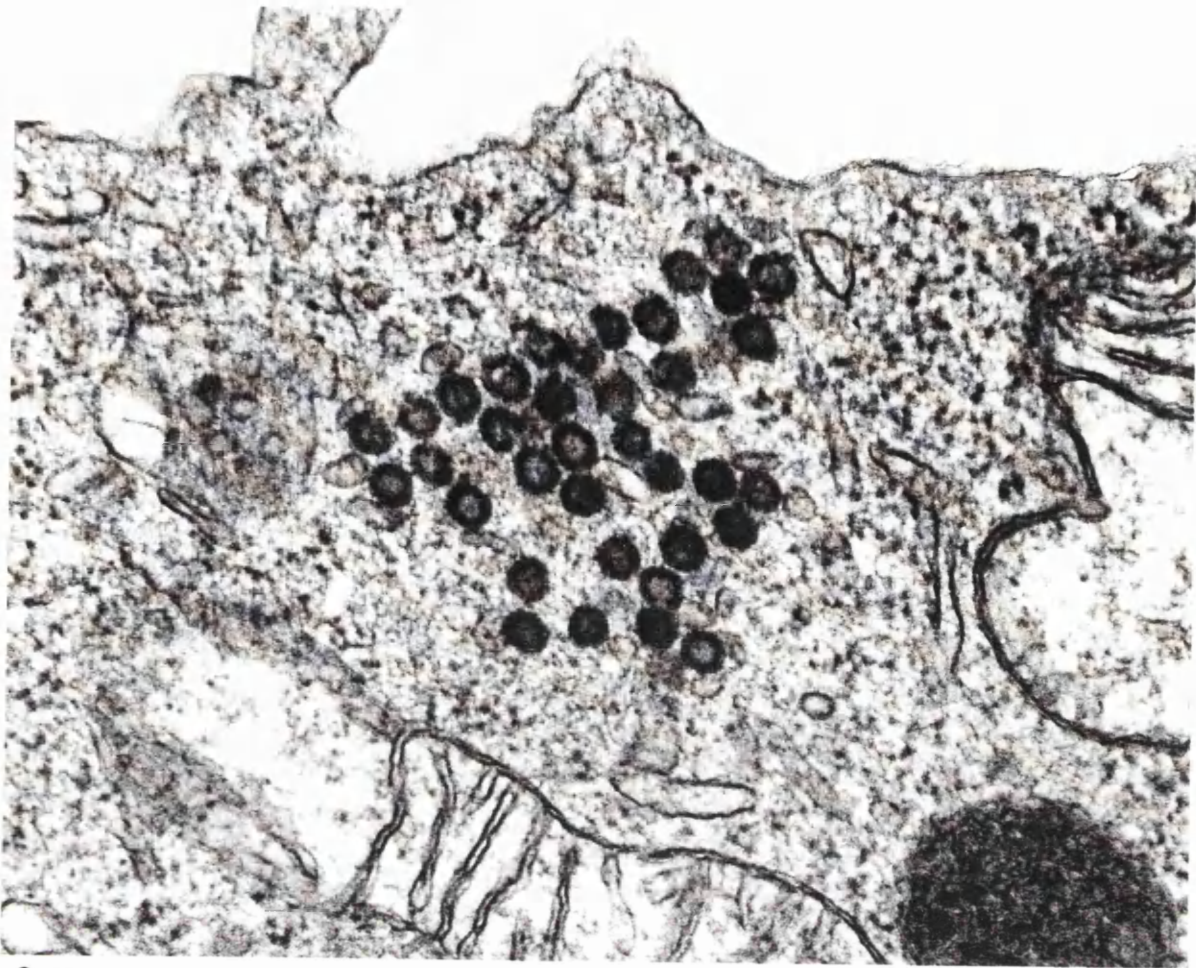


Fig 2.1a



2.1b

2.1 c

2.6.2 The CTLL cell

The CTLL is a murine T cell line that requires IL-2 to maintain its viability and growth (Gillis et al 1978).The CTLL is unresponsive to stimulation or priming by IL-1. CTLLs were grown at a concentration of 2 x 10^4 cells ml^{-1} for 2 days and 1 x 10^4 over the weekend. The cells were grown in supplemented RPMI-1640 containing 5% HIFCS and 10% MLA-144 (an IL-2 producing cell line) supernatant for 2 days growth or 20% MLA-144 supernatant for growth over the weekend. DM-RPMI-1640 was found to be unsuitable for growing CTLLs due to a very slow toxic effect of the HEPES.

2.6.2 The MLA-144 Cell

This is a gibbon T cell line which constitutively produces IL-2 (Rabin et al 1981).MLA-144 cells were grown, at a concentration of 1 x 10^5 cells ml^{-1} in supplemented RPMI-1640, to which 15% HIFCS, 5 x 10^{-5}M 2-mercaptoethanol (2-ME) and 5 x 10^{-6}M indomethacin had been added (to enhace IL-2 production by affecting glutathione levels and removing feedback inhibition by prostaglandins), for 5 days before harvesting the cell-free supernatants by centrifuging at 300g for 10 min. The supernatants were freeze-thawed and filtered through a 0.22μm sterile Millipore filter and stored at -20°C until needed. Every batch of MLA-144 supernatant produced was tested for IL-2 activity by its ability to maintain CTLL viability. The ability of a batch of supernatant produced on 18-5-88 to support CTLL viability is shown in Fig.2.2

2.6.4 Cryo-preservation of cell-lines.

A batch of all cell-lines were cryo-preserved and stored under liquid nitrogen for use when infection or abnormalities were detected in the culture. The cryo-preservative mixtures for the cells is outlined in table 2.1.

Table 2.1 Cryo-preservative Mixtures

% V / V	CONSTITUENT
FOR EL4 NOB1s AND MLA-144s	
50	RPMI-1640
40	HIFCS
10	DMSO
FOR CTLLs	
50	MLA-144 SUPERNATANT
40	HIFCS
10	DMSO

The cells were suspended at 5 x 10^6 cells ml^{-1} in their respective mixtures and 1.8ml placed into a plastic freeze-tube (Nunc-Gibco). The tube was then transferred to a polystyrene freezer box (Gibco) and the box was placed at -75°C for 2 days before transferring to a liquid nitrogen store. For reanimation the cells were quickly defrosted at 37°C and placed into supplemented RPMI-1640 containing 10% HIFCS and for CTLLs 20% MLA-144 supernatant.

2.7 IL-1 ASSAY

IL-1 was quantitated using a modification of a bioassay developed by Gearing et al (1987). The assay relies on the dose-dependent production of IL-2 from the NOB1 cell line in response to IL-1. The IL-2 so produced is then quantitated by using the CTLL cell line. CTLL viability and proliferation was measured using the MTT dye method (Tada et al 1986). Thus there were 2 stages involved until the completion of the assay:

STAGE 1. dilution of IL-1 samples onto NOB1s

a. The NOB1s were washed twice in DM-RPMI-1640 by centrifuging at 300g for 10 min. and resuspended at 2 x 10^6 cells ml^{-1} in supplemented RPMI-1640 containing 5 % HIFCS.

b. IL-1 test-samples were diluted serially in triplicate in supplemented RPMI-1640 containing 5% HIFCS and 100μl of the diluted sample in a sterile 96 well plate.

c. 100μl of the prepared NOB1s were added to each well (final of 1 x 10^5 NOB1s per well) and cultured for 24hr at 37°C in a CO_2 incubator at 5% CO_2 , 95% air and 100% humidity.

d. After 24hr 50μl of the NOB1 cell supernatant was removed from each well and placed into a new 96 well "copy" plate. The contents of the new plate were freeze-thawed at -75°C to kill any NOB1s carried over.

STAGE 2. addition of CTLLs.

e. The CTLLs were washed, to remove IL-2 from the cells, by centrifuging twice at 300g in DM-RPMI-1640. The pellet of cells was resuspended in RPMI-1640 and placed into the incubator for 30 mins. The cells were then washed twice more in DM-RPMI-1640 and resuspended at 8 x 10^5 cell ml^{-1} in supplemented RPMI-1640 containing 5% HIFCS. This procedure was found to be optimal for removing IL-2 bound to the receptors of CTLLs and thus reducing their basal viability without IL-2 (Michalski & McComb, 1986).

f. 50μl of the washed CTLLs were added to 50μl of the supernatants from the NOB1 cells in the copy plate. The plate was allowed to incubate for 24hr at 37°C in a CO_2 incubator.

g. After 24hr 20μl of MTT* at 5mg.ml^{-1} in PBS was added to each well including a control well containing just medium (usually A_1 (the first well) in a 96 well plate). After a further 4½hr incubation at 37°C in a CO_2 incubator 100μl of SDS in 0.01N HCL was added to each well. The plates were returned to the incubator for a further 12hr or overnight.

** MTT is converted by mitochondrial enzymes of living cells from a yellow compound in solution (max absorbance of 400nm) to a dark blue crystalline MTT formazan precipitate which when dissolved in SDS exhibits a maximum absorbtion at 570-590nm (Denizot & Lang 1986, Tada et al 1986.). The MTT compares favourably with the tritiated-thymidine method for measuring cell proliferation (Tada et al 1986) with the added advantages of:*

1. *no need for radioactive material and the associated problems of handling and disposal*
2. *MTT assay is quick: takes less than 1min. to add MTT to a 96 well plate.*
3. *The MTT formazan in SDS is stable for up-to 5 days.*
4. *It takes less than 10 seconds to read a 96 well plate in a Multiskan reader*

h. The absorbance of each well was measured using a MAXline Vmax microplate reader incorporating Softmax 2.01 programme(Molecular Devices Corp. California USA). A test wavelength of 570nm and a reference of 630nm was used. The resultant absorbtion of each well is proportional to the dose-dependently released IL-2 from the NOB1 cells by IL-1.

i. A control of rhIL-1α and 10% MLA-144 supernatant were employed at the NOB1 and CTLL stage respectively to test the cells responsiveness.

j. The supernatants from the peritoneal macrophages were also tested directly on the CTLL cells to see if they could directly maintain the viability or proliferation of this cell line.

k. All results obtained with the assay system were expressed as a percentage of the respective control employed to give % increases from controls ie.

$$\frac{(\text{Response}) - (\text{Basal})}{(\text{Control-response})-(\text{Basal})} \times 100$$

Fig.2.3 shows a typical dose-response curve for rhIL-1α on EL4 cells grown in normal RPMI-1640 and fig. 2.4 shows a dose response obtained for EL4 cells grown in DM-RPMI-1640.

2.8 RIA for PGE_2

A radioimmunoassay (RIA) (from New England Nuclear Labs) was used to quantitate PGE_2 in supernatants from macrophages stimulated with LPS. All freeze-thawed samples were immediately assayed for PGE_2 prior to storage at -75°C. The kit employs ^{125}I-PGE_2 as the radioactive antigen which competes for a fixed number of antibody binding sites for binding with the native PGE_2 to be assayed . The assay was performed on duplicate samples and standards. The antigen-antibody complex was separated from the free antigen by precipitation of the antibody-bound tracer with polyethelyene glycol in the presence of a carrier immunoglobulin. After centrifugation, the supernatant containing the unbound antigen was decanted and the pellet containing the antigen-antibody complex was counted in a gamma counter (Beckman LS 1801) until a significant count was reached. After counting, the concentration of PGE_2 in the samples was determined from a standard curve by :

1. Correcting all raw counts to counts per min. (CPM).

2. Averaging the counts for each set of duplicates.

3. Calculating the average net counts for all the standards and samples by subtracting from each the average blank counts ie.those to which tracer and buffer had been added to determine non-specific binding

4. The normalised % bound (% B/B_o) was calculated for each standard and sample as follows:

$$\% B/B_o = \frac{\text{Net CPM of Standard or Sample}}{\text{Net CPM of "0" Standard*}} \times 100$$

   * "0" standard were the tubes to which only the PGE_2 tracer and the antibody were added.

5. % B/B_o was plotted against the log of the standard PGE_2 (pg per tube) and the quantity PGE_2 in the samples was determined by interpolation from the standard curve an example of which is given in fig 2.5.

6. The quantity of PGE_2 in the original supernatant was calculated by multiplying the quantity obtained in 5 by the dilution factor of the original supernatant used. A dilution of 1:2000 was employed for macrophage supernatants where the macrophages were not incubated with a NSAID and 1:1000 for NSAID-treated macrophages.

The dilution steps and containers used in the above protocol were in accordance with the manufacturers instructions. Due to the high concentration of PGE_2 in the macrophage supernatants, extraction and concentration of the PGE_2 was not required . The effect of the NSAIDs on the RIA system and on antigen-antibody binding were also investigated. The RIA was also used

to determine the ability, if any, of indomethacin-treated macrophages to metabolise exogenously added PGE_2 (see chapter 4). The cross-reactivity of the assay is given in table 2.2. The percentages are calculated at the 50% B/B_o point (from New England Nuclear (NEN) booklet).

Table 2.2 Cross-reactivity of RIA kit.

COMPOUND	% CROSS-REACTIVITY	COMPOUND	% CROSS-REACTIVITY
PGE_2	100	AA	<<0.01
PGE_1	3.7	$DHKF_{2\alpha}$	<<0.01
$DHKPGE_2$	0.4	PGA_1	<<0.01
PGA_2	0.4	PGB_2	<<0.01
PGF_1	0.03	$6KPGF_1$	<<0.01
THROMBOXANE B_2	0.02	LINOLEIC ACID	<0.001
PGF_2	>0.01	PGD_2	<0.001

KEY: AA arachidonic acid; K keto; DH dihydroxy

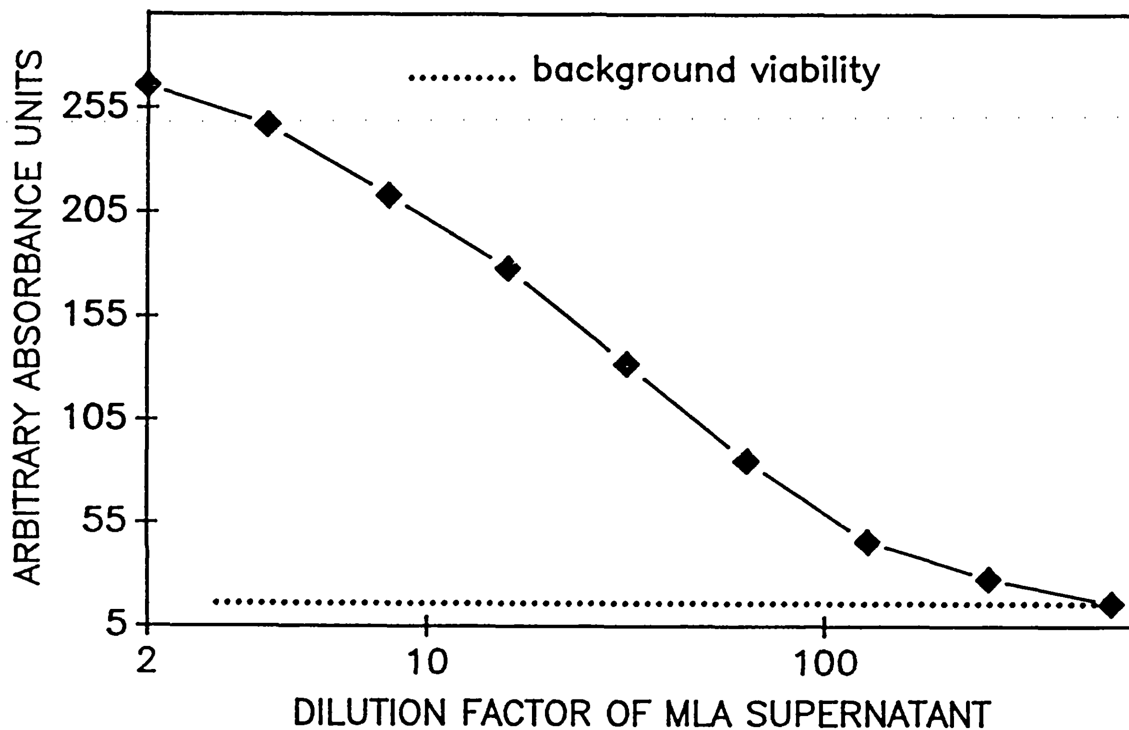


Figure 2.2 The effect of MLA supernatant on CTLL viability. The CTLLs were cultured for 24hr. in the presence of varying concentrations of MLA supernatant. The MTT method was used to assess cell viability. Each point represents the mean of triplicate readings.

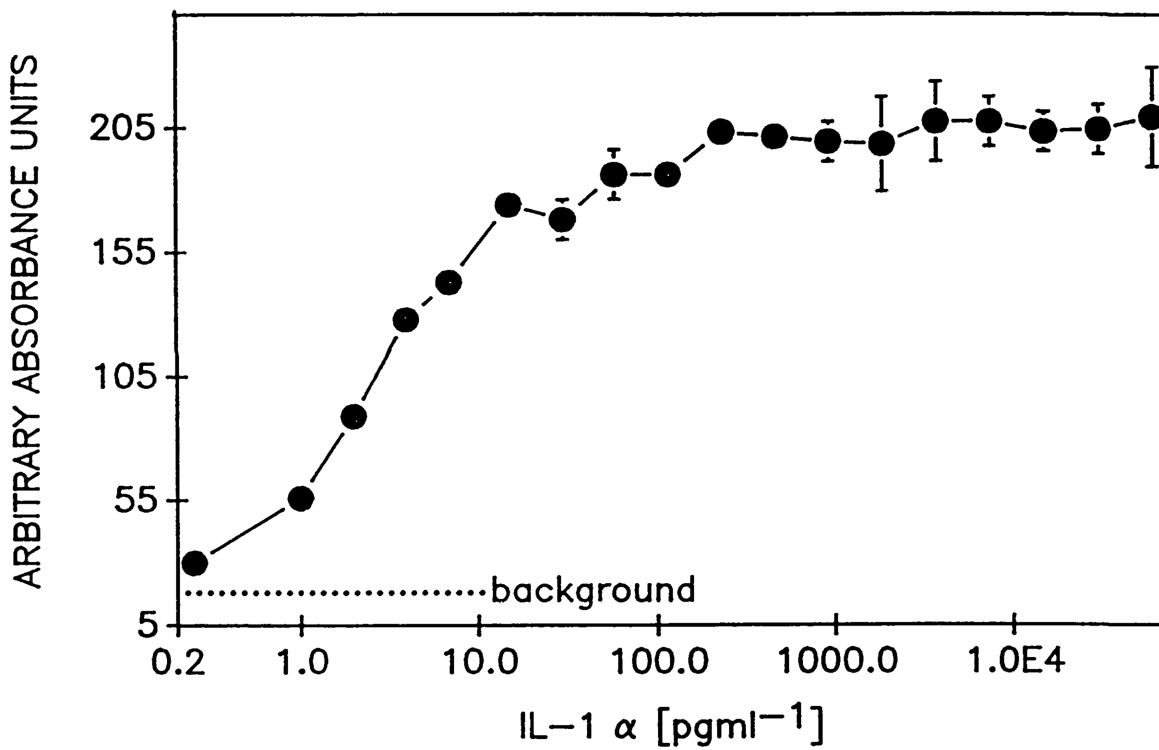


Figure 2.3 A typical dose-response curve for rhIL-1α. Each point represents the mean ± standard error for triplicate readings. The EL4 NOB1 and CTLLs were grown in normal supplemented RPMI-1640

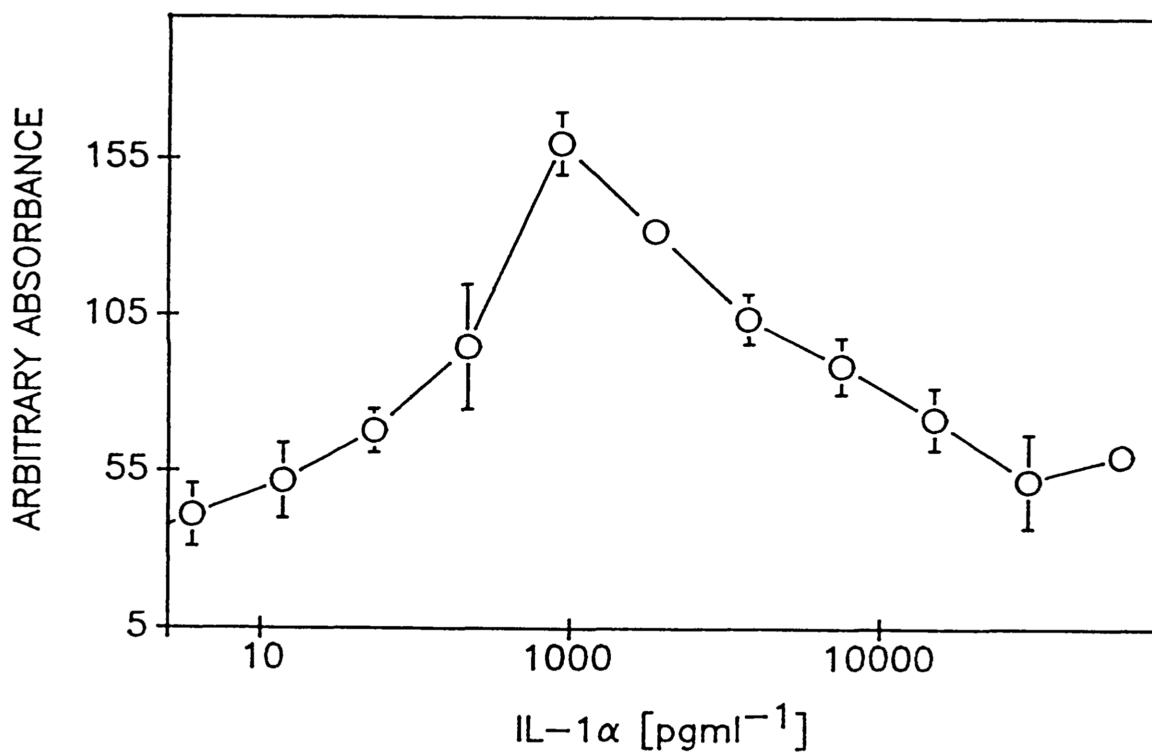


Figure 2.4 A typical dose-response curve for rhIL-1α. Each point represents the mean ± standard error for triplicate readings. The EL4 NOB1s were grown in supplemented Dutch-modification-RPMI-1640 and CTLLs were grown in normal supplemented RPMI-1640. The Dutch-modification medium adversely affects the ability of the EL4 cells to respond to rhIL-1α.

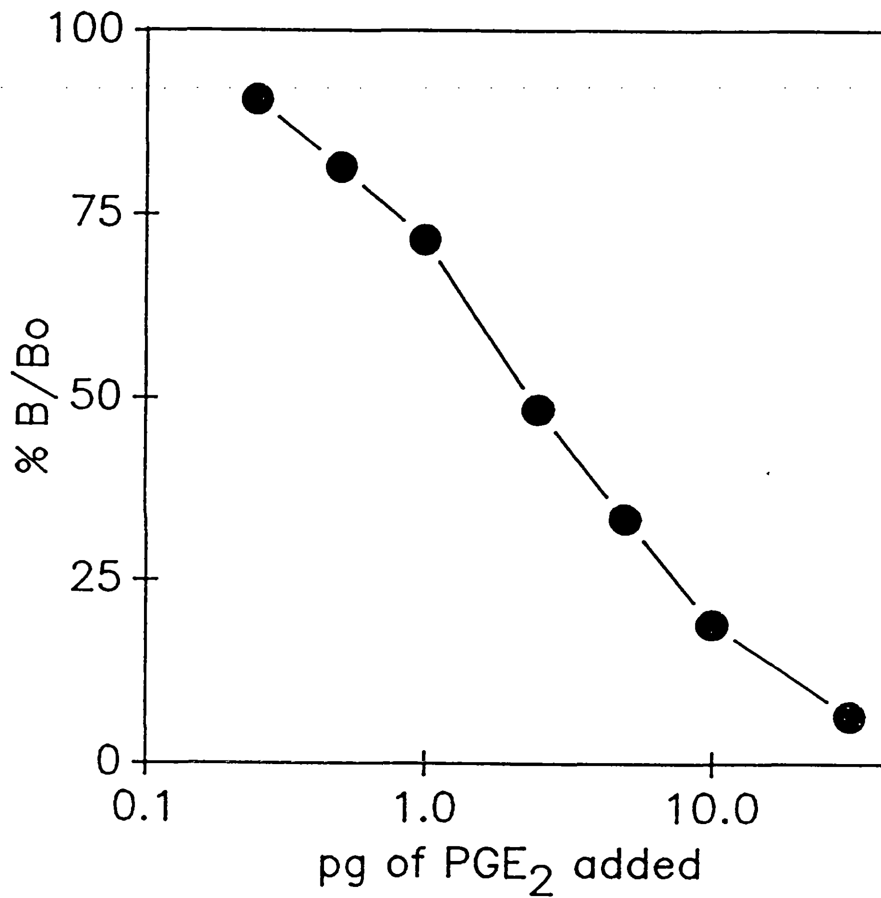


Figure 2.5 A standard curve for PGE_2 using the commercial radioimmunoassay kit from New England Nuclear. Concentrations of PGE_2 in macrophage suppernatants were determined from such standard curves

CHAPTER THREE

STIMULI USED TO INDUCE IL-1 PRODUCTION

3.1 INTRODUCTION

A number of stimuli have been reported to induce the synthesis and secretion of IL-1 *in vitro*. A range of stimuli were used in the present study. The following section will provide background information on each stimulus used, and present a dose-response profile for each stimulus. The effect of the stimuli on the assay system will also be presented.

3.1.1 Lipopolysaccharide (LPS)

Both vertebrates and invertebrates have evolved complex mechanisms to respond to infection by Gram-negative bacteria. The response is characterised by initiation of various inflammatory mechanisms that rely on the recognition of LPS. These include:

i. Stimulation of cytokine secretion.
ii. Stimulation of superoxide production and enzyme release from neutrophils.
iii. Activation of the complement cascade.
iv. Stimulation of platelets and B cells .

Although the pro-inflammatory effects of LPS are beneficial when localised to a site of infection, the entry of small quantities of LPS into the circulation may result in a systemic inflammatory response leading to septic shock. Chemical characterization of LPS from a number of enterobacterial species has revealed the following common structural features:

i. Lipid A moiety.
ii. Core oligosaccharide.
iii. O-specific polysaccharide.

Lipid A has the same basic structure in practically all Gram-negative bacteria (see Fig.3.1). The core oligo-saccharide constitutes a domain of limited structural diversity within the same bacterial genera (Paulsen & Unger 1986). The core oligosaccharide and lipid A are linked through a 3-deoxy-D-manno-heptose and 3-deoxy-D-manno-2-octulosonic acid (KDO) residue, via an acid-labile bond, in all LPS molecules. The O-specific polysaccharide is the outer-most of the endotoxin molecule and is exposed on the surface of the intact bacterium. This structure is often referred to as the O-specific antigen and is the major determinant to which the host makes antibody.

It was originally thought that the lipid A fraction of LPS was responsible for most of the stimulatory activities of LPS (Rietschle et al., 1987). However, the polysaccharide moiety has also been found to express certain biological activities, such as induction of colony-stimulating factors (Behling & Nowotny, 1982), interferon (Nowtony et al., 1986), adjuvanticity (Scibienski, 1980), B cell mitogenicity (Williamson et al., 1984) and IL-1 secretion (Haffner-Cavaillon et al., 1984; Haffner-Cavaillon et al., 1989).

Given the ubiquitous nature of LPS and its potential toxicity, it is not surprising that several mechanisms of cellular detoxification exist. One such mechanism is deacylation by acyloxyacyl hydrolase an enzymatic activity which has been identified in human polymorphonuclear cells and murine macrophages (Hall & Munford, 1983; Munford & Hall, 1985). Acyloxyacyl hydrolase removes fatty acyl side-chains from Lipid A. The enzymatically deacylated LPS retains some of its immunostimulatory response, as determined by its activity in the dermal Shwarzman reaction (Munford & Hall, 1986).

The molecular basis for the interaction of LPS with immune cells has been the subject of much investigation. LPS appears to interact with membranes in two distinct steps (Hamilton & Adams, 1987):

1. A rapid temperature-independent attachment to cells

2. A time- and temperature-dependent event which may represent intercalation of the lipid moiety of LPS into the membraneous bilayer.

A number of cellular LPS-binding proteins have been described (Kirkland et al., 1990) but such proteins have yet to be shown to be functional receptors.

Throughout the present study a single batch of phenol extracted LPS from *Salmonella minnesota* (Re59s), was used to eliminate possible inter-batch variation in the stimulatory activity of the LPS on IL-1 production. Fig. 3.1 shows the structural components and localization of LPS in bacterial cell-walls.

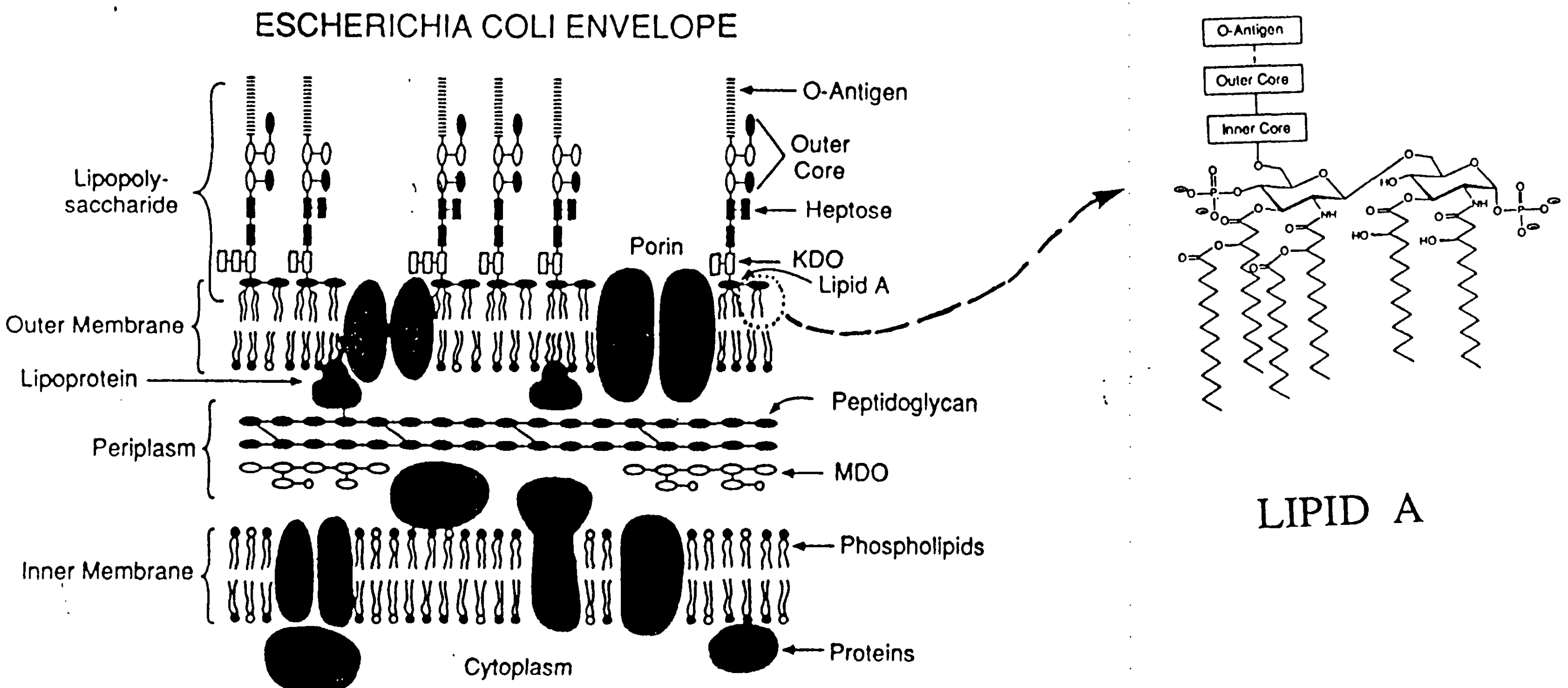


Figure 3.1 Structural components of lipopolysaccharide (LPS) (Adapted from Raetz 1990)

3.1.2 **Neuropeptides**

The ability of neuropeptides to modulate and/or initiate inflammatory responses has been addressed by a number of authors (Payan et al., 1984; Foreman 1987; Barnes, 1987). There is compelling evidence which suggests a role for the nervous system and neuropeptides in the arthritic process. The main points of which are summarised below:-

- Patients who develop RA after sustaining paralysing lesions to the nervous system are spared joint inflammation in the paralysed limb (Levin et al., 1985).

- Substance P (SP) has been shown to amplify experimental adjuvant arthritis in rats (Levin et al., 1984).

- SP has been shown to induce proinflammatory effects in macrophages, neutrophils, mast-cells, T and B cells (reviewed by McGillis et al., 1987).

- SP, neurokinin A and neurokinin B have been shown to induce IL-1 secretion from macrophages and macrophage cell-lines (Lotz et al., 1987; Lotz et al., 1988; Kimball et al.,1988a).

- Neuropeptides have been shown to enhance the proliferative effect of IL-1 on fibroblasts (Kimball & Fisher, 1988b).

- Intra-articular injection of IL-1 produced elevated SP levels in the knee joints of rabbits compared to contralateral knee joints injected with saline (O'Bryne et al., 1990).

In consideration of the above, the ability of SP, NK-A, NK-B and cGRP to modulate IL-1 production from resident murine macrophages has been investigated.

3.1.3 Platelet Activating Factor (PAF)

PAF (1-alkyl-2(R)-acetyl-glycero-3-phosphocholine) is an ether-phospholipid synthesized endogenously from arachidonic acid. PAF was originally characterized in 1972 as a platelet aggregating factor released from immunoglobulin-E activated rabbit basophils. PAF has since been shown to be an important regulator of the cellular immune response and an important inducer of acute and chronic inflammatory processes (Braquet & Rola-pleszczynski., 1987). Pipric et al. (1988) have shown the existence of PAF receptors on murine peritoneal macrophages with a Kd of $\sim10^{-10}$M. The stimulation of such receptors was shown to activate phosphatidylinositol turnover in the macrophage membrane. The EC_{50} for initiating such responses was $5x10^{-9}$M. The ability of PAF to stimulate IL-1 secretion from monocytes/macrophages has been reported by a number of authors:-

- Pignol et al. (1987) showed that low doses (pM) augment IL-1 secretion and high doses (μM) inhibit secretion.

- Salem et al. (1990) have recently shown that PAF (1nM to 5μM) can potentiate LPS-stimulated IL-1 secretion from monocytes. PAF alone had no effect on IL-1 secretion.

The ability of PAF (10^{-10}M to 10^{-6}M) to stimulated and potentiate IL-1 secretion from resident murine macrophages has been investigated.

3.1.4 Tumour necrosis factor:-

TNFα was originally described by Carswell et al (1975) as an endotoxin-induced serum factor that caused haemorrhagic necrosis of certain tumours *in vivo*. TNFα is produced by activated macrophages/monocytes and to a lesser extent by lymphocytes, keratinocytes and endothelial cells. The ability to inhibit lipoprotein lipase and induce a wasting syndrome in

mice lead to the early descriptive term **cachectin** for TNFα. TNFβ, a structurally related peptide, historically called lymphotoxin, has similar biological properties to TNFα but is mainly synthesized by mitogen-stimulated lymphocytes (Nedwin et al., 1985). Both TNFα and TNFβ are able to act at the same cell-surface receptor. The biological properties of TNF and IL-1 overlap in many respects (for review see Dinarello, 1989). TNFα has been shown to stimulate the production of IL-1 in monocytes and macrophages (Dinarello et al., 1986, Bachwic et al., 1986) and vascular endothelial cells (Nawroth et al., 1986; Libby et al., 1986). Indeed Brennan et al;. (1989) have shown that incubation of synovial cells, isolated from RA patients, with anti-TNFα antibodies (but not anti-TNFβ) abrogates the spontaneous production of IL-1 from such cells. This has led to TNFα being implicated as a chronic stimulus for IL-1 production in the RA joint. The ability of human recombinant TNFα (rhTNFα) to stimulate the production of IL-1 from murine macrophages has been investigated.

3.1.5 Calcium Ionophores

A23187 and ionomycin were used as the calcium ionophores. A23187 is a carboxylic acid ionophore which complexes Ca^{2+} and subsequently transfers the cation into the cell from the extracellular medium. Monovalent cations such as Na^{+} and K^{+} are not complexed by A23187. Ionomycin is a polyether antibiotic isolated from *Streptomyces conglobatus*. Ionomycin is highly specific for divalent cations, it complexes with Ca^{2+} in one-to-one stoichiometry and is capable of extracting Ca^{2+} ions from an aqueous phase into an organic phase. The following order of ion specificity has been observed for ionomycin: $Ca^{2+} > Mg^{2+} >> Sr^{2+} = Ba^{2+}$ in this respect ionomycin is a more selective Ca^{2+} ionophore than A23187 which complexes both Mg^{2+} and Ca^{2+} with similar affinities (Liu & Herman, 1978).

A number of studies have looked at the ability of Ca^{2+} ionophores to stimulate IL-1 secretion:-

- Matsushima and Oppenheim (1985) have shown that A23187 can stimulate IL-1 secretion from monocytes.

- Simon (1985) found that A23187 enhanced IL-1 production by P388D1 cells.

- Newton (1987) failed to observe IL-1 secretion or intracellular accumulation of IL-1 in human monocytes stimulated with A23187 or ionomycin (20nM to 2μM).

- Shinomyia & Nakano (1987) showed that 1μM A23187 markedly stimulated the accumulation of intracellular IL-1 and moderately stimulated its secretion from thioglycollate-elicited murine peritoneal macrophages.

- Suttles et al. (1990) showed that A23187 and ionomycin were unable to stimulate IL-1 production from P388D1 cells or thioglycollate-elicited murine peritoneal macrophages. However, the ionophores did potentiate the secretion of intracellular stores of IL-1 stimulated by low concentrations of LPS. Furthermore FS-4 cells, a human fibroblast cell-line, which synthesize but do not secret IL-1, accumulated IL-1ß in response to stimulation by TNFα 10ng.ml^{-1} but failed to secrete IL-1 upon subsequent stimulation with Ca^{2+} ionophores. This suggests that the ionophores potentiate an existing mechanism for IL-1 secretion rather than cause a non-specific leakage of intracellular IL-1.

- Brandwein (1990) showed that 1μM A23187 induced both membrane IL-1 expression and IL-1 secretion from thioglycollate-elicited murine macrophages. However, this stimulation could not be dissociated from cell-toxicity. Lower, non-cytotoxic concentrations of A23187 (0.1μM) failed to stimulate IL-1 secretion from the macrophages. However, a slight increase in intracellular IL-1 accumulation was observed.

Thus, conflicting data exists regarding the ability of Ca^{2+} ionophores to stimulate IL-1 production.

3.1.6 **4ß-PDBu and diC8**

Tumour-promoting phorbol esters, such as 4ß-PDBu have been shown to bind to protein kinase C (PKC) intracellularly and activate PKC by mimicking the structure of the endogenous PKC activator, diacylglycerol (DAG) (Nishizuka, 1984; Yaminishi et al., 1983). The use of PKC activators has led to the implication of a regulatory role for PKC in the endocrine, exocrine and immune systems (for review see Nishizuka, 1986). Generally, the phorbol esters are poorly metabolized by cells and produce a prolonged activation of PKC. The endogenous activators of PKC, the diacylglycerols, are only produced transiently in membranes and are rapidly metabolized by specific enzymes. Thus phorbol esters represent unphysiological stimulators of PKC. A much closer approximation to physiological PKC activation can be achieved using the synthetic DAG: 1,2-dioctanoyl-sn-glycerol (diC_8). The use of diC_8 was preferred for the following reasons:-

1) Diacylglycerols derived from receptor-mediated hydrolysis of phosphoinositides (1-stearoyl, 2-arachidonylglycerol being one of the main naturally occurring species) do not readily intercalate into intact cell membranes and thus cannot be used to activate cells *in vitro*.

2) DiC_8 showed similar activity (as judged by comparable Vmax values) in activating PKC when compared with the endogenous DAG, 1-stearoyl, 2-arachidonylglycerol (Go et al., 1987).

3) DiC_8 competes for binding with ^3H 4ß-PDBu in intact neutrophils thus demonstrating the accessibility of the analogue to the PKC *in situ* (Cox et al., 1986).

4) Cox et al.,(1986) have found diC_8 to be the most potent DAG analogue with respect to stimulating the respiratory burst in neutrophils.

5) DiC_8 shows a similar profile of metabolism to the endogenous DAG ie. both are metabolized mainly by DAG kinase (Muid et al. 1987).

4ß-PDBu was used as the phorbol ester of choice since it is relatively hydrophillic compared with phorbol myristate acetate (PMA) and can be washed off treated cells, unlike PMA (Rodriguez-Pena & Rozengurt, 1984; Collins & Rozengurt, 1984). Structurally the active 4ß-PDBu molecule possesses a relatively polar head group (the phorbol moiety) with lipophilic ester substituent in the 12- and 13-positions of the phorboid nucleus. Thus, to some extent it represents a non-ionic detergent. In order to distinguish between receptor-mediated activity of 4ß-PDBu and non-specific effects, due possibly to membrane perturbation caused by its detergent like property; the inactive 4α-PDBu isomer was used as a control. The 4α position of the hydroxy group in 4α-PDBu renders it inactive as a PKC stimulator whilst maintaining the

same physico-chemical properties of the 4ß isomer. Katakami et al. (1986) have reported that 4-ßPDBu can dose-dependently stimulate IL-1 secretion from cycloheximide treated, thioglycolate elicited, murine peritoneal macrophages.

Brandwein (1990) showed that 1-oleoyl-2-acetylglycerol (OAG) a DAG analogue was unable to stimulate IL-1 secretion or intracellular accumulation. However, OAG is a relatively unstable DAG analogue compared to diC_8 and is not as effective as diC_8 at stimulating PKC.

The dose-dependent effects of 4ß-PDBu and diC_8 on IL-1 secretion and accumulation were investigated in the present study.

3.2 RESULTS

3.2.1 The effect of LPS on IL-1 production

LPS from *Salmonella minnesota* was found to stimulate IL-1 production and secretion of IL-1 in a dose-dependent manner (Fig. 3.2). Low doses of LPS (less than $10\mu g.ml^{-1}$) were more effective by about 100-fold at stimulating cellular accumulation of IL-1 than IL-1 secretion. LPS did not stimulate EL4 NOB1 cells to release IL-2 or inhibit the ability of the cells to respond to a sub-optimal dose of rhIL-1α (0.01 Uml^{-1}) (Fig.3.3). LPS was not cytotoxic to the cells at the concentrations use.

3.2.2 The effect of neuropeptides on IL-1 production

SP, NK-A, NK-B and cGRP were without effect at stimulating IL-1 production. The neuropeptides did not potentiate or inhibit IL-1 production stimulated by LPS $1\mu g.ml^{-1}$ (Fig 3.4ab, 3.5ab, 3.6ab, 3.7ab)

3.2.3 The effect of PAF on IL-1 production

PAF did not stimulate IL-1 production. PAF was also without effect on IL-1 production stimulated by LPS $1\mu g.ml^{-1}$ (Fig. 3.8ab).

3.2.4 The effect of TNFα on IL-1 production

rhTNFα dose-dependently stimulated the accumulation of cell-associated IL-1. However, rhTNFα did not induce IL-1 secretion at the concentration employed (Fig. 3.9). At high doses rhTNFα was shown to stimulate EL4 NOB1 cells to release IL-2, but did not directly stimulate CTLL proliferation (Fig 3.10). The maximal stimulation of EL4-NOB1 cells with rhTNFα was approximately 40% of the maximal stimulation observed with

rhIL-1α. The highest concentrations of rhTNFα likely to be found in the diluted supernatants placed onto EL4-NOB1 cells (< 0.03 $\mu g.ml^{-1}$) did not directly stimulate or synergize with rhIL-1α to stimulate EL4-NOB1 cells (Fig.3.11) . When the TNFα sample was heated at 100°C for 10 mins the ability of TNFα to stimulate IL-1 production was lost (Fig 3.9.)

3.2.5 The effect of calcium ionophores on IL-1 production

The calcium ionophores A23187 and ionomycin dose-dependently stimulated the accumulation of cell-associated IL-1. The ionophores did not stimulate the secretion of IL-1 (Fig 3.12 and 3.13). Ionomycin did not stimulate EL4-NOB1 cells at concentrations likely to be present in the diluted macrophage supernatants (10^{-11}M - 10^{-8}M). Ionomycin at a concentrations of 10^{-8}M did not affect the stimulation of EL4 NOB1 cells with rhIL-1α. Fig. 3.14 shows a typical dose-response curve to IL-1α in the presence of 10^{-8}M ionomycin. Concentrations above 10^{-6}M for ionomycin and $5x10^{-7}$M for A23187 were shown to be cytotoxic to the macrophages as judged by uptake of trypan blue and lysis of the cell.

3.2.6 The effect of 4ß PDBu, 4α PDBu and diC8 on IL-1 production

DiC_8 12.5-200 μM did not significantly stimulate the accumulation of cell-associated IL-1 or secretion of IL-1 (Fig 3.15). DiC_8 at concentrations likely to be present in the diluted macrophage supernatants (0.01-0.2μM) did not affect the ability of EL4 NOB1s to respond to a sub-optimal dose of IL-1 (Fig. 3.16). The inactive phorbol-ester isomer 4α PDBu did not stimulate IL-1 production (Fig 3.17b). 4ß PDBu 10^{-10} - 10^{-7}M had no effect on IL-1 production. At 10^{-6}M 4ß-PDBu stimulated the secretion of IL-1 but did not stimulate cell-associated IL-1 accumulation (Fig 3.17a). The phorbol esters were allowed to incubate for 4hr. with the macrophages prior to washing off with three rinses of RPMI-1640. When 4ß PDBu

was allowed to remain on the macrophages for the full duration of the incubation (20hr) to generate IL-1, there was still no detectable accumulation of cell-associated IL-1 (Fig.3.18) . 4ß PDBu dose-dependently stimulated EL4 NOB1 cells to release IL-2 (Fig 3.19) but did not directly effect CTLL viability.

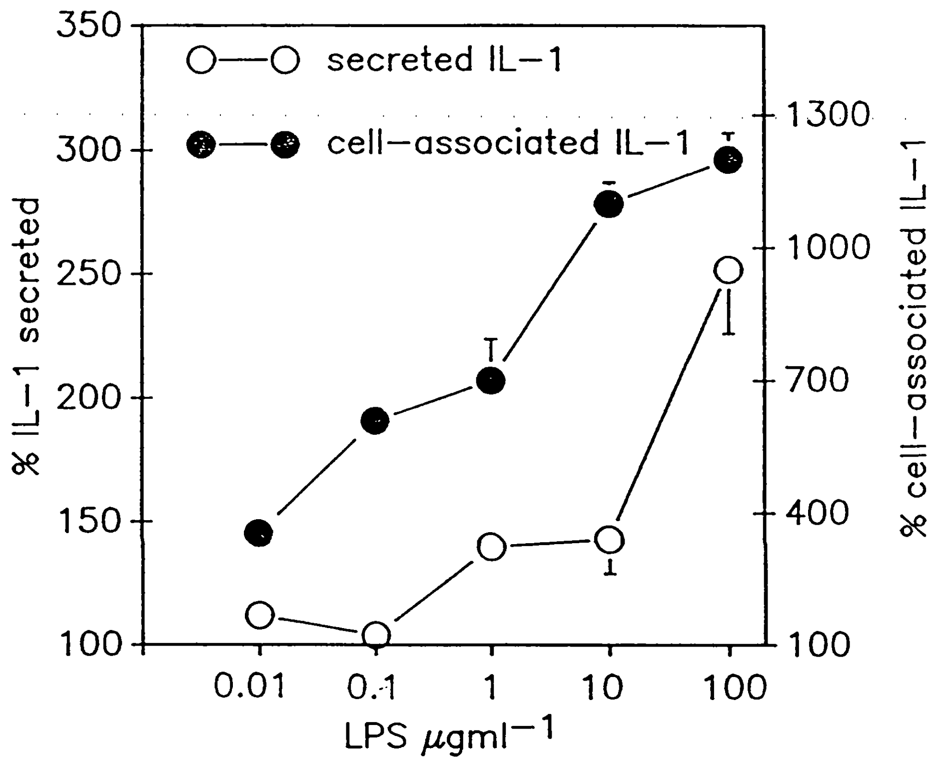


Figure 3.2 The effect of LPS on secreted and cell-associated IL-1 from resident murine macrophages. The macrophages were incubated for 20hr. at 37°C in serum free RPMI-1640 supplemented with glutamine, antibiotic and varying concentrations of LPS. Basal level of IL-1 production (corresponding to 15 ± 10 and 40 ± 19 abs. units for supernatants and lysates) was arbitrarily set at 100% . Each value is the mean ± standard error (n=3)

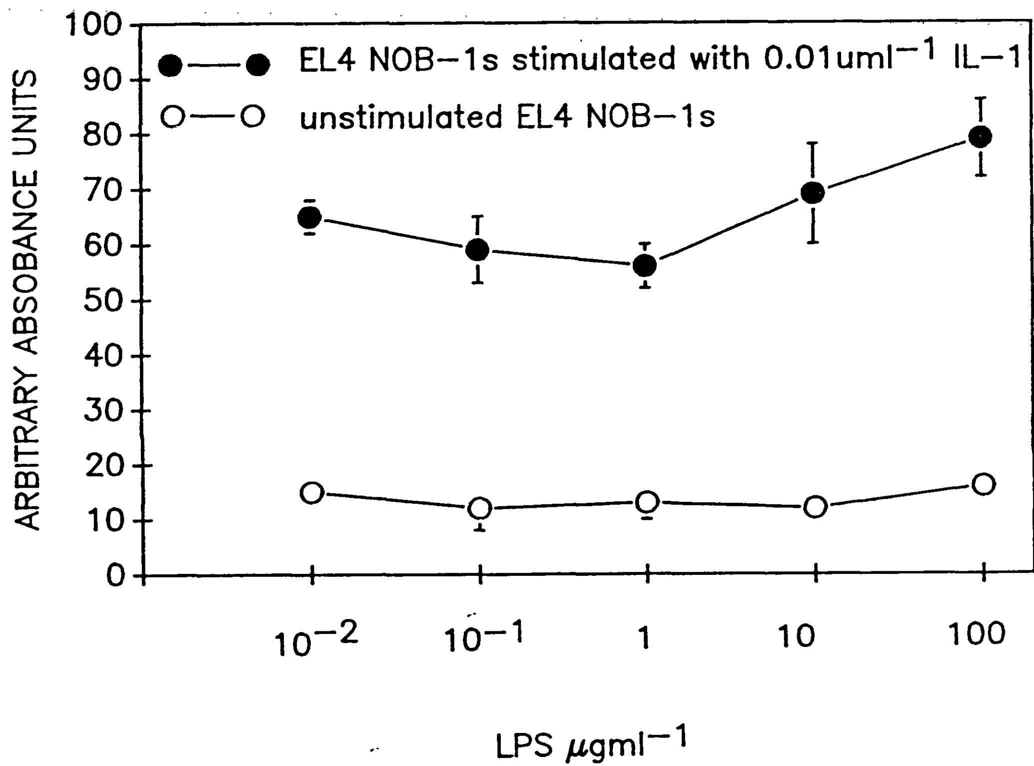


Figure 3.3 The effect of LPS on the stimulation of EL4 NOB1 cells. The cells were cultured in normal supplemented RPMI-1640 for 24hr. as outlined in chapter 2. Each point represents the mean ± standard error (n=3)

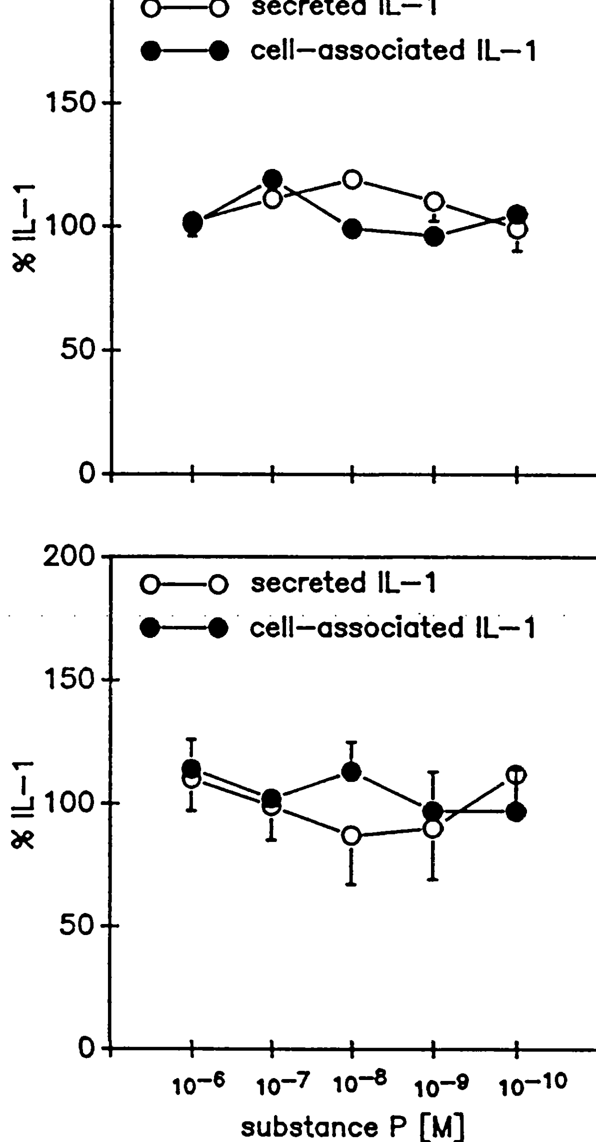


Figure 3.4 (a) The effect of substance P on IL-1 production from resident murine macrophages.(b) The effect of substance P on IL-1 production from resident murine macrophages stimulated with $1\mu g.ml^{-1}$ LPS .

Supernatants were diluted 100 to 400 fold for the determination of secreted IL-1 and 1000 to 4000 fold for the determination of cell-associated IL-1 from LPS stimulated cells. In cells not stimulated with LPS, dilutions of 25 to 100 and 250 to 1000 were used respectively for IL-1 secretion and cell-associated IL-1. Basal level of IL-1 production (corresponding to 10±5 and 29±16 in unstimulated and 19±7 and 70±20 abs.units in stimulated supernatants and lysates respectively) was arbitrarily set at 100% . Each point represents the mean ± standard error (n=3)

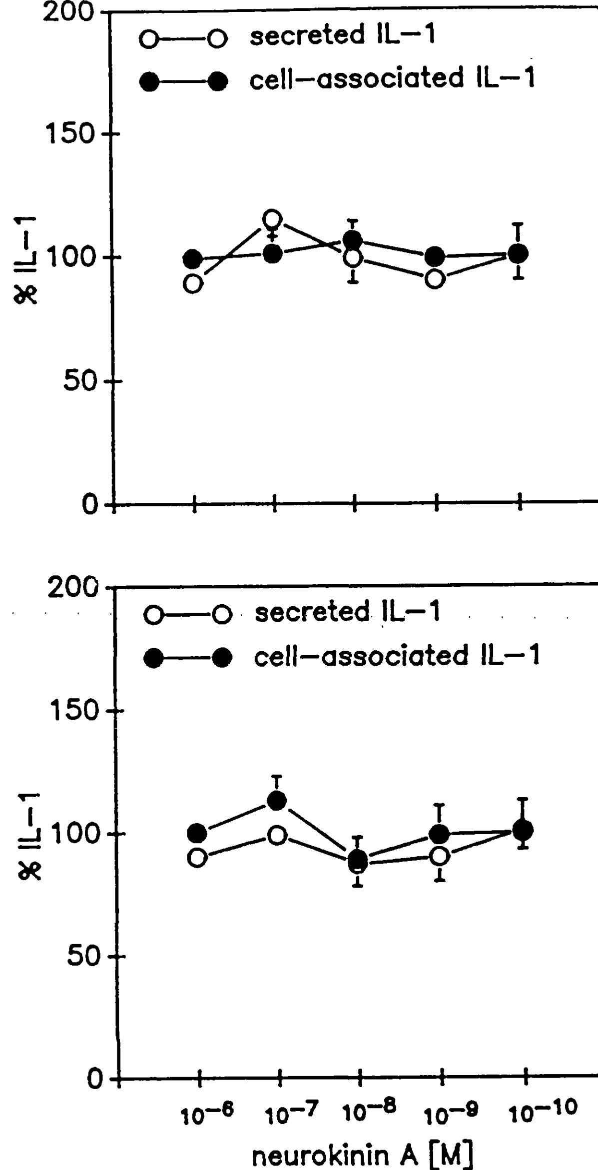
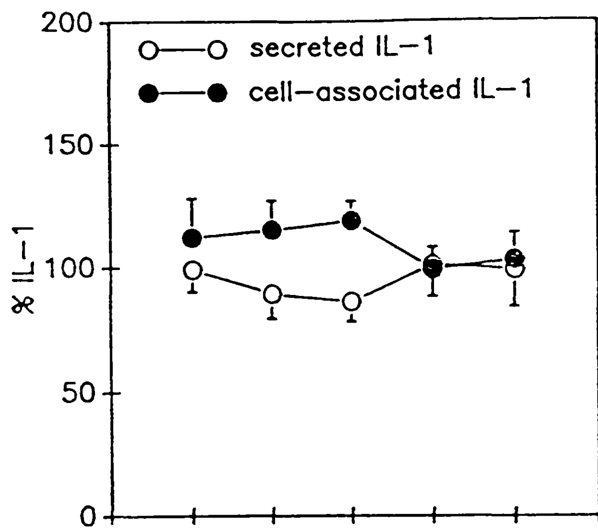


Figure 3.5 (a) The effect of neurokinin A on IL-1 production from resident murine macrophages. (b) The effect of neurokinin A on IL-1 production from resident murine macrophages stimulated with $1\mu g.ml^{-1}$ LPS

Supernatants were diluted 100 to 400 fold for the determination of secreted IL-1 and 1000 to 4000 fold for the determination of cell-associated IL-1 from LPS stimulated cells. In cells not stimulated with LPS, dilutions of 25 to 100 and 250 to 1000 were used respectively for IL-1 secretion and cell-associated IL-1. Basal level of IL-1 production (corresponding to 15±5 and 41±11 in unstimulated and 21±7 and 120±12 abs.units in stimulated supernatants and lysates respectively) was arbitrarily set at 100% . Each point represents the mean ± standard error (n=3)



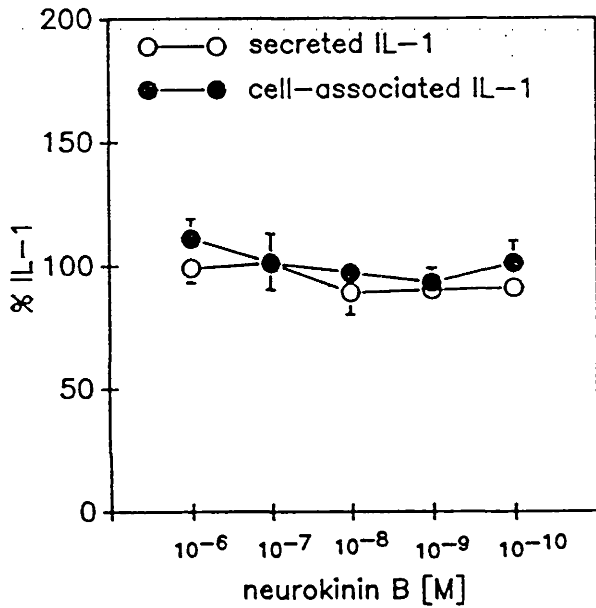


Figure 3.6 (a) The effect of neurokinin B on IL-1 production from resident murine macrophages.(b) The effect of neurokinin B on IL-1 production from resident murine macrophages stimulated with $1\mu g.ml^{-1}$ LPS.

Supernatants were diluted 100 to 400 fold for the determination of secreted IL-1 and 1000 to 4000 fold for the determination of cell-associated IL-1 from LPS stimulated cells. In cells not stimulated with LPS, dilutions of 25 to 100 and 250 to 1000 were used respectively for IL-1 secretion and cell-associated IL-1. Basal level of IL-1 production (corresponding to 5±3 and 15±6 in unstimulated and 10±7 and 60±15 abs.units in stimulated supernatants and lysates respectively) was arbitrarily set at 100% . Each point represents the mean ± standard error (n=3)

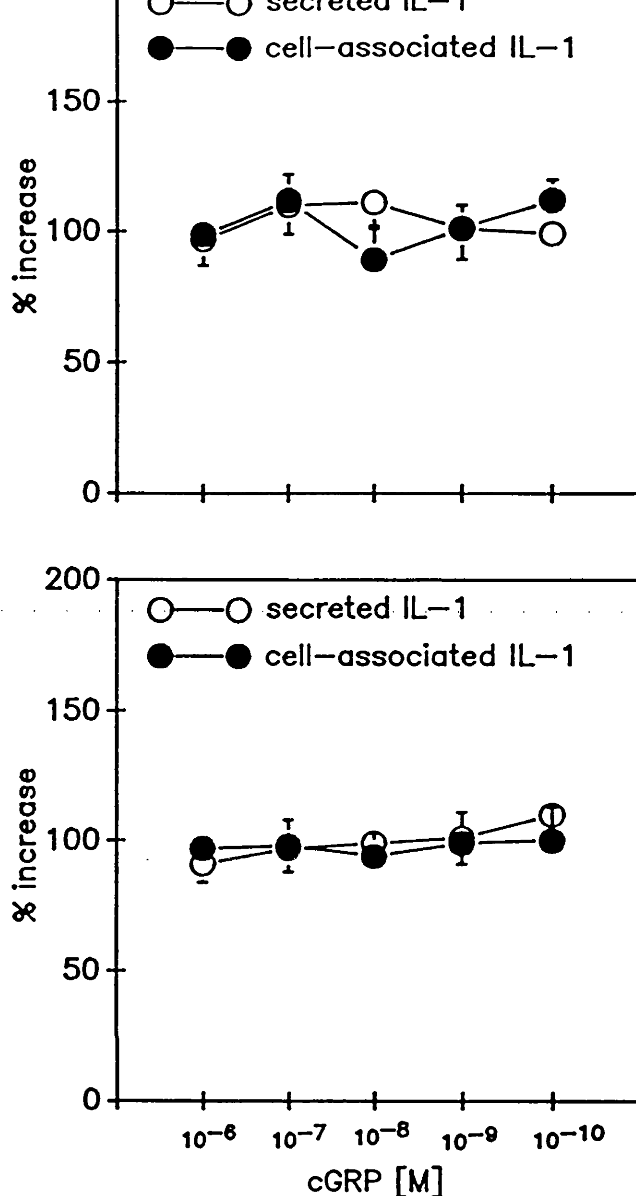


Figure 3.7 (a) The effect of calcitonin gene-related peptide on IL-1 production from resident murine macrophages. (b) The effect of calcitonin gene-related peptide on IL-1 production from resident murine macrophages stimulated with $1\mu g.ml^{-1}$ LPS .
The cells were cultured for 20hr. in supplemented RPMI-1640 as outlined in chapter 2. Supernatants were diluted 100-400 fold for the determination of secreted IL-1 and 1000-4000 fold for the determination of cell-associated IL-1 from LPS stimulated cells. In cells not stimulated with LPS, dilutions of 25-100 and 250-1000 were used respectively for IL-1 secretion and cell-associated IL-1. Basal level of IL-1 production was arbitrarily set at 100% . Each point represents the mean ± standard error (n=3)

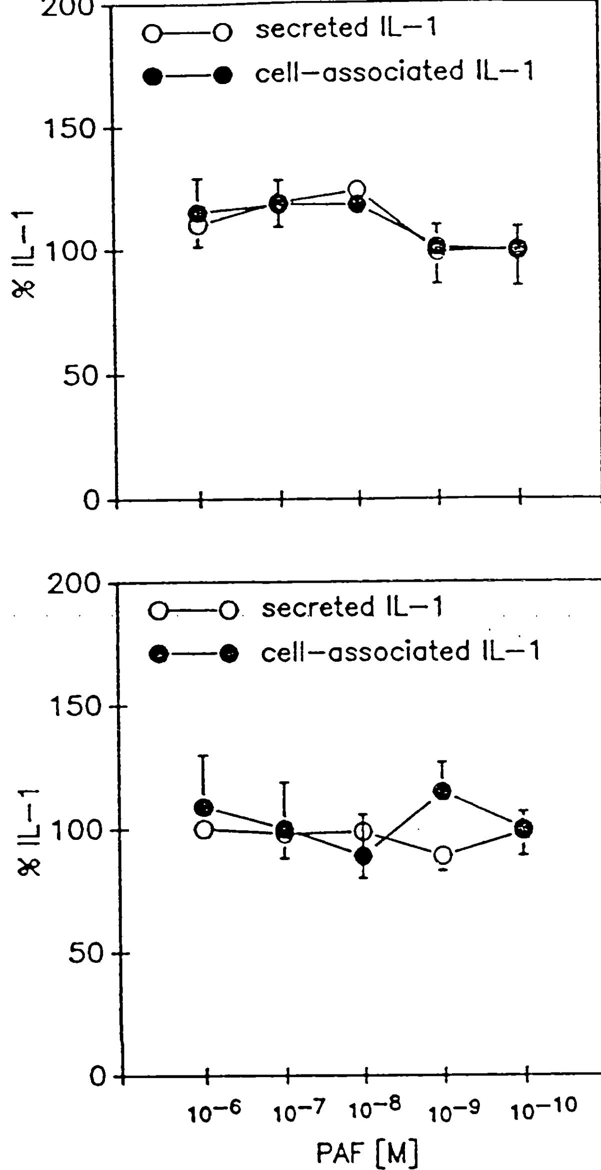


Figure 3.8 (a) The effect of platelet activating factor on IL-1 production from resident murine macrophages. (b) The effect of platelet activating factor on IL-1 production from resident murine macrophages stimulated with 1μg.ml^{-1} LPS.
Supernatants were diluted 100 to 400 fold for the determination of secreted IL-1 and 1000 to 4000 fold for the determination of cell-associated IL-1 from LPS stimulated cells. In cells not stimulated with LPS, dilutions of 25 to 100 and 250 to 1000 were used respectively for IL-1 secretion and cell-associated IL-1. Basal level of IL-1 production (corresponding to 14±9 and 21±11 in unstimulated and 21±3 and 72±8 abs.units in stimulated supernatants and lysates respectively) was arbitrarily set at 100% . Each point represents the mean ± standard error (n=3)

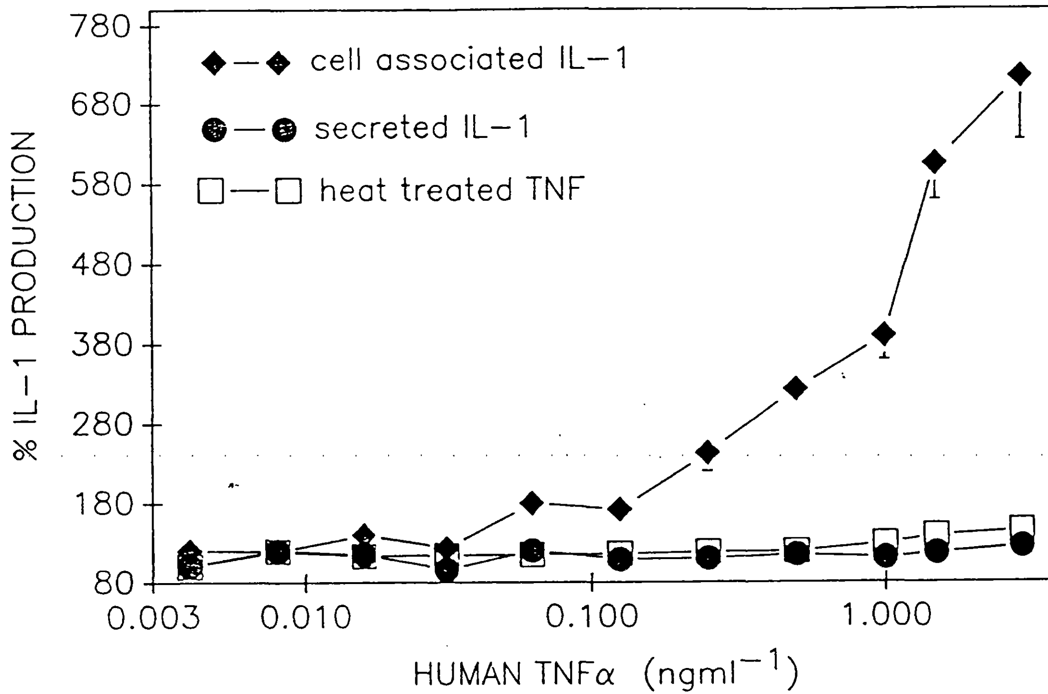


Figure 3.9 The effect of rhTNFα on IL-1 production from resident murine macrophages. The cells were cultured for 20hr. in the presence of varying concentrations of TNF. Dilutions of 100 to 400 and 1000 to 4000 fold were respectively employed for the determination of secreted and cell-associated IL-1. Basal level of IL-1 production (corresponding to 12 ± 6 and 37 ± 13 abs. units for supernatants and lysates) was arbitrarily set at 100%. Each point represents the mean ± standard error (n=4)

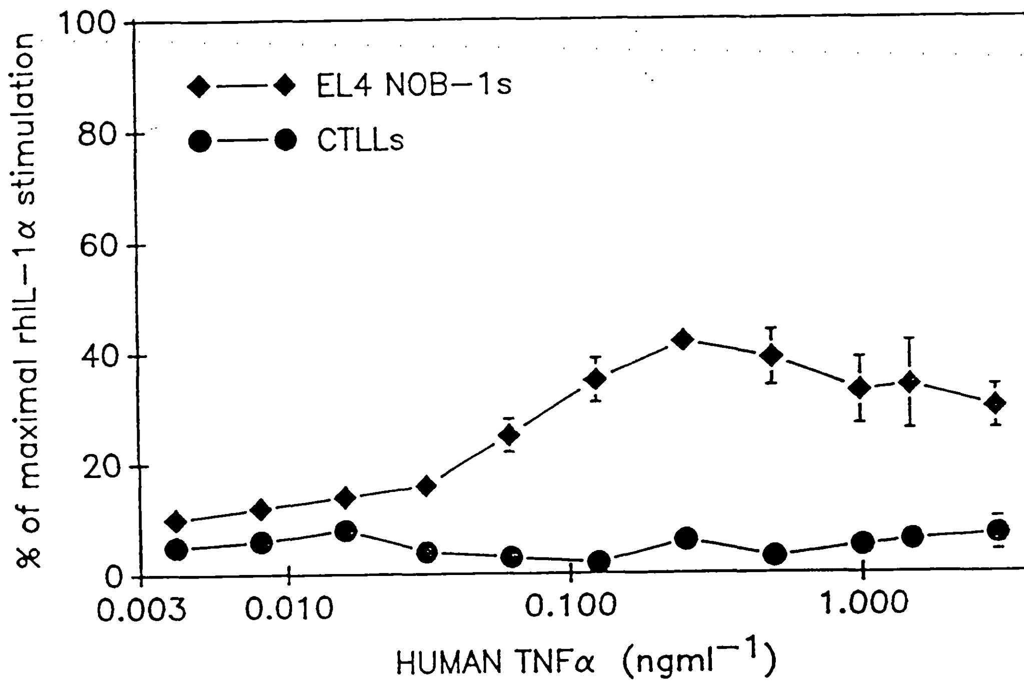


Figure 3.10 The effect of rhTNFα on the stimulation of EL4 NOB1 and CTLL cells. The stimulation of EL4 NOB1 cells with rhTNFα was expressed as a percentage of the maximum response produced by IL-1. CTLLs were not stimulated to maintain their viability by either IL-1α or rhTNFα. Each point represents the mean ± standard error (n=3)

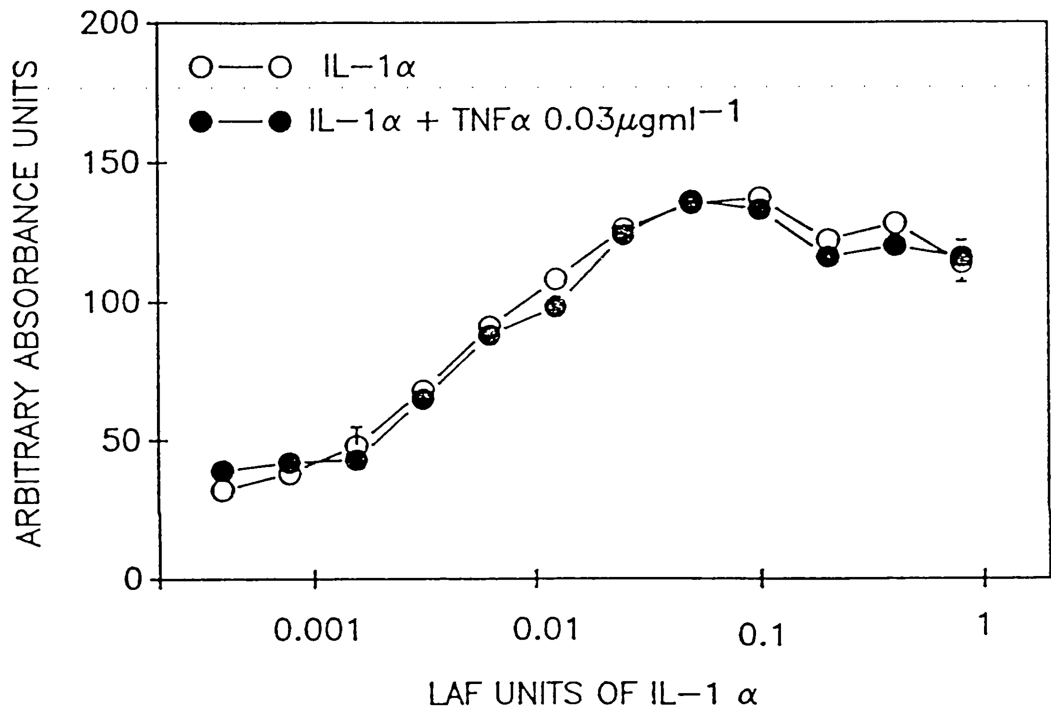


Figure 3.11 The effect of rhTNFα on the dose-response to IL-1α in EL4 NOB1 cells. The above figure is representative of a typical result. Each point represents the mean of triplicate readings ± standard error.

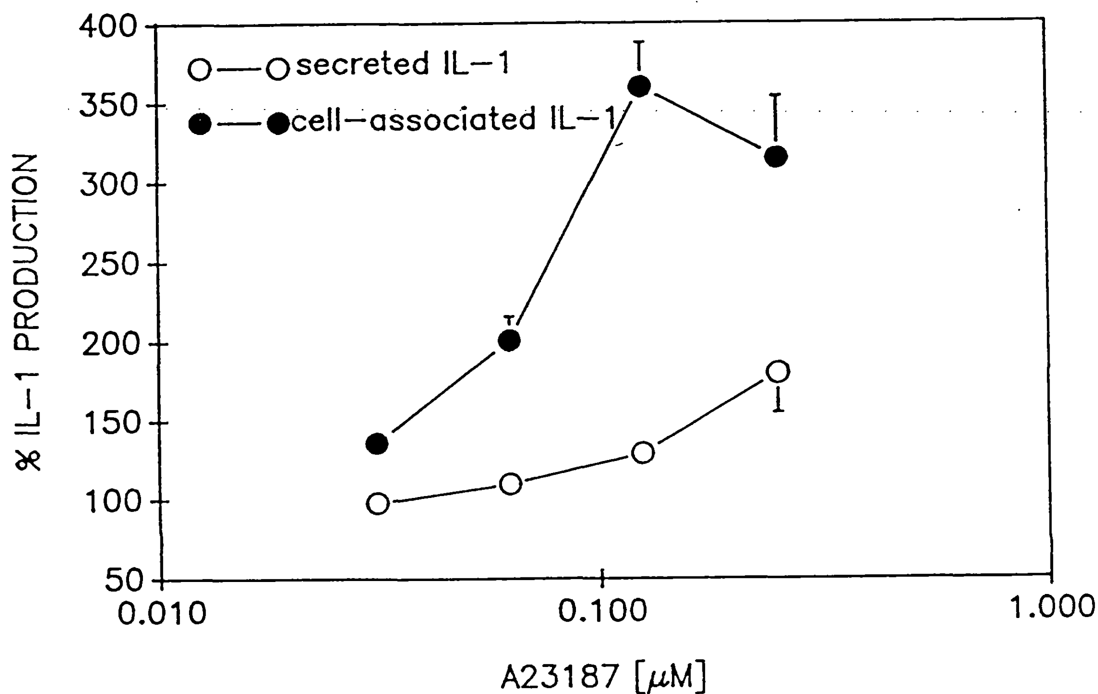


Figure 3.12 The effect of the calcium ionophore A23187 on IL-1 production from resident murine macrophages. The cells were incubated for 20hr. in the presence or absence of ionophore. Dilutions of 100 to 400 and 1000 to 4000 fold were respectively employed for the determination of secreted and cell-associated IL-1. Basal level of IL-1 production (corresponding to 18 ± 10 and 45 ± 18 abs. units for supernatants and lysates) was arbitrarily set at 100%. Each point represents the mean ± standard error (n=3)

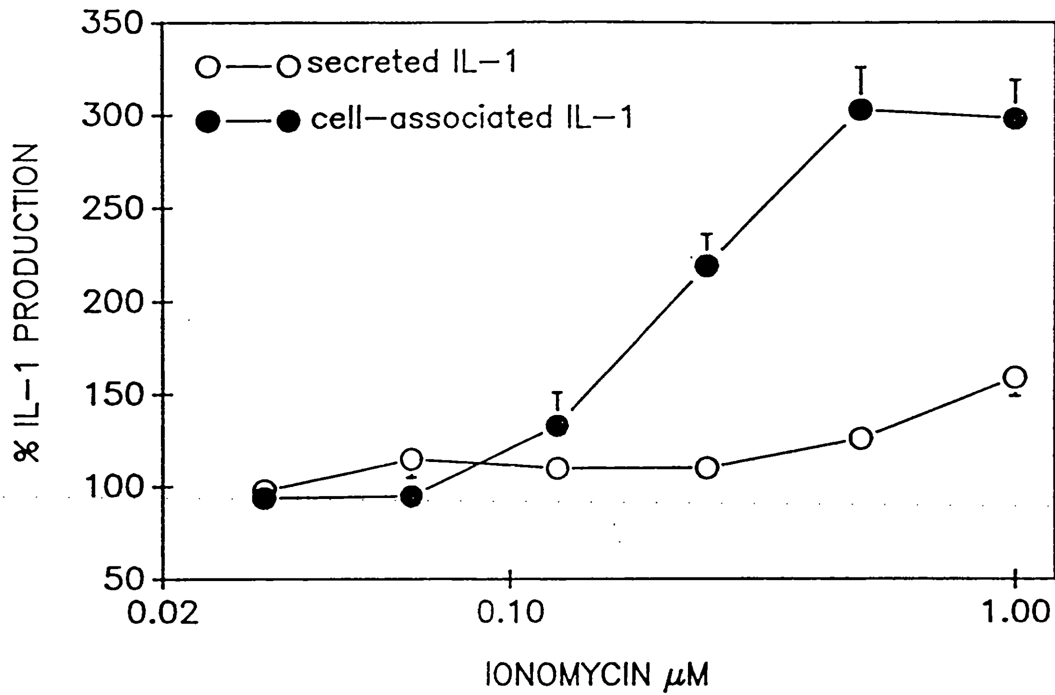


Figure 3.13 The effect of the calcium ionophore Ionomycin on IL-1 production from resident murine macrophages. The cells were incubated for 20hr. in the presence or absence of ionophore. Dilutions of 100 to 400 and 1000 to 4000 fold were respectively employed for the determination of secreted and cell-associated IL-1. Basal level of IL-1 production (corresponding to 11 ± 4 and 43 ± 11 abs. units for supernatants and lysates) was arbitrarily set at 100%. Each point represents the mean ± standard error (n=4)

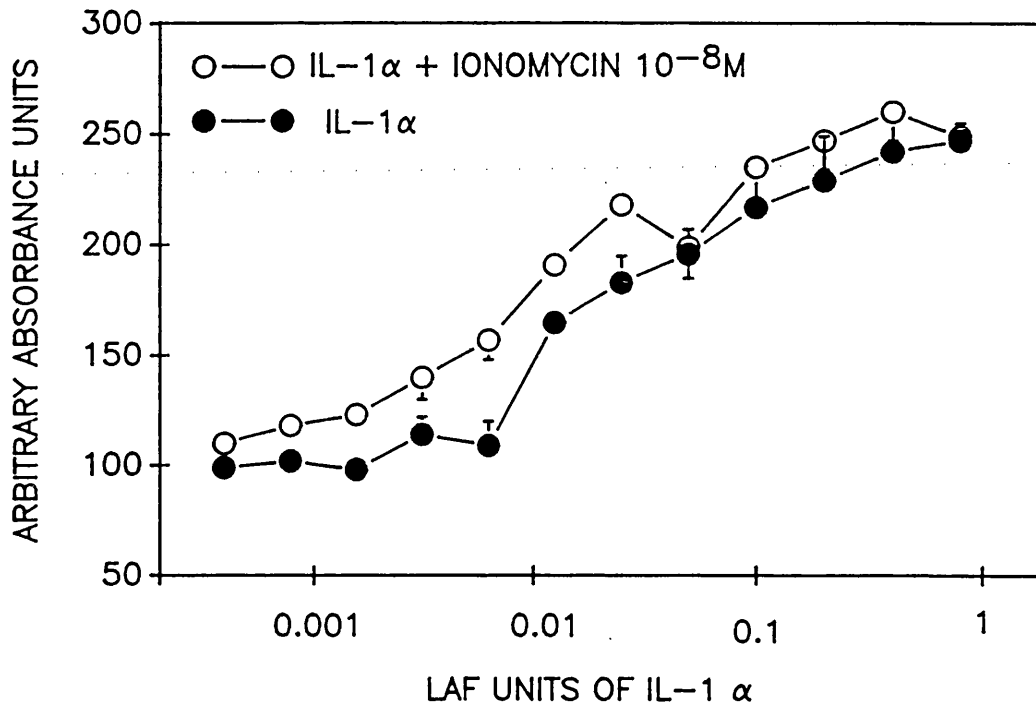


Figure 3.14 The effect of ionomycin on the response of EL4 NOB1 cells to IL-1α. A dose-response curve to rhIL-1α was conducted in the presence and absence of 10^{-8}M ionomycin. The above is a typical effect. Each point is the mean ± standard error of a triplicate determination.

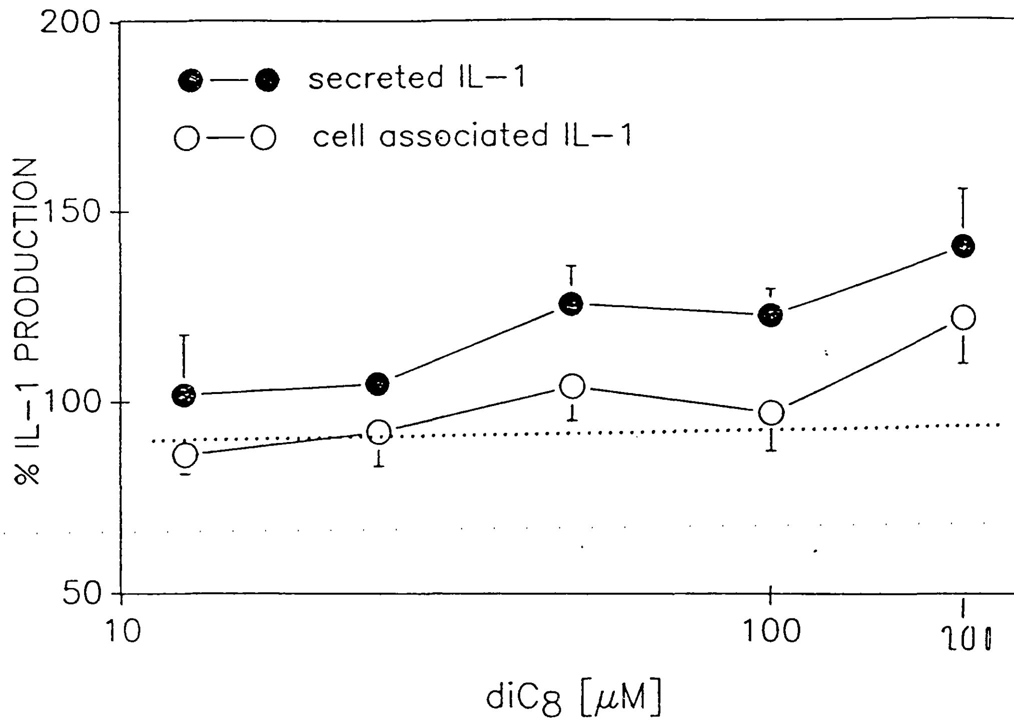


Figure 3.15 The effect of diC_8 on IL-1 production. Resident peritoneal macrophages were incubated with diC_8 for 20hr. Dilutions of 100 to 400 and 1000 to 4000 fold were respectively employed for the determination of secreted and cell-associated IL-1. Basal level of IL-1 production (corresponding to 9 ± 4 and 29 ± 16 abs. units for supernatants and lysates) was arbitrarily set at 100%. Each point represents the mean ± standard error (n=3)

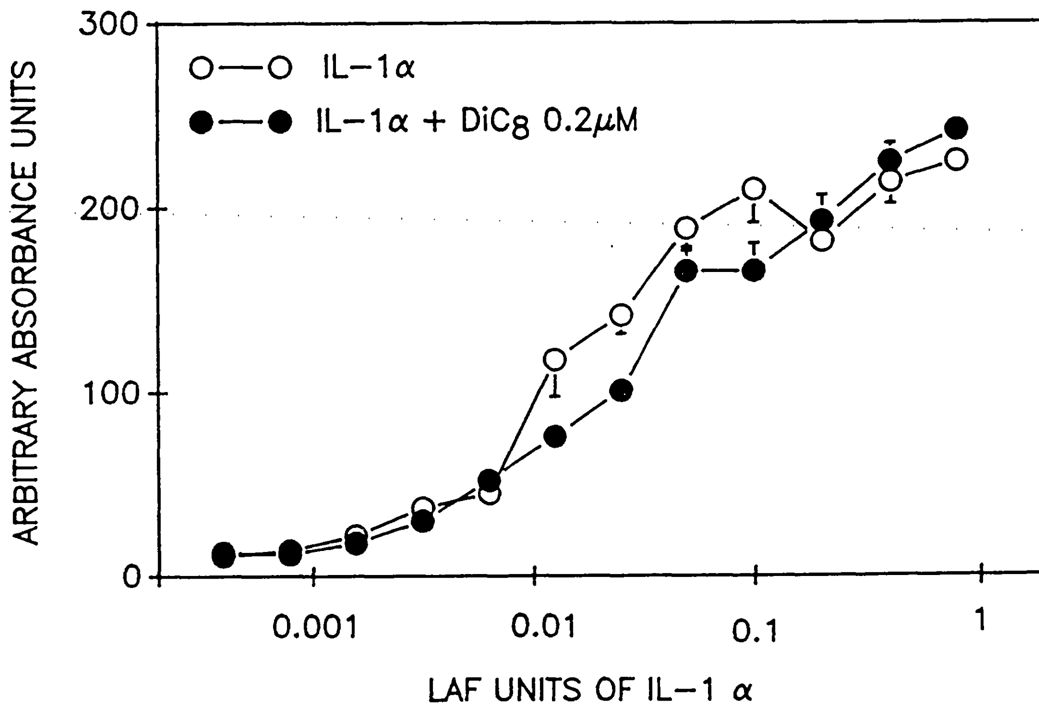


Figure 3.16 The effect of diC_8 on the response of the assay system to IL-1α. A dose-response curve to rhIL-1α was conducted in the presence and absence of 0.2μM diC_8. The above is a typical effect, each point is the mean ± standard error of a triplicate determination.

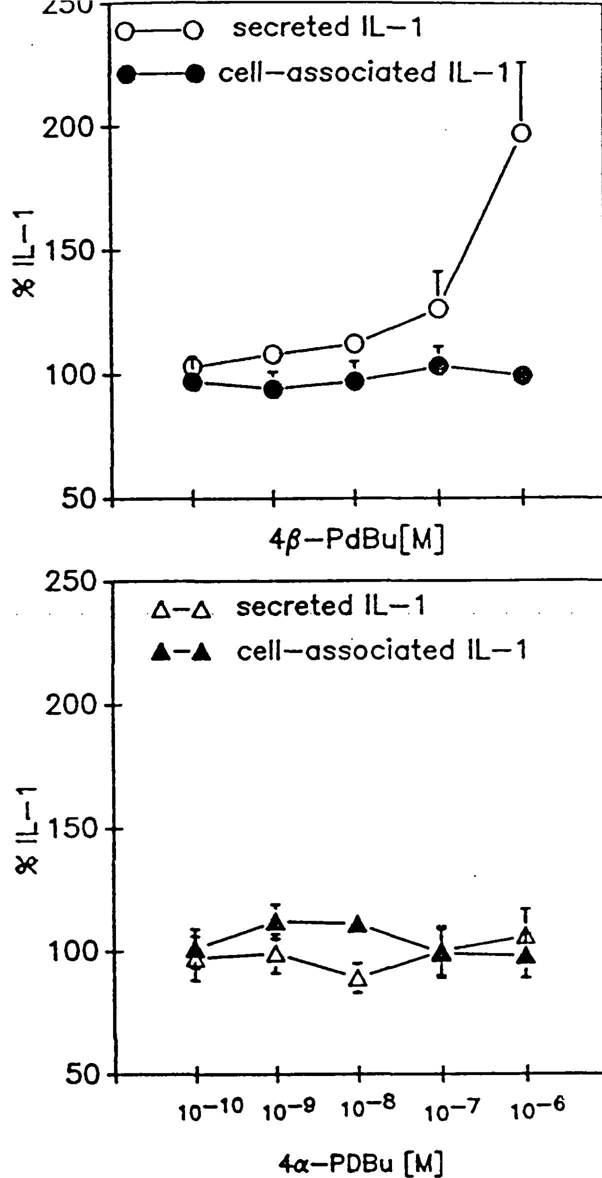


Figure 3.17

a. The effect of 4ß-PDBu on IL-1 production

b. The effect of 4α-PDBu on IL-1 production

The phorbol esters were allowed to incubate with the resident murine macrophages for 4hr. and were then washed off with three rinses of RPMI-1640. The cells were allowed to incubate in serum-free supplemented RPMI-1640 for 18hr. Dilutions of 100 to 400 and 1000 to 4000 fold were respectively employed for the determination of secreted and cell-associated IL-1. Basal level of IL-1 production (corresponding to 13 ± 6 and 39 ± 12 abs. units for supernatants and lysates) was arbitrarily set at 100%. Each point represents the mean ± standard error (n=3)

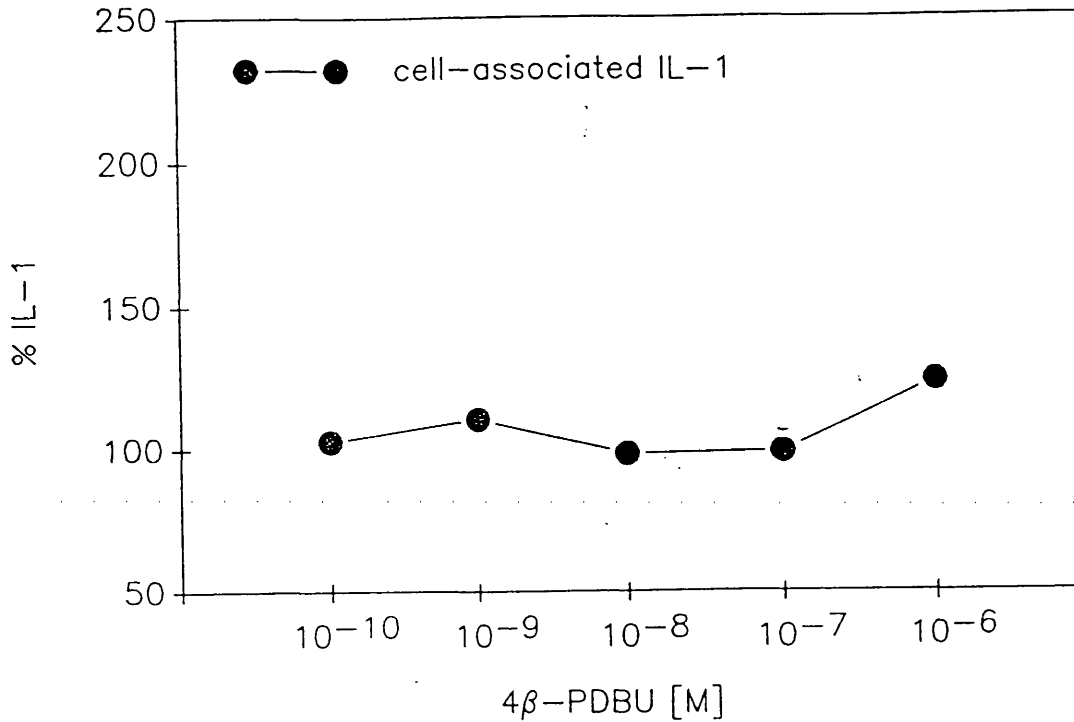


Figure 3.18 The effect of 4ß-PDBu on cell associated IL-1. The phorbol ester was allowed to incubate with the resident murine macrophages for 20hr. The resulting accumulation of cell-associated IL-1 was then assessed. The original macrophage lysates were diluted 1000 to 4000 fold before being assayed. Basal level of IL-1 production (corresponding to 13 ± 6 and 39 ± 12 abs. units for supernatants and lysates) was arbitrarily set at 100%. Each point represents the mean ± standard error (n=3)

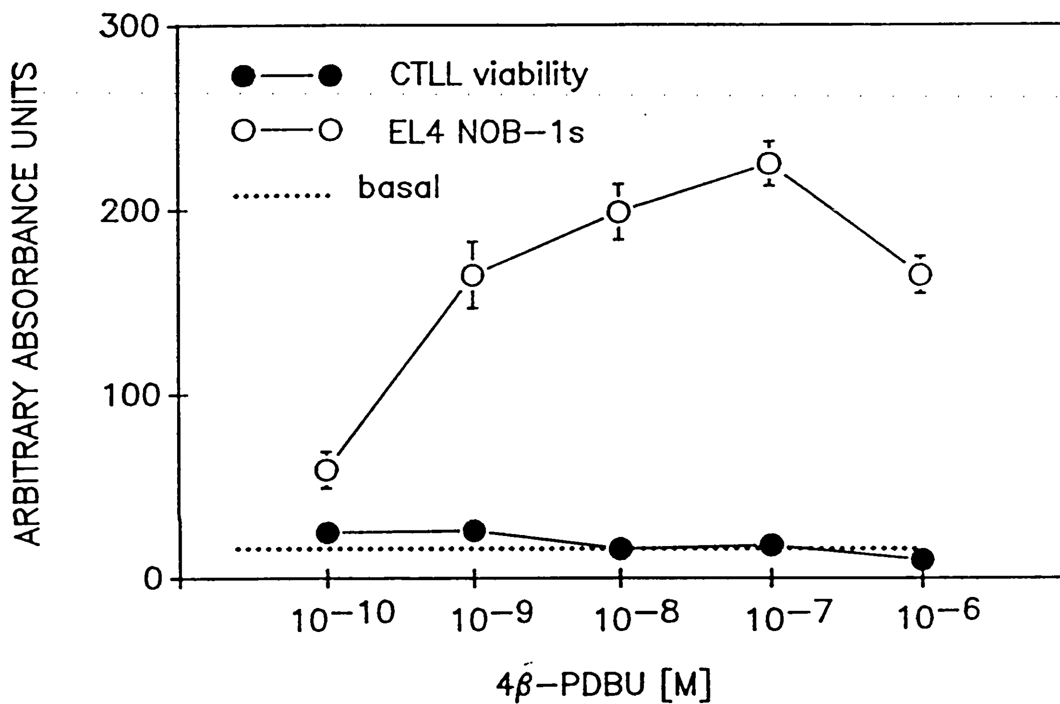


Figure 3.19 The effect of 4ß-PDBu on EL4 NOB1 activation and CTLL viability. The basal is that for both cell-lines. The figure represents the mean ± standard error of triplicate readings.

3.3 DISCUSSION

The data presented in this chapter have described the effects of a number of stimuli on IL-1 secretion and cell-associated IL-1 accumulation. With the bioassay employed throughout this study, the differential analysis of IL-1ß and IL-1α secretion was not possible since all biologically active forms of IL-1 are detected. Even with the measurement of cell-associated IL-1, where only the biologically active pro-peptide form of IL-1α is found intracellularly, the disruption of the cell and the subsequent exposure of the intracellular IL-1ß pro-peptide (biologically inactive) to membrane associated enzymes could result in its conversion to a biologically active form (see Introduction). An attempt to quantify the IL-1 produced in terms of weight per ml^{-1} of active peptide or units ml^{-1} (where 1 unit is the amount of IL-1 required to produce 50% maximal stimulation of thymocyte proliferation) was not attempted for the following reasons.

(i) The requirement for the construction of a calibration curve, for each assay, would have been wasteful of the limited supply of rhIL-1α.

(ii) The construction of calibration curves would have added to an already very lengthy and laborious assay procedure.

(iii) Because of the biological variation in the absolute quantities of IL-1 produced by cells, normalisation of the data against a control is usually employed in the analysis of results. Thus, there would have been no advantage conferred by normalising IL-1 in terms of absolute units.

However, an attempt was made to use a "reference stimulus" by which the potency of other stimuli could be compared.

The effect of each stimulus on IL-1 production will be discussed individually.

LPS

Of the stimuli used, LPS was the most effective agent at stimulating cell-associated IL-1 and IL-1 secretion. However, it should be noted that the potency of LPS at stimulating IL-1 production from macrophages is dependent both on the source of LPS and the source of the cell. For instance Newton (1986) by comparing the ability of a variety of LPSs to stimulate a defined quantity of IL-1 from peripheral blood monocytes found the following order of potency for LPS strains: *Salmonella* > *E. Coli* >> *V. Cholera* and *P. aeroginosa*. Whilst LPS is effective at concentrations of pg to $ng.ml^{-1}$ at stimulating IL-1 production from human peripheral blood monocytes (Schindler & Dinarello,1989; Newton,1986), concentrations of $0.1\mu g$ to 100 $\mu g.ml^{-1}$ are required to produce detectable IL-1 production from murine peritoneal macrophages (Shinomiya & Nakano, 1987). The present study shows that low doses of LPS, ($1-2\mu g.ml^{-1}$), effectively stimulate cell-associated IL-1 accumulation whilst possessing weak stimulatory activity for IL-1 secretion. This suggests that the accumulation and secretion of IL-1 may be differentially regulated. However, it may also be that significant secretion of IL-1 does occur at lower doses of LPS, but at such concentrations only the IL-1ß pro-peptide is secreted, without being processed into its biologically active form. This would imply that higher concentrations of LPS are required to induce the enzyme(s) responsible for processing IL-1. Indeed, this argument is supported by the work of Beuscher et al. (1990) who showed that IL-1ß is secreted mainly as the biologically inactive pro-peptide form from murine peritoneal macrophages stimulated with LPS $0.1-1\mu g.ml^{-1}$.

Neuropeptides

The neuropeptides SP, NK-A, NK-B and cGRP were unable to stimulate IL-1 production from murine peritoneal macrophages or enhance IL-1 production from LPS-stimulated cells. The

observation by Lotz et al (1987,1988) and Kimball et al. (1988a) that SP, NK-A and NK-B can stimulate IL-1 production may be explained by

(i) A differential effect on the cell-type. Lotz et al. used peripheral blood monocytes and synoviocytes and Kimball et al. used the murine cell-line P338D1. These cells may be more responsive to the neuropeptides mentioned than murine peritoneal macrophages.

(ii) LPS contamination of the peptides was not ruled out by Lotz et al. Since peripheral blood monocytes are particularly sensitive to LPS, the IL-1 production observed may well have been caused by contaminating LPS.

(iii) The LAF assay was used by the above authors to quantitate IL-1. The inadequacies of the LAF assay at quantitating IL-1 have been dealt with in Chapter 1. Thus, the authors may have been measuring TNFα, IL-6 and the synergistic effects that the neuropeptides may have with such cytokines in stimulating thymocyte proliferation. This is a consideration not addressed by the authors and is of importance in light of the observation made by Kimball & Fisher (1988) who showed that neuropeptides synergize with IL-1 to stimulate fibroblast proliferation. I have shown that the neuropeptides cannot augment IL-1 production stimulated with LPS. Therefore, they appear to have no effect either on the production of IL-1 or the ability of the assay to detect IL-1.

PAF

PAF was unable directly to stimulate IL-1 production. PAF neither enhanced or inhibited IL-1 production stimulated by

LPS, $1\mu g.ml^{-1}$. This is in contrast with the observations of Salem et al. (1990) who showed that PAF 1nM to $5\mu M$ dose-dependently potentiated LPS-stimulated IL-1 secretion and cell-associated IL-1 accumulation from human peripheral blood monocytes. The authors showed that this effect could be inhibited by the PAF receptor antagonist L-652,731 and that PAF did not affect the ability of recombinant IL-1 to stimulate thymocyte proliferation (the LAF assay was used to quantitate IL-1). Thus, the lack of effect of PAF on IL-1 production from LPS-stimulated murine macrophages may reflect a difference in the activation requirements for IL-1 production in the different cell-types/species used.

TNF

Bachwic et al. (1986) showed that rhTNFα $1\mu g.ml^{-1}$ produced an 8-fold increase in IL-1 secretion in resident murine peritoneal macrophages. Indeed, rhTNFα, $1\mu g.ml^{-1}$ stimulated similar levels of IL-1 secretion as did LPS, (salmonella) $10\mu g.ml^{-1}$. I was unable to observe any stimulation of IL-1 secretion with rhTNFα, $1ng.ml^{-1}$ - $3\mu g.ml^{-1}$. However, rhTNFα 0.05 to $3\mu g.ml^{-1}$ dose-dependently stimulated the accumulation of cell-associated IL-1. That this was not due to contaminating LPS in the TNF samples was shown by the ablation of the TNF stimulatory effect by heating rhTNFα for 10 min at 100°C. This would have denatured the TNFα but left the LPS intact, as temperatures greater than 180°C maintained for several hours are required to destroy LPS. The difference in the observation made by Bachwic et al. and myself may be explained by the fact that Bachwic et al. used the LAF assay to quantitate IL-1. TNFα has been shown to stimulate T-cells and to synergize with IL-1 in inducing thymocyte proliferation. Thus, it may well have been the synergistic action of exogenously added TNF with the low levels of basal IL-1 secretion that contributed to increased thymocyte proliferation that was eventually misinterpreted as an increase in the secretion of IL-1. The lack of synergism

between rhTNFα and rhIL-1α was shown for the concentrations of TNFα likely to be carried over in the diluted macrophage supernatants placed onto the EL4 NOB1 cells. However, concentrations of 0.1 to $3\mu g.ml^{-1}$ of TNFα were able to directly stimulate EL4 NOB1 cells to secrete IL-2 which is contrary to the initial report by Gearing et al. (1987) claiming that TNFα was without effect on the assay. Gearing & Thorpe (1989) have subsequently reported a stimulatory effect of TNFα on EL4 NOB1 cells. The authors showed that, at maximum, TNFα produced only 40-50% of the maximal stimulation of EL4-NOB1s produced by IL-1 which is in agrement with my observation. Gearing & Thorpe also showed that murine TNFα was a more potent stimulator than human TNFα. TNFα Concentrations greater than $3\mu g.ml^{-1}$ were not used to induce IL-1 production as this would represent an unrealistically high concentration of TNF which is unlikely to be produced by cells *in vivo or in vitro*.

That rhTNFα did not produce a detectable stimulation of IL-1 secretion does not preclude that rhTNFα may well have been stimulating the secretion of the biologically inactive IL-1ß pro-peptide. This would be analogous to the situation where low doses of LPS only stimulate the secretion of the IL-1ß pro-peptide (Beuscher et al., 1990). The use of an ELISA system in the detection of IL-1ß and IL-1α could be used to resolve this uncertainty since the assay would only detect IL-1 and would also detect all antigenic forms of the IL-1 whether biologically active or inactive. The finding that rhTNFα was biologically active *in vivo* and *in vitro* in heterologous species has led to the assumption that TNFα was not species-specific in its mode of action (Smith et al., 1986). Thus, although studies have demonstrated that rhTNFα competes with recombinant murine (rm)TNFα for receptor binding on murine cells (Aggarwal et al.,1985), recent work has shown that , unlike some cytokines which exhibit species specificity, the inter-species activities of rTNFα vary depending on the cell type under study (Ehrke et al.,1988, Ranges et al., 1989). Therefore, It would be interesting to

compare the effects of rhTNFα and rmTNFα on IL-1 production in light of the growing number of reports on the species specificity of certain actions of TNF. The effect of rmTNFα on IL-1 production was not investigated due to the lack of availability.

4α PDBu, 4β PDBu and diC_8

DiC_8 12.5 to 200μM was without significant effect at stimulating IL-1 production. This may well have been due to the rapid metabolism of the diC_8 by the macrophages. Although diC_8 is primarily metabolized by diacylglycerol kinase in the neutrophil (Muid et al., 1987; Twomey, 1990), the kinetics and pathways of diC_8 metabolism in macrophages remain to be reported in the literature. The rapid metabolism of diC_8 by macrophages is likely since they have a high membrane turnover. Indeed, Steinman et al.(1976) showed that unstimulated macrophages interiorize an area of plasma membrane equivalent to their surface area every 35 minutes. Furthermore, racemization of the active 1,2-sn-diC_8 into its inactive isomer 2,3-sn-diC_8 can occur in warm aqueous environments. Thus, although physiologically more desirable than phorbol-esters as a PKC activator the chemical and metabolic instability is a major drawback in the use of diC_8 to investigate events that require a long time course of stimulation such as IL-1 production.

The ability of 4β-PDBu to stimulate IL-1 production was only observed at 10^{-6}M. At this concentration no stimulation of cell-associated IL-1 was observed and the stimulatory effect appeared to be solely on secretion and was comparable to the secretion of IL-1 observed with LPS $1\mu g.ml^{-1}$. The inactive isomer 4α-PDBu had no stimulatory effect between 10^{-10} - 10^{-6} M. A 4hr incubation step was employed with 4β-PDBu, after which the macrophage monolayers were washed by rinsing with RPMI-1640 three times to remove as much 4β-PDBu as possible. The effectiveness of this washing step was shown by the fact that

although 4ß-PDBu (10^{-10} - 10^{-7}M) stimulates EL4 NOB1 cells to produce IL-2 the supernatants from washed, 4ß-PDBu treated macrophages, were without stimulatory effect on EL4 NOB1 cells. A 4hr pre-incubation with 4ß-PDBu was shown to be effective at stimulating IL-1 secretion from thioglycollate-elicited murine peritoneal macrophages (Katakami et al., 1986). Using such a protocol the authors observed a dose-dependent stimulation of IL-1 secretion by 4ß-PdBu. When Katakami et al. employed longer incubation times with 4ß-PDBu downregulation of PKC occurred and the cells became refractory to further stimulation by phorbol esters but not LPS. In the present study when 4ß-PDBu was left on the macrophage monolayers for 20hrs there was still no observable stimulation of cell-associated IL-1 (the supernatants could not be assayed due to the contaminating 4ß-PDBu). The use of the LAF assay to quantitate IL-1 by Katakami et al. may well have led to the detection of the production of TNFα, IL-6, GM-CSF and the synergistic effects of such cytokines with IL-1 on thymocyte proliferation. The modest levels of secretion observed by myself at high concentrations of 4ß-PDBu may well be due to :-

1. A transitory stimulation of IL-1 production that results in most of the IL-1 being secreted, hence there is no observable accumulation of cell-associated IL-1 with a 4hr or 20hr pre-incubation.

2.Residual levels remaining after washing monolayers treated with 4ß-PDBu 10-6M are sufficient to synergize with undetectable levels of IL-1 at stimulating EL4 NOB1 cells.

The ability of 4ß-PDBu to synergize with IL-1 in stimulating EL4 NOB1 cells was unfortunately not carried out to investigate this possibility further. However, it appears that PKC activation as produced by diC_8 and 4ß-PDBu is a poor stimulus for IL-1 production from peritoneal macrophages. It would be of interest to investigate the effects PKC stimulation on IL-1 message. Although PKC activation can activate gene transcription for a variety of proteins the possibility exists that certain inhibitory proteins may also be produced that can inhibit IL-1 gene transcription and/or mRNA translation.

Ca^{2+} ionophores

Contrary to the reports of Newton (1987), Brandwein (1990) and Suttles et al, (1990) the present study has shown that both A23187 and ionomycin can dose-dependently stimulate the accumulation of cell-associated IL-1. It should be noted that Brandwein (1990) only measured the IL-1 activity associated with the membranes of paraformaldehyde fixed macrophages and may therefore have overlooked the intracellular accumulation of the IL-1ß pro-peptide, which is biologically inactive unless the cells are lysed.

A report by Shinomyia & Nakamo (1987), showed that A23187 can stimulate IL-1 secretion. The authors employed concentrations in excess of 10^{-6}M over a 48hr incubation. Since I have shown that, over a 20hr. period, concentrations in excess of $5x10^{-7}$M A23187 are cytotoxic; the above authors may well have been measuring the leakage of intracellularly accumulated IL-1 into the extracellular supernatant as a result of cell-death. The authors observed only a stimulation of cell-associated IL-1 with A23187 at lower concentrations ($< 10^{-6}$M) which subsequently gave rise to IL-1 secretion after incubation periods of greater than 24hr. Again cell-death may have contributed to the secretion of cell-associated IL-1 over incubation periods longer than 24hr.

Matsushima & Oppenheim (1985) have reported that A23187 10^{-6}-10^{-7}M can synergize with IL-1 and Con-A in stimulating the proliferation of thymocytes in the LAF assay. Ionomycin, 10^{-6}-10^{-7}M and A23187, 10^{-6} - 10^{-8}M directly stimulated EL4 NOB1 cells and also maintained CTLL viability, however, at concentrations likely to be found in the assay system ($<10^{-8}$M for ionomycin and $< 10^{-9}$M for A23187) the ionophores did not synergize with rhIL-1α in stimulating EL4 NOB1 cells.

The possibility that the Ca^{2+} ionophores can stimulate the secretion of the IL-1ß pro-peptide, without stimulating its processing, cannot be ruled out using the assay system employed in the present study because of the lack of biological activity of the IL-1ß pro-peptide.

The observation of Suttles et al. (1990) that ionophores can potentiate LPS-stimulated IL-1 secretion and processing was not investigated. From the results presented it appears that a Ca^{2+} signal is sufficient to initiate the synthesis of IL-1 pro-peptides but is insufficient for the processing and secretion of IL-1. Can Ca^{2+} initiate the transcription of IL-1 mRNA ? Schindler & Dinarello (1989) have shown that the low concentrations of LPS found in culture media are sufficient to induce maximal transcription of IL-1 mRNA without inducing translation. Thus, in the present study the ionophores may only be stimulating the translation of IL-1 mRNA accumulated as s result of LPS contamination. Measurements of mRNA levels during such experiments could be used to resolve the precise level at which Ca^{2+} ionophores stimulate IL-1 production.

On reflection the EL4 NOB1 bioassay was too complex to investigate the effects of compound such as phorbol-esters which have multiple effects all the way down the bioassay cascade. Perhaps the way forward in analyzing the effects of phorbol esters would be to use the selective ELISA techniques in quantitating the IL-1, peptides in conjunction with a selective bioassay. Indeed, using specific monoclonal

antibodies to IL-1 together with RNA analysis by northern blot and dot blot techniques, Fenton et al. (1988) have shown that phorbol-esters stimulate IL-1ß gene expression, transcription of IL-1 mRNA and accumulation of IL-1ß pro-peptide in the THP-1 and HL-60 human myelocytic cell-lines. Whether the phorbol-esters have similar effects on freshly isolated cells remains to be investigated using such techniques.

CHAPTER 4

THE EFFECT OF NSAIDs ON IL-1 PRODUCTION

4.1 INTRODUCTION

The ability of NSAIDs and PGE_2 to affect the secretion of IL-1 from macrophages has been the subject of conflicting reports. Table 4.1 and 4.2 summarises the findings of such reports to date. The authors that report an increase in IL-1 secretion upon incubating stimulated cells with NSAIDs also observe PGE_2 to be inhibitory with respect to NSAID-potentiated, and LPS-stimulated IL-1 secretion. This has lead to a widely accepted proposal for PGE_2 as an endogenous down-regulator of IL-1 production. Kunkel et al. (1986) showed that PGE_2, 10^{-9} - 10^{-6}M ($30ng.ml^{-1} = 9x10^{-8}M$) dose-dependently inhibited IL-1 secretion from LPS-stimulated macrophages, with 99% inhibition produced by PGE_2 10^{-6}M (a ten-fold greater concentration than produced by the macrophages upon stimulation with LPS *in vitro*). Knusden et al (1986) also observed a dose-dependent inhibition of IL-1 secretion with PGE_2 10^{-9} - 10^{-5}M. However, only 60% inhibition of IL-1 secretion was observed with PGE_2 10^{-5}M. The inhibitory effect of PGE_2 on IL-1 secretion has been attributed to its ability to stimulate the accumulation of intracellular cAMP. Indeed, agents that stimulate cAMP production in macrophages/ monocytes such as histamine and forskolin, can inhibit IL-1 secretion. Whilst agents that potentiate the accumulation of cAMP such as theophylline, isobutylmethylxanthine (IBMX) and cholera toxin all enhance the inhibitory effect of agonists that inhibit IL-1 secretion via the generation of cAMP (Dohlsten et al., 1988; Brandwein, 1986; Knudsen, 1986). Analogues of cAMP such as dibutryl cAMP have also been shown to inhibit IL-1 secretion (Knudsen et al., 1986; Brandwein, 1986; Brandwein, 1990). Whilst analogues of cGMP are without effect on IL-1 production (Hurme, 1990). Knudsen et al. (1986) and Tannenbaum & Hamilton (1988) have shown that an increase in intracellular cAMP can inhibit the translation of IL-1 mRNAs into their pro-peptides but it has no effect on the

transcription or stability of the mRNAs.

In contrast, some studies have shown that PGE_2 and NSAIDs are without effect on IL-1 secretion (see Table 4.1). Indeed, Kassis & Hanna (1989) have found that PGE_2 and dibutryl cAMP can dose-dependently potentiate IL-1 secretion from LPS-stimulated macrophages. Furthermore, Ohmori et al. (1990) showed that PGE_2 $2ng.ml^{-1}$ to $1000ng.ml^{-1}$ and dibutryl cAMP selectively increased the steady state levels of IL-1β mRNA (>10 fold) without affecting IL-1α mRNA levels. When a bioassay (LAF assay) was used to quantitate IL-1 activity in supernatants from stimulated cells treated with agents that elevate cAMP Ohmori et al. (1990) found that dibutryl cAMP and PGE_2 suppressed IL-1 activity. However, when an IL-1 radioreceptor binding assay was used, IL-1 levels appeared to be elevated. The drawbacks involved in the use of bioassays for IL-1 has been dealt with in Chapter 1. Much of the work to date that has examined the effects of PGE_2 and NSAIDs on IL-1 secretion has relied on the use of non-selective bioassays for IL-1. The ability of PGE_2 to inhibit such bioassays has also proved problematic, especially when they are used to examine the effects of NSAIDs on IL-1 secretion. An increase in IL-1 activity may represent the removal of the inhibitory effect of PGE_2 by the NSAID.

In 1987 a report by Gearing et al. described an extremely sensitive and selective bioassay for IL-1. This provided an impetus to reexamine the reported effect of NSAIDs, on IL-1 production using this new bioassay. Since IL-1 was known to accumulate intracellularly and possibly reside in or on the membranes of cells, the bioassay was used to look at the effect of NSAIDs, not only on IL-1 secretion but also IL-1 accumulation associated with the cell.

TABLE 4.1 SUMMARY OF STUDIES SHOWING THAT NSAIDs POTENTIATE IL-1 SECRETION AND THAT PGE_2 INHIBITS IL-1 SECRETION

CELL TYPE	STIMULUS	EFFECT OF NSAID ON IL-1 SECRETION	EFFECT OF EXOGENOUS PGE2 ON IL-1 SECRETION	REFERENCE
eMpMØ	LPS $10\mu g ml^{-1}$	INCREASED	DECREASED	Kunkel et al 1986,1987
rMpMØ	ZYMOZAN $500\mu g ml^{-1}$	INCREASED	DECREASED	Kunkel et al 1985
U973/HpbMØ	LPS $1\mu g ml^{-1}$	INCREASED	DECREASED	Knudsen et al 1986
rMpMØ	TNFα 6nM	INCREASED	DECREASED	Bachwich et al 1986
HpbMØ	LPS $5\mu g ml^{-1}$	INCREASED	DECREASED	Rola-Pleszczynski 1986
HpbMØ	LPS $100ng ml^{-1}$	---------	DECREASED	Hurme 1990

eMpMØ: elicited murine peritoneal macrophages
rMpMØ: resident murine peritoneal macrophages
HpbMØ: human peripheral blood monocytes

TABLE 4.2 SUMMARY OF STUDIES SHOWING THAT NSAIDs HAVE NO EFFECT ON IL-1 SECRETION AND THAT PGE_2 CAN INHIBIT OR POTENTIATE IL-1 SECRETION.

CELL TYPE	STIMULUS	EFFECT OF NSAID ON IL-1 SECRETION	EFFECT OF EXOGENOUS PGE2 ON IL-1 SECRETION	REFERENCE
eMpmØ	LPS $10\mu g ml^{-1}$	NO EFFECT	DECREASED	Brandwein 1986,1990
rMpMØ	LPS $20\mu g ml^{-1}$	NO EFFECT	NO EFFECT	Oterness et al 1988
eMpMØ	LPS $10\mu g ml^{-1}$	NO EFFECT	---------	Phadke et al 1986
HaMØ	LPS $10\mu g ml^{-1}$	NO EFFECT	---------	Eden & Turino 1986
HpbMØ	LPS $10 ng ml^{-1}$	---------	INCREASED	Kassis et al 1989
HpbMØ	PMA $10 ng ml^{-1}$	---------	NO EFFECT	Hurme 1990
eMpMØ	LPS $10 ng ml^{-1}$	---------	INCREASED	Ohmori et al 1990

HaMØ: human alveolar macrophages

4.2 RESULTS

4.2.1 The effect of NSAIDs on PGE_2 production

The NSAIDs dose-dependently inhibited the production of PGE_2 from murine macrophages stimulated with LPS, $1\mu g.ml^{-1}$ (Fig 4.1). Ablation of PGE_2 synthesis was observed at indomethacin (IND) $10^{-6}M$, sodium meclofenamate (NaM) $10^{-7}M$ and Piroxicam (PIR) $10^{-5}M$. Aspirin (ASP) up-to $10^{-4}M$ failed to ablate PGE_2 synthesis, but a dose-dependent inhibition was observed. The NSAIDs at the concentrations used were shown to be non-cytotoxic: a greater than 95% cell viability was observed after a 20hr incubation . NaM at $10^{-4}M$ was cytotoxic to the macrophages (less than 20% viability resulted after a 20hr incubation.) and therefore was excluded from use at this concentration.

4.2.2 The effect of NSAIDs on IL-1 secretion and cell-associated IL-1

IND produced a significant increase in IL-1 secretion at concentrations of $10^{-6}M$ and above. Although PGE_2 synthesis was ablated with IND $10^{-6}M$ a dose-dependent increase in IL-1 secretion was still observed with concentrations of IND above $10^{-6}M$. PIR although ablating PGE_2 synthesis at $10^{-5}M$, only produced a significant increase in IL-1 secretion at $10^{-4}M$. NaM produced a dose-dependent increase in IL-1 secretion at concentrations greater than those required to inhibit PGE_2 synthesis. ASP failed to produce a significant increase in IL-1 secretion at the concentrations used, Fig 4.2a.

All NSAIDs potentiated the accumulation of cell-associated IL-1 in a dose-dependent manner Fig 4.3a. The ability of NSAIDs to potentiate cell-associated IL-1 parallelled their ability to inhibit PGE_2 synthesis. Maximum accumulation of cell-associated IL-1 was seen at the minimum concentrations of NSAIDs required to ablate PGE_2 synthesis ie. IND $10^{-6}M$, PIR

10^{-5}M and NaM 10^{-7}M. ASP produced a maximum accumulation of cell-associated IL-1 at 10^{-4}M ie. at the concentration producing maximum PGE_2 inhibition. IND 10^{-5}-10^{-4}M, NaM 10^{-6}-10^{-5}M and PIR 10^{-4}M each produced a significant reduction in cell-associated IL-1 from the respective maximum.

4.2.3 Effect of NSAIDs alone on IL-1 secretion and cell-associated IL-1

The NSAIDs had no effect on the secretion or cell-associated accumulation of IL-1 in macrophages cultured without LPS-stimulation (Fig 4.2b, 4.3b).

4.2.4 Effect of PGE_2 on the stimulation of EL4 NOB1 cells with rhIL-1α

PGE_2 dose-dependently inhibited the stimulation of IL-2 secretion from EL4-NOB1 cells stimulated sub-optimally with rhIL-1α. An IC_{50} of 30 $\pm$ 5 $ng.ml^{-1}$ was observed. No significant effect was seen at concentrations of PGE_2 below $1ng.ml^{-1}$ (Fig 4.4).

4.2.5 The effect of PGE_2 on CTLL viability

PGE_2, $1ng.ml^{-1}$ - $30ng.ml^{-1}$ had no significant effect on CTLL viability maintained with MLA supernatant 10% v/v. At concentrations in excess of 100 $ng.ml^{-1}$ a decrease in the ability of MLA supernatant to maintain CTLL viability was observed (Fig 4.5).

4.2.6 The effect of NSAIDs on the dose-response curve for rhIL-1α

The NSAIDs PIR, IND and NaM, at the maximum concentrations present in dilute macrophage supernatants placed onto EL4 NOB1 cells, (5×10^{-7}M for IND,PIR and 5×10^{-9} for NaM) were without effect on the dose-response profile of rhIL-1α on EL4 NOB1 cells (Fig 4.6).

4.2.7 The effect of paracetamol on IL-1 secretion and cell-associated IL-1

Paracetamol (PAR) 10^{-4}-10^{-8}M was unable to potentiate or inhibit the secretion or cell-associated accumulation of IL-1 in macrophages stimulated with LPS, 1μg.ml-1 (Fig 4.7).

4.2.8 The effect of exogenous PGE_2 on NSAID-potentiated IL-1 production

PGE_2 0.1-30ng.ml^{-1} dose-dependently inhibited the increase of cell-associated IL-1 produced by IND, 10^{-6}M and NaM, 10^{-7}M (Fig 4.8). Exogenous PGE_2, 20ng.ml^{-1} also inhibited the potentiated secretion observed with IND, 10^{-4}M and NaM, 10^{-5}M and returned it to the levels obtained with LPS alone (Fig 4.9).

4.2.9 The time-dependent inhibitory effect of PGE_2 on IL-1 production

PGE_2 20ng.ml^{-1} exerted a maximum inhibitory effect on IL-1 production when added concomitantly with IND and NaM to macrophages stimulated with LPS. The inhibitory effect was time-dependently reduced by addition of PGE_2 prior to or after LPS addition to macrophages incubated with a NSAID. Addition of PGE_2 4hr after LPS gave no inhibitory effect (Fig 4.10).

4.2.10 The effect of indomethacin on rhTNFα-stimulated IL-1 production

IND 10^{-6}M did not potentiate the ability of rhTNFα, 1μg.ml^{-1} to stimulate the accumulation of cell-associated IL-1. IND at 10^{-4}M did not trigger the secretion of cell-associated IL-1 increased by rhTNFα, 1μg.ml^{-1} (Fig 4.11).

4.2.11 The effect of indomethacin on calcium ionophore-stimulated IL-1 production

IND, 10^{-4} - 10^{-6}M was without effect on ionomycin-stimulated IL-1 secretion or increase of cell-associated IL-1 (Fig 4.12). With A23187 $5x10^{-7}$M, IND 10^{-4}M decreased the accumulation of cell-associated IL-1 whilst concomitantly increasing IL-1 secretion. However, this was shown to be due to a cytotoxic effect produced by IND and A23187, as judged by Trypan blue exclusion .

4.2.12 The effect of murine macrophages on exogenous PGE_2

The ability of murine macrophages to metabolise or potentiate the breakdown of exogenously added PGE_2 was studied. Macrophages were incubated in culture medium with IND 10^{-5}M and PGE_2 20ng.ml^{-1} for 20hr. This did not lead to a reduction in the amount of PGE_2 as measured by RIA. This was also shown for PIR 10^{-4}M and NaM 10^{-6}M.(Fig 4.14).

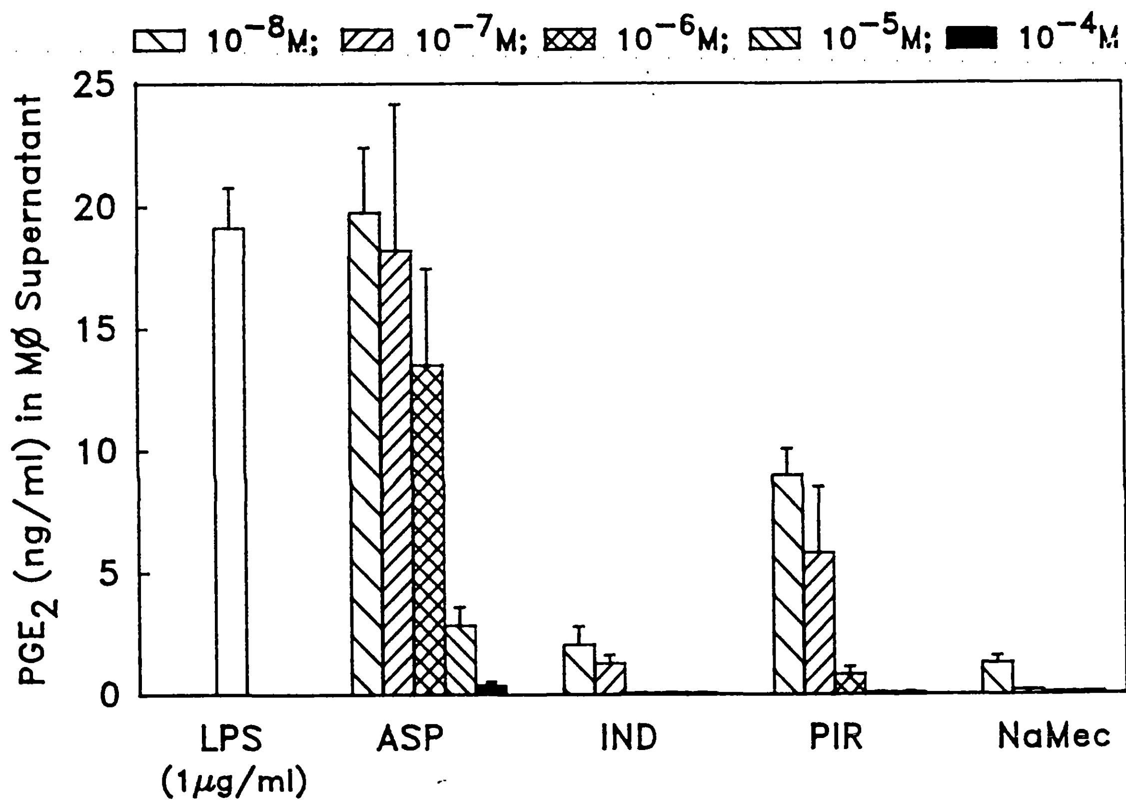


Figure 4.1 The effect of NSAIDs on PGE_2 synthesis from resident murine peritoneal macrophages stimulated with LPS 1μg.ml^{-1}. The PGE_2 content was determined after a 20hr. incubation using a radioimmunoassay. Each bar represents the mean ± standard error (n=4).

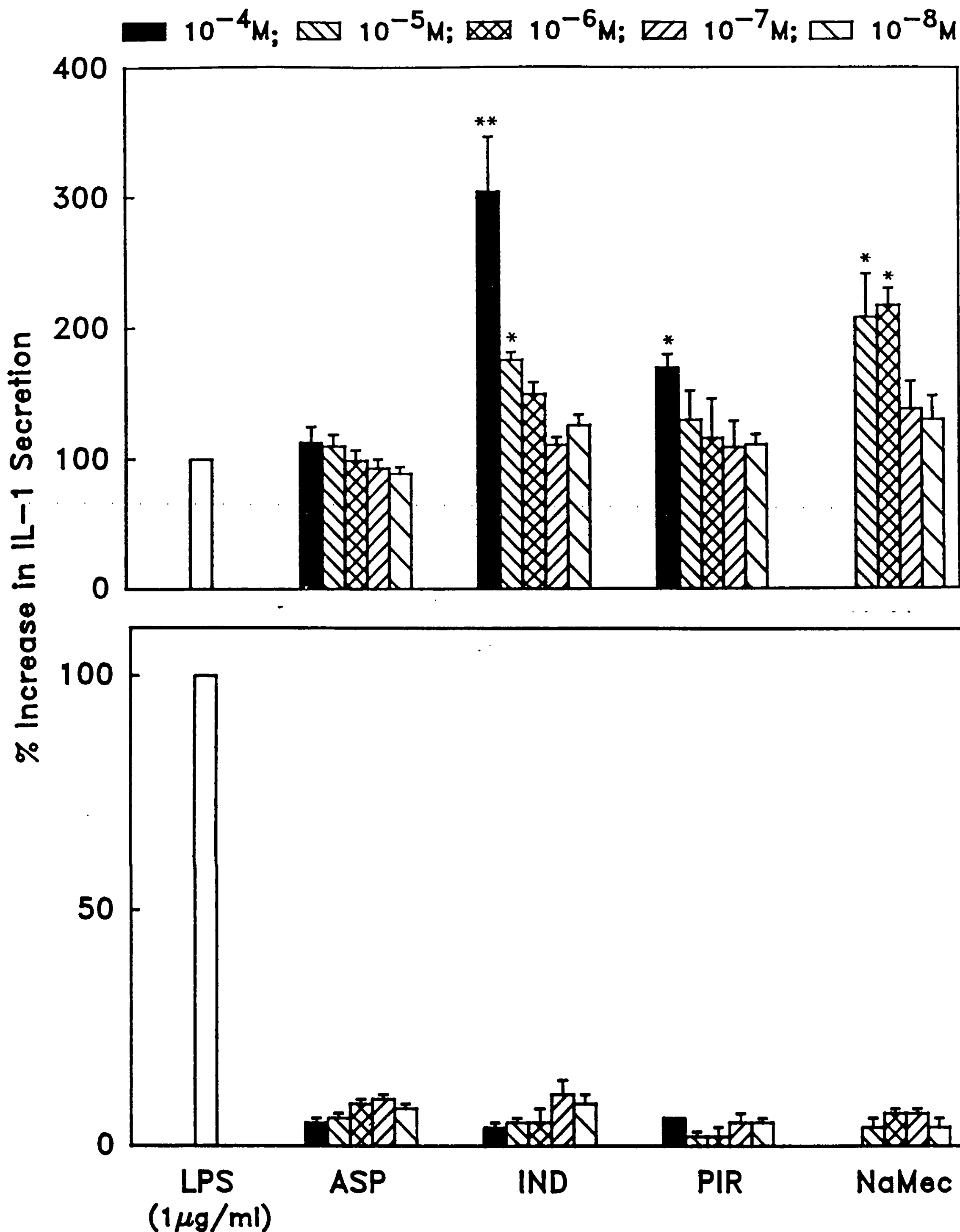


Figure 4.2 The effect of NSAIDs on IL-1 secretion.
(a). NSAIDs incubated with LPS 1μg.ml^{-1}.
(b). NSAIDs alone.
The cells were incubated for 20hr. and the supernatants diluted 100 to 400 fold before being assayed. Each bar represents the mean ± standard error (n=4). Statistical significance was determined using the Students t-test. ** $p < 0.01$ * $p < 0.05$.

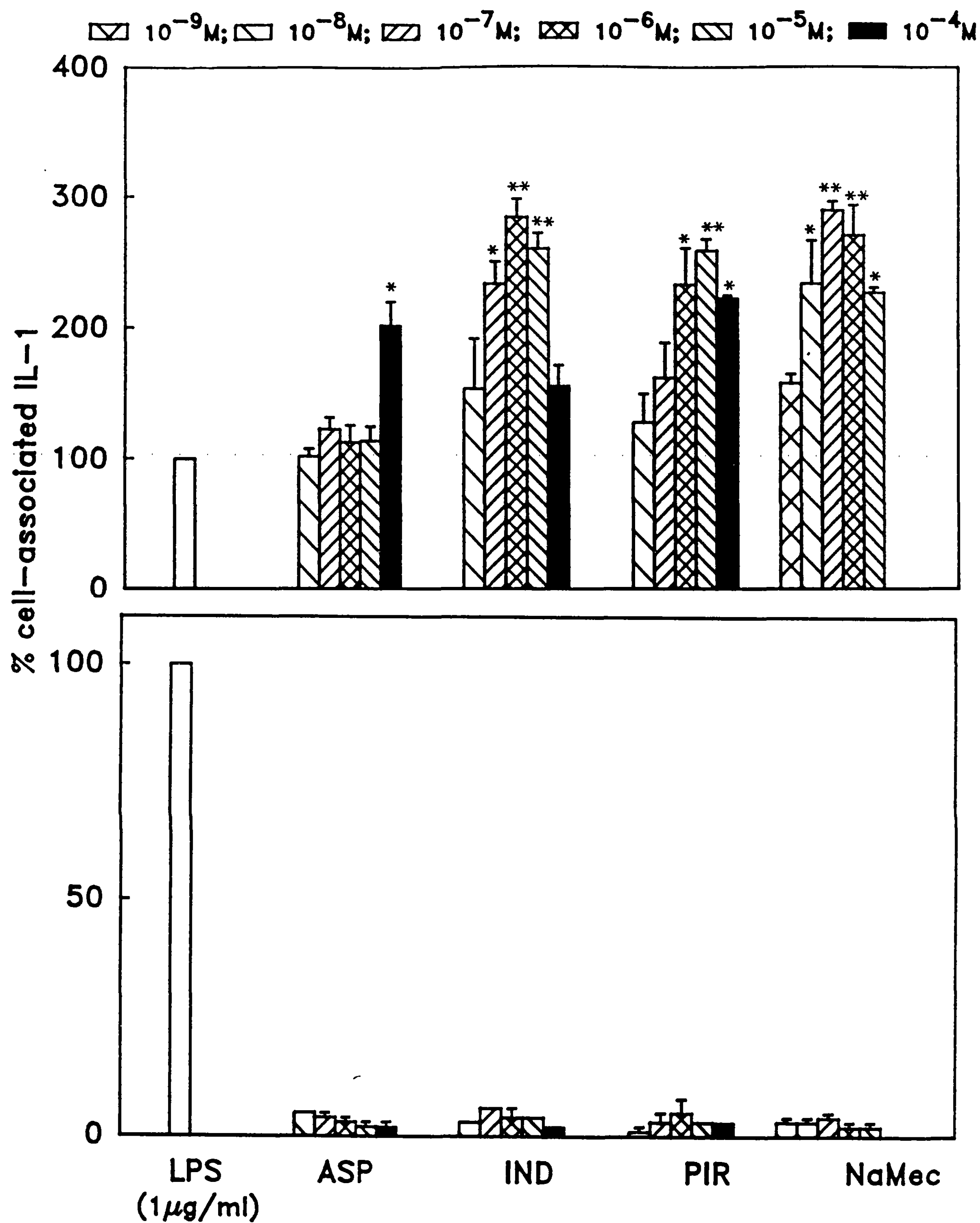


Figure 4.3 The effect of NSAIDs on cell-associated IL-1
(a). NSAIDs incubated with LPS 1μg.ml^{-1}.
(b). NSAIDs alone.
The cells were incubated for 20hr. and the lysates diluted 1000 to 4000 fold before being assayed. Each bar represents the mean ± standard error (n=4). Statistical significance was determined using the Students t-test. ** $p< 0.01$ * $p<0.05$.

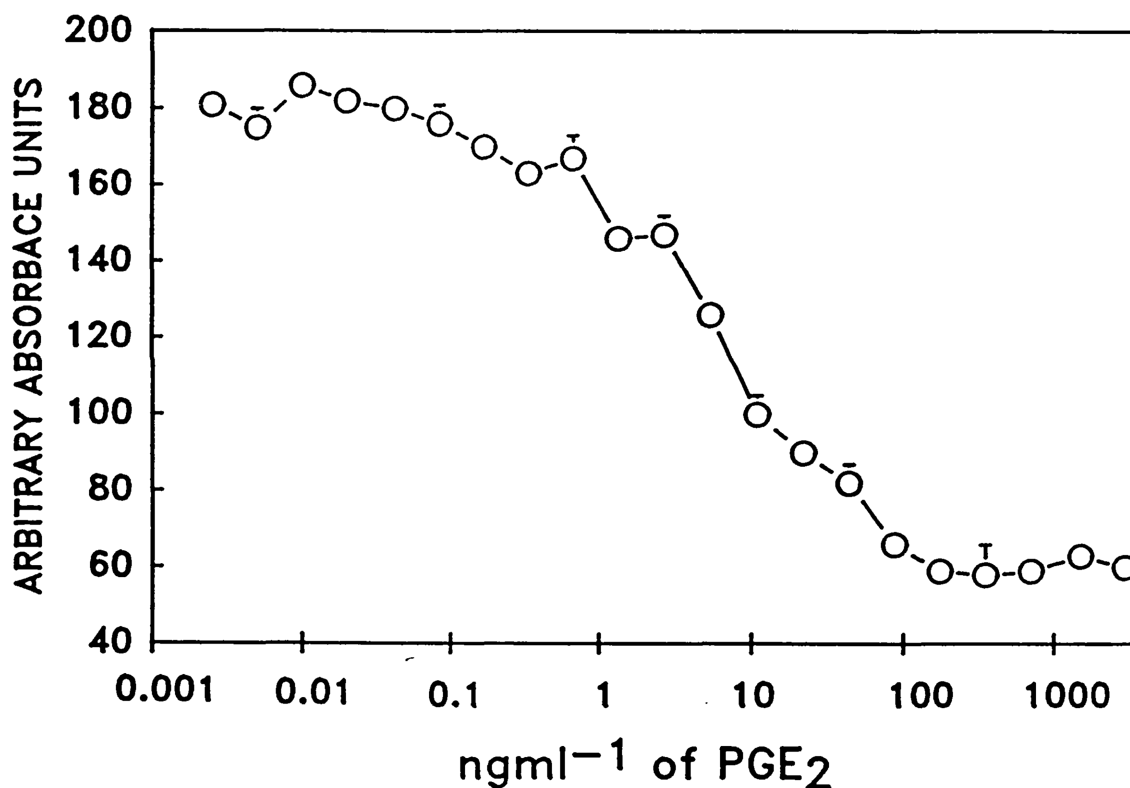


Figure 4.4 The effect of PGE_2 on the sub-optimal stimulation of EL4 NOB1 cells with rhIL-1α. Exogenous PGE_2 was added to the cells 30min. prior to the addition of IL-1. The above is a typical dose-response each point is the mean of triplicate readings.

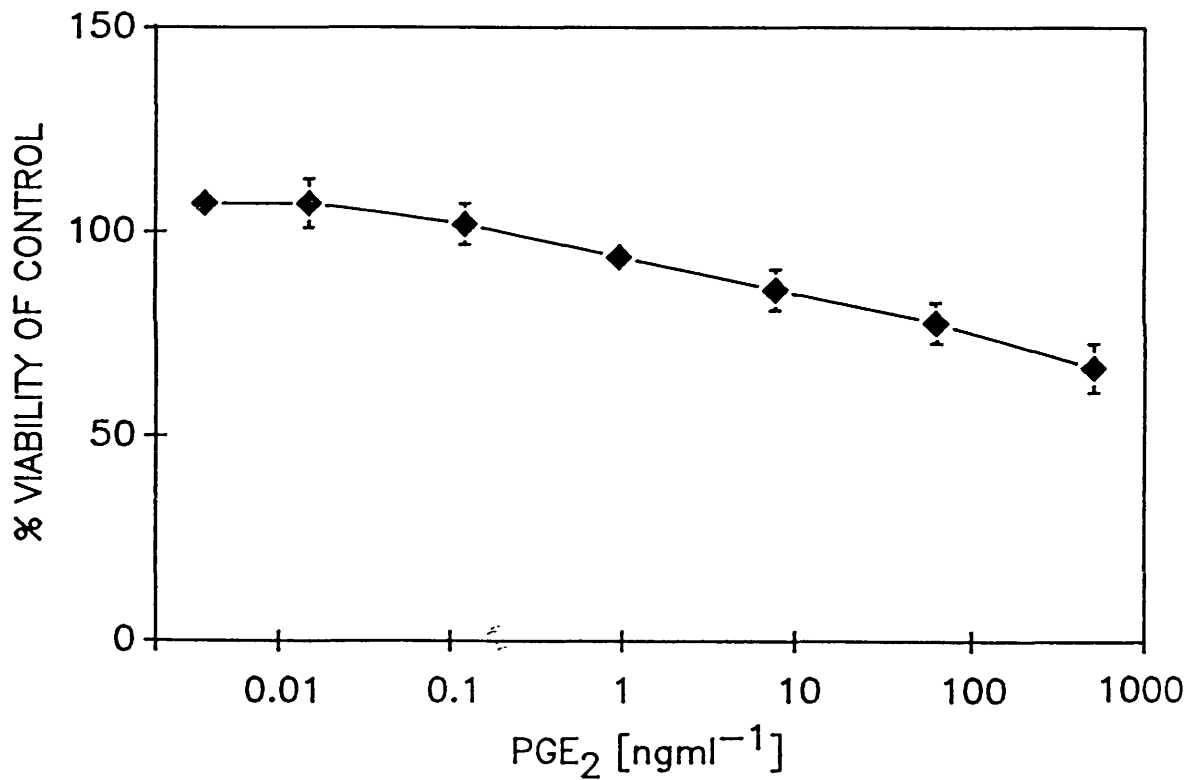


Figure 4.5 The effect of PGE_2 on CTLL viability . PGE_2 was added 30min. prior to the addition of 10% v/v MLA supernatant. Each point represents the mean ± standard error (n=3).

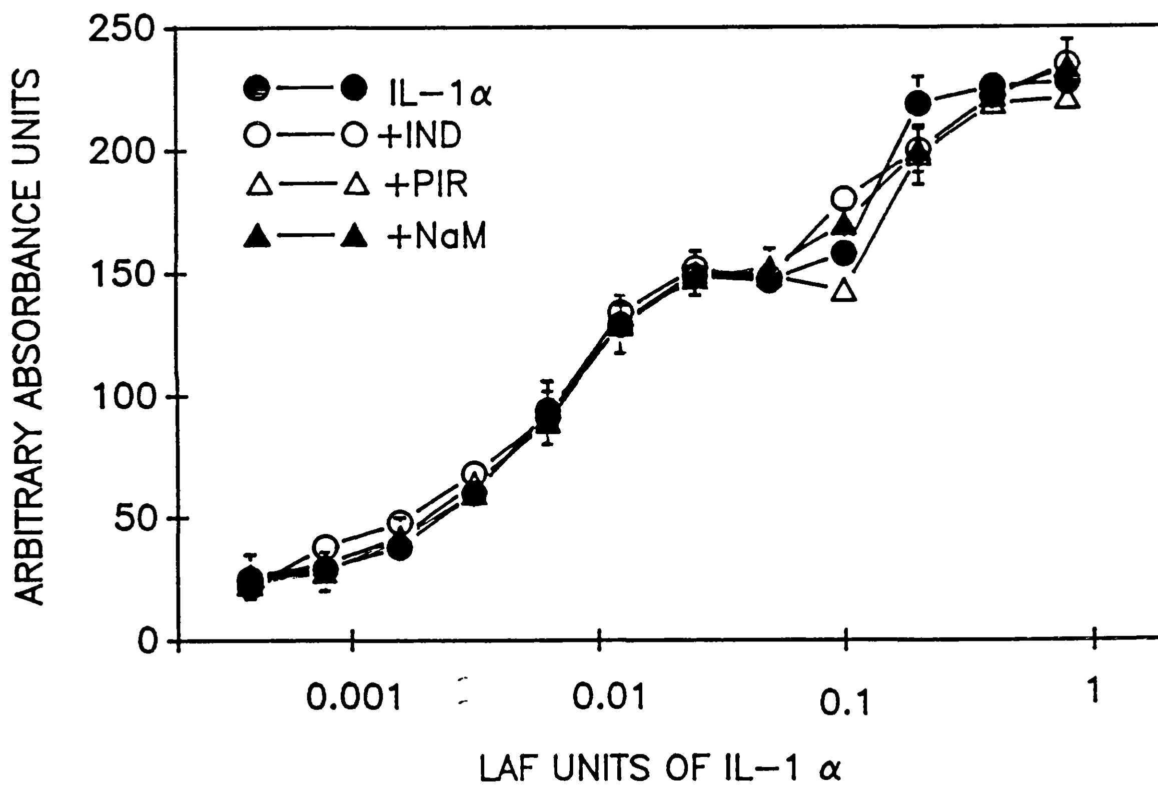


Figure 4.6 The effect of NSAIDs on the response of EL4 NOB1 cells to rhIL-1α. The above is a representative experiment. Each point is the mean of a triplicate reading.

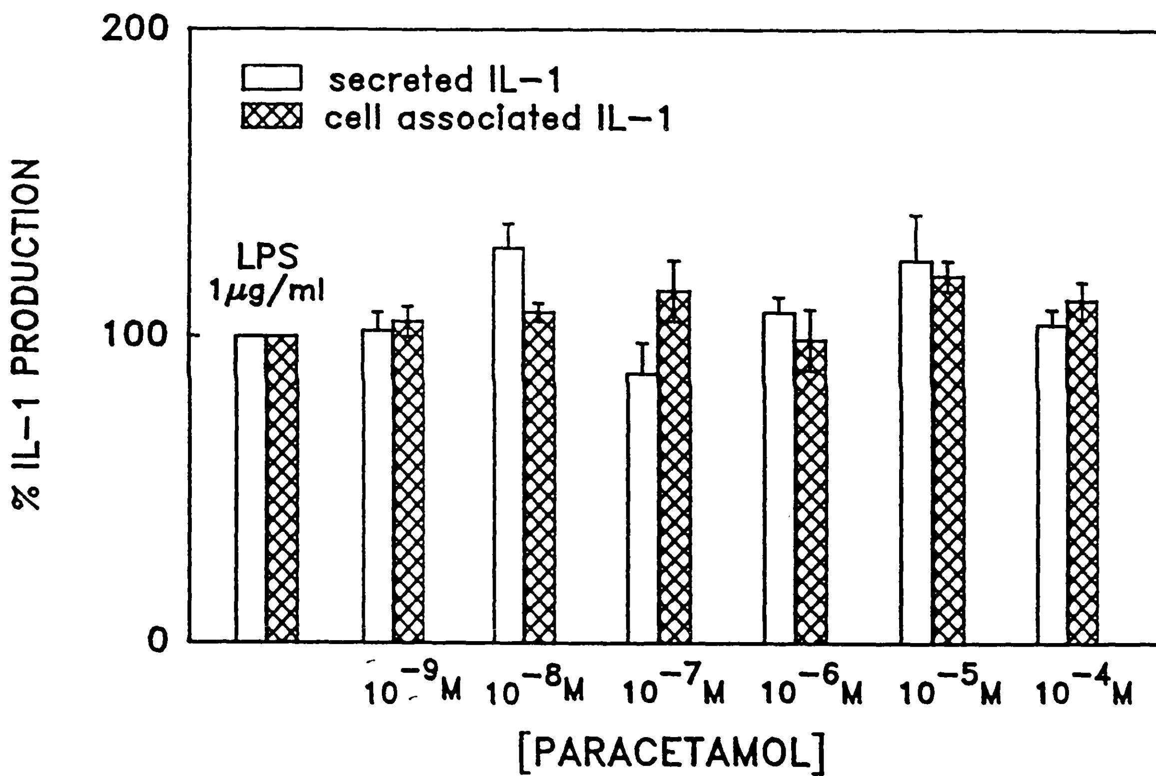


Figure 4.7 The effect of paracetamol on IL-1 secretion and cell-associated IL-1. Supernatants were diluted 100 to 400 fol for the determination of secreted IL-1 and 1000 to 4000 fold for the determination of cell-associated IL-1 . Each point represents the mean ± standard error (n=3).

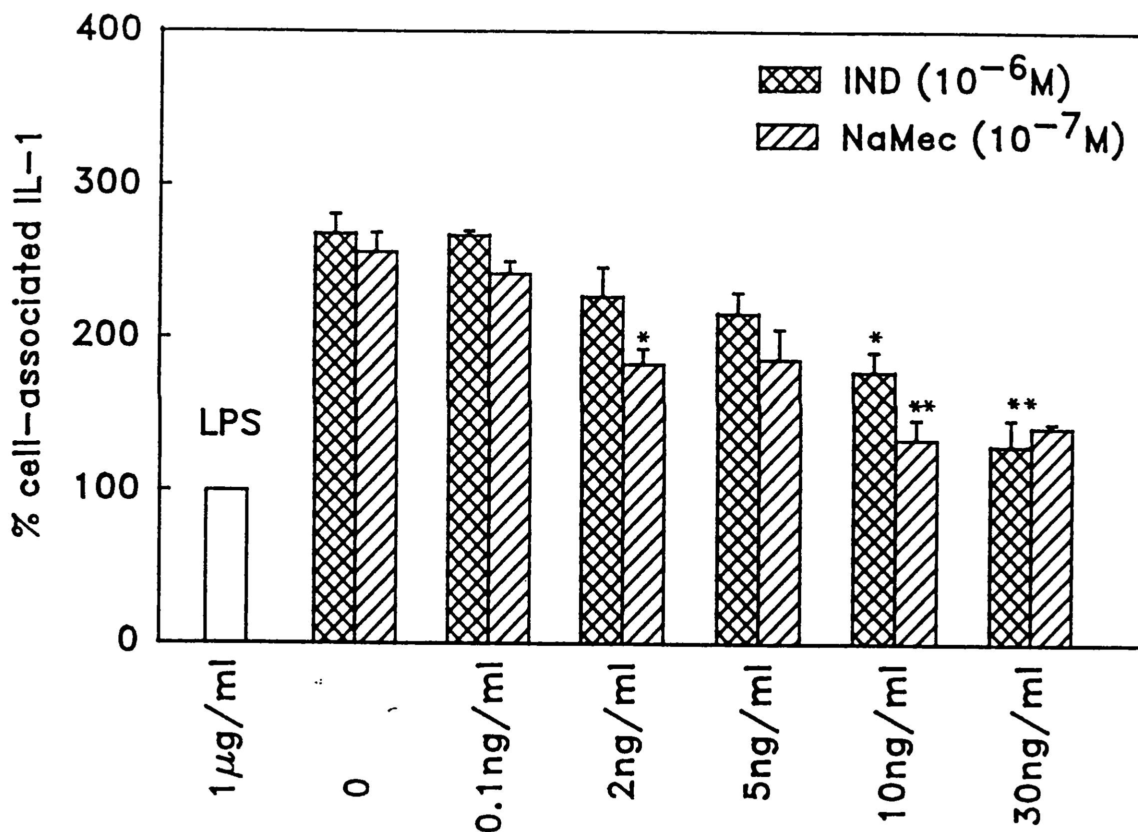


Figure 4.8 The effect of exogenous PGE_2 on NSAID-potentiated IL-1 production. The PGE_2 was added 30min after the addition of the NSAID. Each bar represents the mean ± standard error (n=4). Statistical difference from control was determined using the Students t-test. ** $p < 0.01$ * $p < 0.05$.

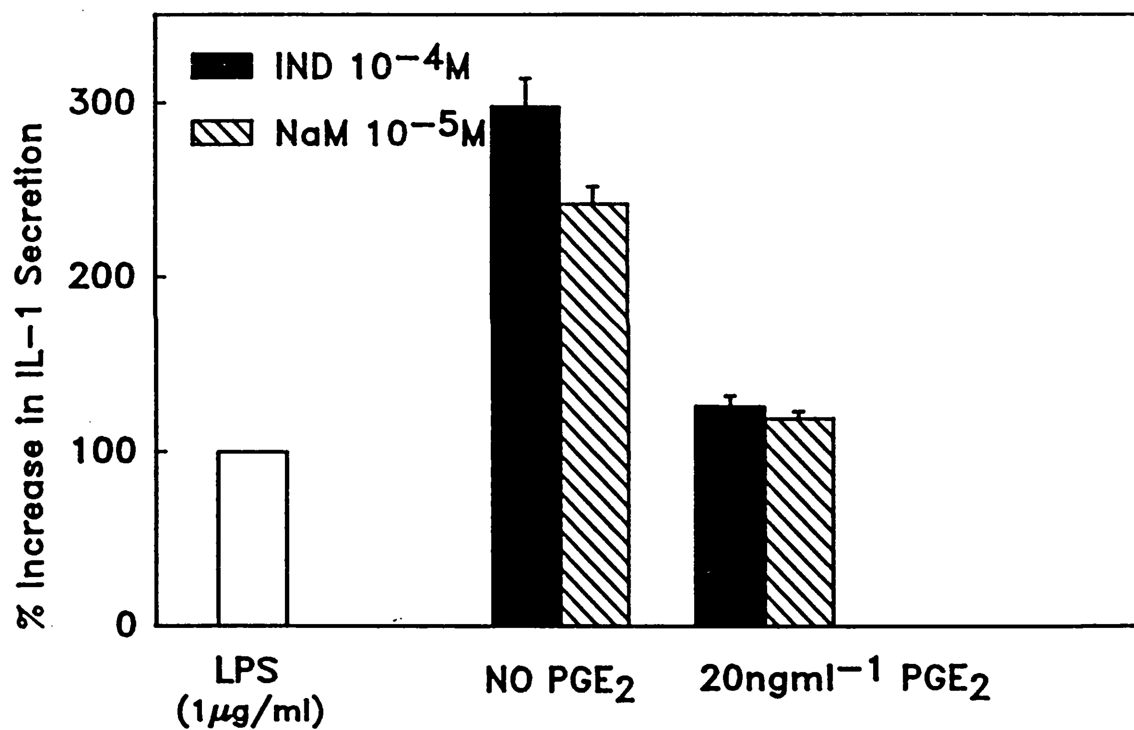


Figure 4.9 The effect of PGE_2 on NSAID potentiated IL-1 secretion. The PGE_2 was added 30min after the addition of the NSAID. The above is a representative experiment. Each bar is the mean of triplicate readings.

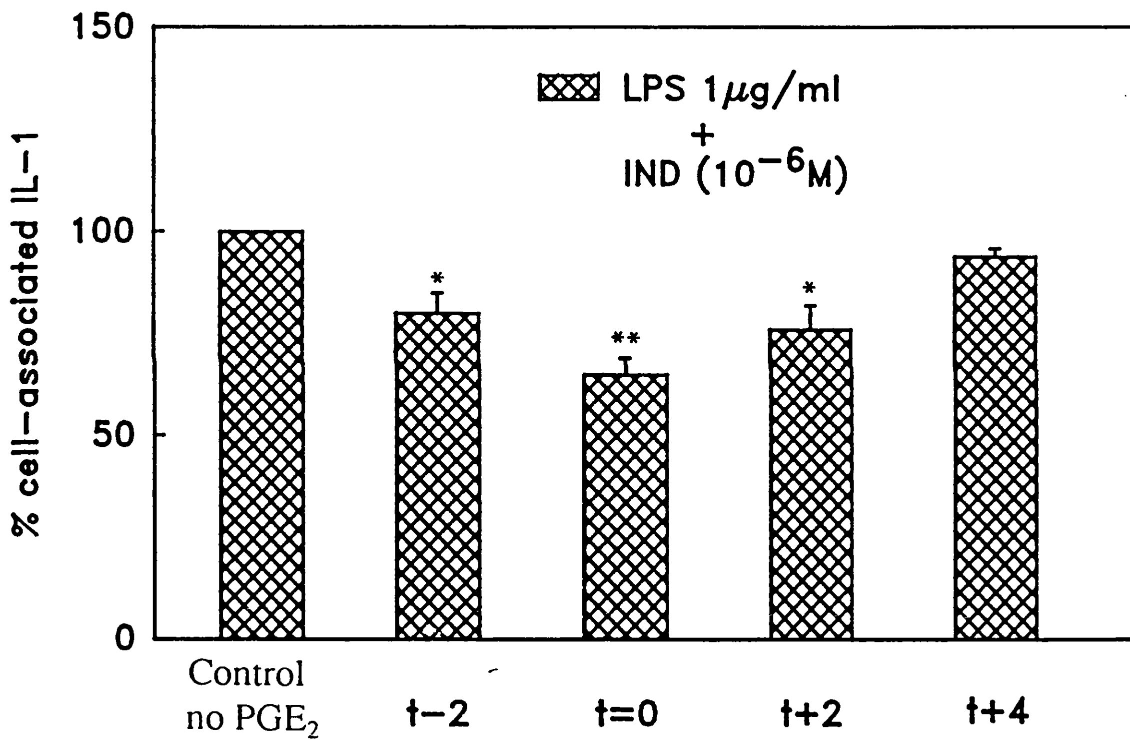


Figure 4.10 The time-dependent inhibition of cell-associated IL-1 by PGE_2 ,20ng.ml^{-1} . PGE_2 was added before (-) or after (+) the addition of IND + LPS at the times shown. Each bar represents the mean ± standard error (n=6). Statistical difference from control was determined using the Students t-test. ** $p< 0.01$ * $p<0.05$.

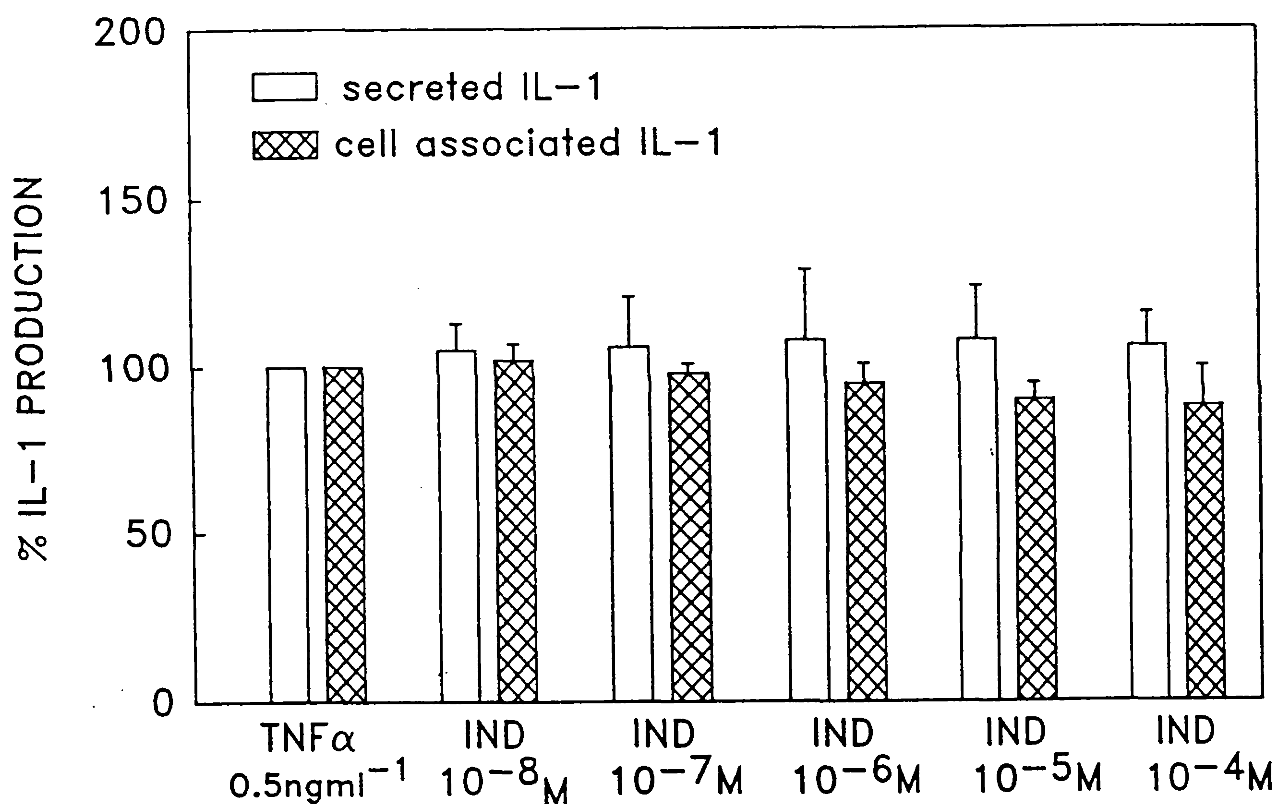


Figure 4.11 The effect of indomethacin on rhTNFα-stimulated IL-1 production. TNFα, 0.5ng.ml^{-1} increased cell-associated IL-1 by 250% from untreated cells but did not stimulate IL-1 secretion. This effect was arbitrarily set at 100% and the effect of indomethacin expressed as a % of this response. Each bar represents the mean ± standard error (n=3).

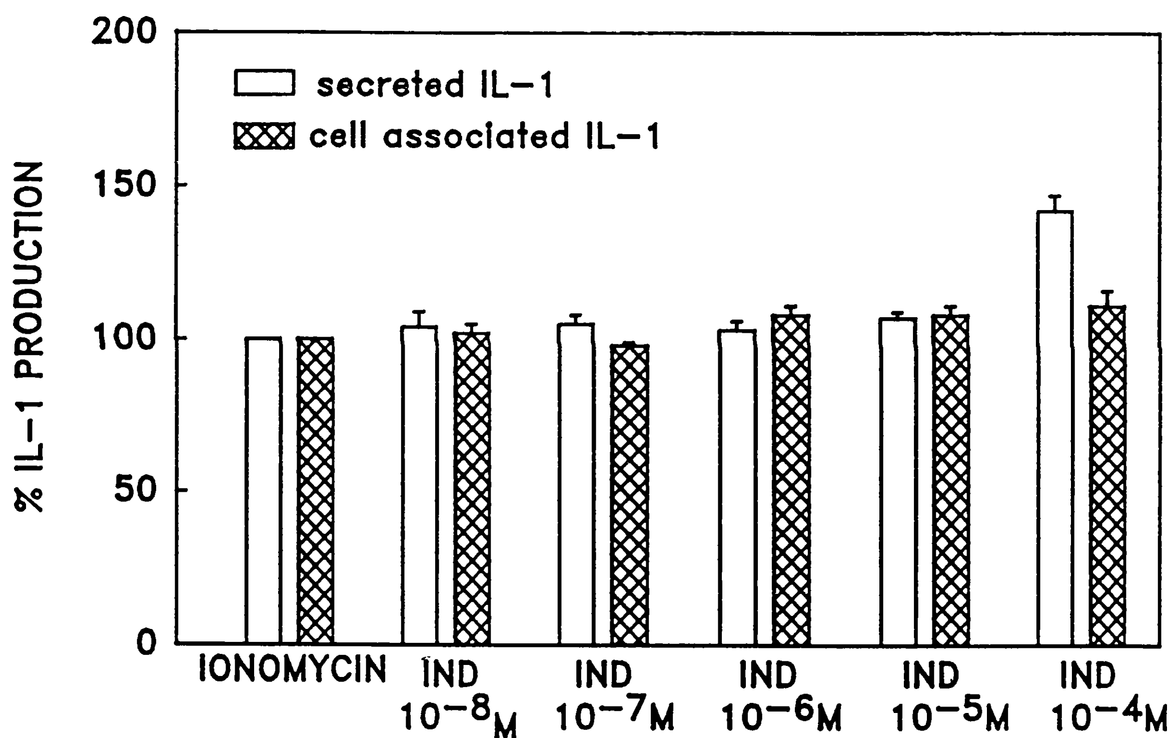


Figure 4.12 The effect of indomethacin on ionomycin-stimulated IL-1 production. Ionomycin, $5x10^{-7}M$ was used and this increased cell-associated IL-1 by 200% compared with untreated cells but did not stimulate IL-1 secretion. This effect was arbitrarily set at 100% and the effect of indomethacin expressed as a % of this response. Each bar represents the mean ± standard error (n=3).

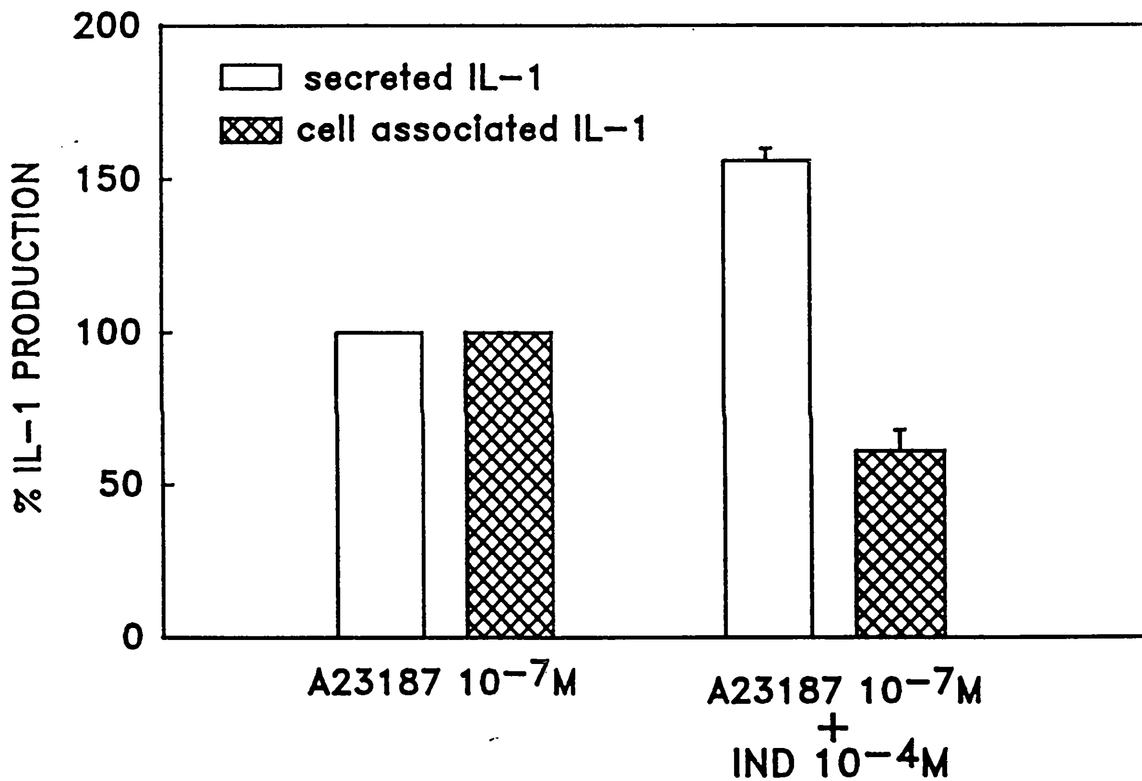


Figure 4.13 The effect of indomethacin on A23187-stimulated IL-1 production. A23187 10^{-7}M was used and this increased cell-associated IL-1 by 350% compared with untreated cells but did not stimulate IL-1 secretion. This effect was arbitrarily set at 100% and the effect of indomethacin expressed as a % of this response. Each bar represents the mean ± standard error (n=3).

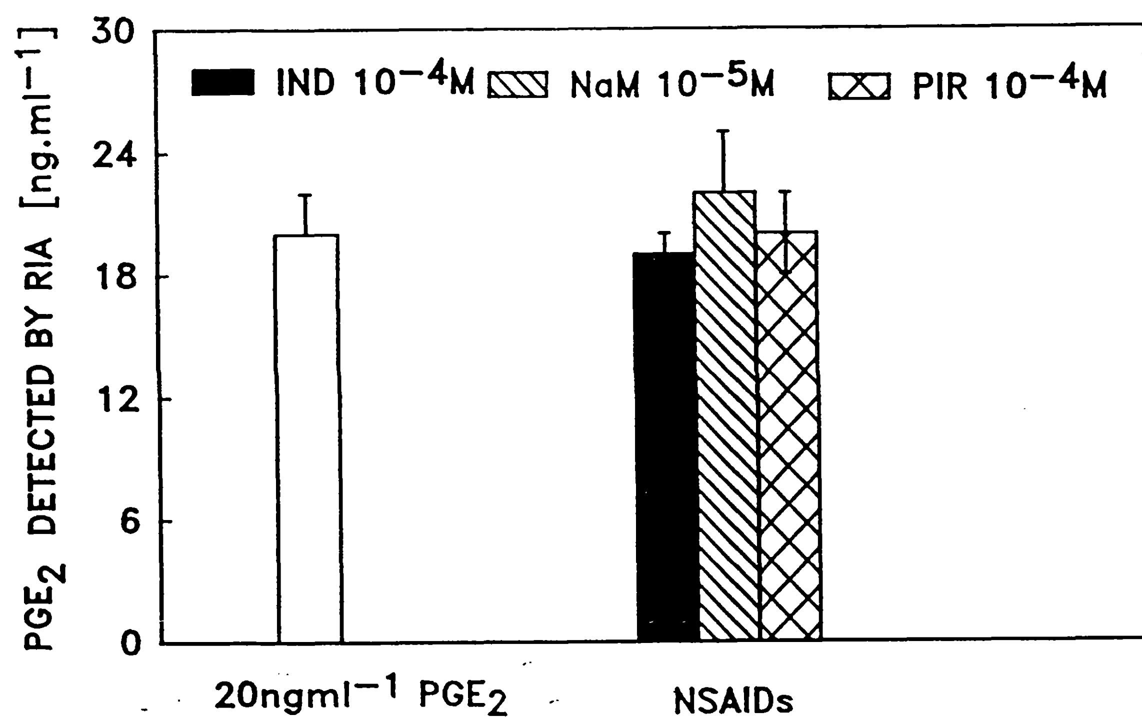


Figure 4.14 The effect of murine macrophages on exogenous PGE_2. PGE_2 20ng.ml^{-1} was added to macrophages that had been treated with a NSAID and allowed to incubate for 20hr. at 37°C before being assayed. PGE_2 20ng.ml^{-1} was also added to medium alone and carried through the same assay. Each bar represents the mean ± standard error (n=3).

4.3 DISCUSSION

4.3.1 Do NSAIDs affect IL-1 production?

The results in this chapter clearly show that NSAIDs (with the exception of ASP within the context of IL-1 secretion) can dose-dependently potentiate both IL-1 secretion and cell-associated IL-1. The concentrations of NSAID required to potentiate cell-associated IL-1 paralleled concentrations required to inhibit PGE_2 synthesis. Indeed, maximum accumulation of cell-associated IL-1 was observed at the minimum concentration of PIR, NaM and IND required to ablate PGE_2 synthesis. At concentrations of NaM, IND and PIR greater than that required to inhibit PGE_2 synthesis, ie. 10^{-6}-10^{-5}M and 10^{-5}-10^{-4}M and 10^{-4}M respectively, a dose-dependent reduction in cell-associated IL-1 was observed. When the ability of NSAIDs to potentiate IL-1 secretion was examined, it was found that concentrations greater than those required to inhibit PGE_2 synthesis were required to produce the most dramatic increases in IL-1 secretion. Interestingly, the concentrations of IND, PIR and NaM that reduced the accumulation of cell-associated IL-1 were also the most effective at potentiating the secretion of IL-1. The NSAIDs alone without LPS stimulation, did not stimulate IL-1 production from macrophages.

4.3.2 Is PGE_2 a down-regulator of IL-1 production?

PGE_2 dose-dependently and time-dependently inhibited the potentiation of both IL-1 secretion and accumulation of cell-associated IL-1 produced by NSAIDs *in vitro*. The inhibitory effect of exogenous PGE_2 was produced over the concentration range generated *in vitro* by stimulation with LPS, $1\mu g.ml^{-1}$. Indeed, the concentration of PGE_2 created by the stimulation with LPS was sufficient to reduce NSAID-potentiated IL-1 production back to the levels produced by LPS alone. Thus, the present study, by using a more sensitive and selective bioassay for IL-1, is in agreement with reports of an inhibitory effect of PGE_2 on IL-1 production. However, a

sweeping statement that PGE_2 inhibits IL-1 production in all cell-types cannot be made on the results of this study alone. It remain to be investigated whether or not the inhibitory activity of PGE_2 is dependent on:-

(i) cell-type
(ii) activation-state of the cell [e.g. macrophages can exist in responsive, primed or fully activated states, Hamilton & Adams (1987)]
(iii) the stimuli used to initiate IL-1 production

4.3.3 **Why is the inhibitory effect of PGE_2 time-dependent**

Cyclic AMP and agents known to mimic or elevate cAMP levels have no effect on the levels of IL-1 mRNA translated or on the stability of mRNA generated. However, the translation of the IL-1 mRNA into IL-1 pro-peptides can be inhibited by cAMP according to Knusden et al. (1986) and Kunkle et al. (1990). This offers an explanation as to how PGE_2 could inhibit IL-1 production ie. inhibition of post transcriptional translation of IL-1 mRNA.

Knudsen et al. (1986) observed that PGE_2 produces a transitory stimulation of cAMP in macrophages. The authors observed a maximum accumulation of cAMP 20 min after PGE_2 addition, which subsequently declined to basal levels 60 min after PGE_2 addition, with no subsequent elevation in cAMP. The transient elevation in intracellular cAMP probably activates a cAMP-dependent protein kinase. The kinase may then phosphorylate a variety of cellular proteins, some of which may inhibit the translation and processing of IL-1. Thus, addition of PGE_2 to macrophages 4hr prior to LPS may result in a return of cAMP to basal levels and the subsequent decline in the inhibitory proteins induced.

The transitory elevation of cAMP by PGE_2 rather than a prolonged elevation may involve various desensitisation mechanisms. Shamma et al. (1988) have investigated the mechanisms which limit the stimulation of adenylate cyclase by PGE_2 in the P388D1 murine-macrophage cell-line. The authors found that:

1) Exposure of the cells to PGE_2 for 60 min induced a PGE_2-specific desensitization of the adenylate-cyclase system. The ability of other ligands such as isoprenaline, 5'-guanylimidodiphosphate or forskolin to stimulate adenylate-cyclase was unaffected over this time period. This early 'homologous' desensitization appeared to be due to the reduction in the number of PGE_2 receptors on the cell surface.

2) Exposure of cells to PGE_2 for 6hr induced a 'heterologous' desensitization, as the responses of adenylate-cyclase to PGE_2 as well as other ligands was significantly reduced. This late 'heterologous' desensitisation was postulated to involve the functional and/or structural alteration of G proteins responsible for transducing the receptor signal.

That a 2hr delay in the addition of PGE_2 following LPS also results in a loss of inhibitory activity, as regards IL-1 production, cannot be explained by the above . Some alternative explanations may include :-

(1) the inhibitory effect of cAMP is directed at the very early stages involved in the transduction mechanisms employed by LPS.

(2) LPS may inhibit the transduction mechanisms employed by prostaglandin receptors and/or down regulate such receptors. For example, Hazeki et al. (1986) showed that exposure of guinea-pig macrophages to LPS for 5h inhibited the ability of PGE_2 and adrenaline to elevate cAMP in such cells.

(3) The IL-1 mRNAs may have been maximally translated into their pro-peptides.

4.3.4 What is the mechanism of NSAID-potentiated IL-1 production ?

The ability of NSAIDs to potentiate the accumulation of cell-associated IL-1 appears to be related to their ability to inhibit PGE_2 synthesis. This is supported by the following evidence obtained during the present study :

(i) The dose-response curve for NSAID-potentiation of cell-associated IL-1 parallels that for the inhibition of PGE_2 synthesis.

(ii) Maximum accumulation of cell-associated IL-1 occurs at the minimum concentration of NSAID required to ablate PGE_2 synthesis.

(iii) Addition of exogenous PGE_2 can dose-dependently inhibit NSAID-potentiated cell-associated IL-1 accumulation.

(iv) Paracetamol, an antipyretic and analgesic which lacks the ability to inhibit peripheral cyclo-oxygenase, had no effect on IL-1 accumulation.

However, by inhibiting PGE_2 synthesis NSAIDs could be potentiating the synthesis of leukotrienes which in-turn could contribute to the accumulation of cell-associated IL-1. The role of the 5-lipoxygenase pathway in potentiating IL-1 production will be dealt with in Chapter 5.

The ability of NSAIDs to potentiate LPS-stimulated IL-1 secretion does not appear to be related to their ability to inhibit PGE_2 synthesis. For instance, PIR 10^{-5}M although ablating PGE_2 synthesis did not significantly increase IL-1 secretion. Concentrations much greater than those required to

inhibit PGE_2 synthesis resulted in a dose-dependent potentiation of IL-1 secretion with IND and NaM. The observation that NSAIDs potentiate IL-1 secretion at concentrations above those required to aboragate PGE_2 production and that such concentrations also result in a decrease in cell-associated IL-1 from the maximum produced by each respective NSAID, suggests that IND, PIR and NaM may enhance IL-1 secretion by triggering the release of cell-associated IL-1. Although IND, NaM, PIR and ASP below 10^{-6}M, 10^{-7}M, 10^{-5}M and 10^{-4}M respectively did not produce an observable increase in IL-1 secretion, this does not rule out the possibility that at such concentrations the NSAIDs may have been potentiating the secretion of the IL-1ß pro-peptide. The IL-1ß pro-peptide if unprocessed would not be detected by the bioassay. The use of an ELISA assay or RIA for IL-1α and IL-1ß could be used to investigate such a possibility since they would detect all antigenic forms of IL-1. To elucidate the mechanism by which NSAIDs can potentiate LPS-stimulated IL-1 secretion requires a knowledge of:

(i) How LPS stimulates IL-1 production

(ii) How IL-1 is secreted

The above two questions represent important stumbling blocks in understanding the mechanisms by which drugs can interfere with IL-1 synthesis and secretion. Chapter 3 dealt with the current opinions regarding the synthesis and secretion of IL-1, but in short the mechanism by which IL-1 is processed and secreted remain unresolved. The structure and biological activity of LPS has been discussed in Chapter 3. The precise nature of the signal transduction events or receptors activated by LPS is unclear. For reviews on LPS activation pathways see Cavillion & Haffner-Cavaillon (1990)

4.3.5 The effect of IND on Ca^{2+}-ionophore and rhTNFα-stimulated IL-1 accumulation

In Chapter 3 it was shown that the Ca^{2+} ionophores A23187, and ionomycin and rhTNFα dose-dependently stimulate the accumulation of cell-associated IL-1 without stimulating IL-1 secretion. IND at 10^{-6}M and 10^{-4}M concentrations previously shown to potentiate cell-associated IL-1 and IL-1 secretion respectively, did not potentiate the accumulation of cell-associated IL-1 with rhTNFα or 'trigger' the secretion of the accumulated IL-1. Similarly, IND was without significant effect in potentiating ionomycin-stimulated cell-associated IL-1 or 'triggering' the secretion of the accumulated IL-1. However, with A23187 ,IND 10^{-4}M enhanced IL-1 secretion with a concomitant decrease in cell-associated IL-1, but this was shown to be due to a cytotoxic effect exerted by IND and A23187 in combination at such concentrations. Thus the ability of NSAIDs to potentiate LPS-stimulated IL-1 secretion is not due to :

(i) direct stimulation of the secretory mechanism for cell-associated IL-1.

(ii) a non-specific effect e.g. changing the fluidity of the macrophage membrane thus allowing the leakage of cell-associated IL-1.

Therefore, IND 10^{-4}-10^{-5}M, serves to potentiate a secretory pathway for IL-1 stimulated by LPS. For stimuli that merely increase cell-associated IL-1 production without inducing IL-1 secretion, IND does not trigger the secretion of such IL-1 . This suggests that IND potentiates, but does not directly activate a secretory mechanism for IL-1. Whether this also applies to the other NSAIDs remains to be investigated. Similarly, whether the effect is specific for LPS or is generally applicable to any stimulus that triggers IL-1 secretion also awaits investigation.

4.3.6 **The possible mechanisms by which NSAIDs potentiate IL-1 secretion stimulated by LPS.**

The suggestions that can be made about the mechanism(s) by which NSAIDs potentiate LPS-stimulated Il-1 secretion are listed below :-

1) Potentiation of the transduction pathway(s) for LPS e.g. enhancing PKC stimulation or the activation of other kinases.

2) Inhibition of the regulatory mechanisms that limit the stimulatory capacity of LPS

3) Potentiation of post-translational translocation of IL-1, stimulated by LPS, (see Chapter 3 for relevance to IL-1 secretion).

4) Potentiation of the processing of IL-1ß pro-peptide into its bioactive form by enhancing the production or activity of processing enzymes.

4.3.7 **How do the concentrations of NSAIDs used in the present study compare with those achieved *in vivo*?**

Although a considerable amount of data has been accumulated concerning the disposition of NSAIDs in normal subjects and in patients with a variety of clinical conditions, current understanding of these drugs indicates that much of the prior data is inaccurate and potentially misleading. Problems with prior studies include:-

(i) **Lack of assessment of unbound pharmacologically active drugs.** All NSAIDs are highly protein bound, in excess of 99%, leaving only 1% or less of the drug free in plasma and available to tissue sites of activity. Many studies of NSAID disposition ignore protein binding and simply report results in terms of total drug concentration. As patients age, their

serum albumin concentrations progressively diminish (Schmuller, 1979). Thus, on average serum concentrations of albumin are lower in the elderly than in the young patients. Similarly the RA processes leads to diminished levels of albumin in both the intravascular and extravascular pools (Vestal et al., 1978) possibly due to increased catabolism caused by cytokines such as IL-1, IL-6 and TNFα. Studies which have compared protein bound and unbound levels in plasma have shown that in the elderly twice the amount of "free" naproxen is found in the systemic circulation compared with a young control group (Upton et al., 1984; Van Den Ouwland et al., 1988).

(ii) **Distribution.** Although measurement of plasma concentrations of NSAID following single and multiple doses of these drugs provides useful data in defining rates of absorbtion and elimination, such studies provide little information as to the efficacious concentrations of the drug at their site of action ie. the synovium. The NSAID concentration in synovial fluid is dependent upon the nature of the exchanges occurring between the synovial fluid and the plasma through the anatomical barriers ie. the synovium and the small blood vessel walls supplying the synovium. The parameters governing the exchange of NSAID between plasma and the synovium are:-

(a) diffusion of free drug across the synovium and blood vessels

(b) leakage of plasma proteins across the synovial blood vessels

Both of the above can be influenced during the course of inflammation. A reduction in pH caused by the influx and metabolic activity of cells recruited to an inflammatory site would favour the existence of the weakly acidic NSAID in an undissociated form. Thus upon entering the synovium the NSAID

would become more lipophilic and associate with lipid pools and proteins thus creating a concentration gradient for more NSAID to diffuse into the synovial fluid. Therefore, although studies of unbound NSAID reveal a close parity between synovial and plasma concentrations they do not take into account the concentration of NSAID that may actually be associated with the plasma membranes of the inflammatory cells. Indeed the possibility that NSAIDs may actually accumulate within cells has been poorly studied. Studies have shown that salicylate-type NSAIDs and indomethacin accumulate within peripheral mononuclear cells *in vitro* (Raghoeber et al., 1988, 1989). Palmoski & Brandt (1985) have demonstrated that concentrations of NSAIDs in canine knee cartilage removed and cultured *ex vivo* exceeded those in the surrounding medium. This raises an important point of whether or not it is the concentration of NSAID associated with inflammatory cells rather than "free-unbound drug" that governs the pharmacodynamics of NSAIDs. This is particularly pertinent for NSAIDs which are planar lipophilic drugs capable of irreversibly binding to proteins/enzymes eg. aspirin irreversibly acetylates serine-residues of cyclo-oxygenase. Measurements of NSAID concentrations associated with cells may provide a better indicator for dose-response profiles than measurements of plasma and synovial fluid concentrations. Indeed,Orme et al. (1985) have shown that measurements of NSAID concentrations in plasma and synovial fluid are poor indicators of dose-response profiles.

(iii) **Stereospecificity.** All of the propionic acid NSAIDs are administered as racemic mixtures (except naproxen which is given only as the active S-stereoisomer. That the stereoisomers possess identical chemical characteristics, but exhibit different pharmacokinetics and pharmacodynamics has been shown by a number of studies (Ariens, 1983; Drayer, 1986; Evans et al. 1988). The separate enantiomers of chiral NSAIDs have been shown to differ in protein binding, excretion and metabolism. Indeed for ibuprofen, inversion from the R to the

S-configuration occurs *in vivo* (Evans et al. 1988). Thus studies which measure the combined amount of both stereoisomers do not accurately reflect the disposition of the active NSAID.

(iv) **Dose.** Many of the pharmacokinetic studies with NSAID rely on the measurement of body fluid levels after single or multiple dosing with a NSAID. Taking into consideration the above discussion, such protocols would overlook the ability of NSAID to accumulate in body fluids by virtue of the cells acting as "reservoirs" for chronically administered NSAIDs. Furthermore, a 'standard' NSAID dose is employed in such studies. Taking one such study as an example 50mg of indomethacin was given to arthritic patients as a single dose and 'free'-plasma levels measured with time, resulted in a peak of 1-8μM (Emouri et al., 1973). However, when Hvidberg et al. (1972) administered 25mg of indomethacin to arthritic patients continuously, peak levels of 1.5-8.5μM were obtained; a similar level observed by Emouri et al. but with half the dose. The question of what is a 'standard dose' also needs to be addressed. In many arthritic patients NSAID dose is titrated to produce a desired clinical response. Thus, it is not uncommon for a dose range of 25-300mg of indomethacin to be employed in treating RA patients. The preoccupation of researchers conducting pharmacokinetic studies with the use of a standard 25-50mg dose of indomethacin may overlook the disproportionate increases in free levels of drug that may occur at higher concentrations, due to the saturation of the binding sites on albumin. This is particularly pertinent in patients where albumin levels have been reduced as a result of a disease process . The NSAIDs also vary greatly with respect to half-lives of elimination. Naproxen, phenylbutazone, piroxicam and azapropazone are examples of NSAIDs that have half-lives in excess of 10 hrs. The half-lives of salicylates are dose-dependent but, at the high doses used in the treatment of RA, the half-life tends to be greater than 10hrs. In contrast, the half-lives of most other NSAIDs are in the

range of 1-3 hr. Thus, acute pharmacokinetic studies may overlook the ability of NSAIDs with long half-lives of elimination to accumulate within the synovial fluid.

Although *in vivo* concentrations of 10^{-4}M have not been reported for NSAIDs such as IND, the concentration range of 10^{-6}-10^{-5}M used in the present study occurs in patients taking IND. However, a debate over appropriate concentrations to use *in vitro* should be conducted with caution when the information concerning the pharmacokinetic and pharmacodynamic parameters of NSAID action *in vivo* remains far from clear.

In conclusion, it is necessary to examine the limitations of the findings presented above before extrapolation to an *in vivo* context e.g. RA or animal models of RA where IL-1 production is known to occur and contribute to the pathology. The obvious differences are that the present study:

1) **Looks at an isolated cell-type.**
In vivo, complex interactions between T-cells, B-cells, osteoblasts, osteoclasts etc. are important in regulating mediator production. Thus, effects observed *in vitro* may never occur *in vivo* and *vice versa* due to regulator controls exerted by other cell-types. For instance, NSAIDs may also potentiate the secretion of other cytokines such as TGFβ, IL-10 and IL-4 which serve to inhibit IL-1 secretion. On the other hand, NSAIDs may further exacerbate the production of IL-1 *in vivo* by stimulating the production of pro-inflammatory cytokines from T-cells etc. eg. TNFα, GM-CSF, IL-6 and IFNγ which have been shown to enhance IL-1 production.

2) **Limited time-course.**
Chronic inflammatory conditions *in vivo* are initiated and progress over considerably longer time-courses than the 20hr incubation employed in the present study. Whether or not NSAIDs produce a sustained increase in IL-1 production or whether their effect is transitory is not resolved by the

present study. Longer incubation times with NSAIDs could have been employed, however the ability of isolated macrophages to produce IL-1 decreases upon culturing for more than 1 day.

(3) **The accumulation of PGE_2.**

The present study showed that resident murine peritoneal macrophages do not metabolise exogenously added PGE_2. This was hardly surprising since the metabolism of PGE_2 is known to occur mainly in the lung and liver by specific enzymes. Thus, the *in vitro* model lacks the ability to remove the PGE_2 generated by the macrophages. Therefore, the question arises, does PGE_2 accumulate *in vivo* and if so, how do the concentrations compare to the *in vitro* environment?. That PGE_2 does accumulate in the RA joint has been shown by Higgs et al 1974 who found levels around 5-20ng.ml^{-1} in synovial exudates which are similar to the levels observed in the present study. Pettipher et al. (1989) measured the accumulation of PGE_2 in the synovial fluid from an antigen-induced model of RA in rabbits and found levels of around 10ng.ml^{-1}. Thus, there seems to be little disparity between the levels of PGE_2 accumulation by the cells in the present study and that accumulated *in vivo*.

(4) **Desensitization phenomena.**

It was shown in the present study that macrophages pre-incubated with PGE_2 became desensitised to its inhibitory effect on IL-1 production in a time-dependent manner. Since PGE_2 accumulation does occur *in vivo* the question arises as to whether or not a similar desensitization phenomena occurs *in vivo*. If so, the administration of NSAIDs would not exert an effect on IL-1 production by virtue of inhibiting PGE_2 synthesis since the cells would already be desensitized to the effects of PGE_2. However, this would not rule out the ability of NSAIDs to trigger the secretion of accumulated IL-1 which was shown to be independent from their ability to inhibit PGE_2 synthesis.

The findings of the present study contribute to the growing number of reports that outline the pro-inflammatory effects of NSAIDs (see Chapter 1). It may possibly provide a useful platform to extend observation on IL-1 production from isolated human cells treated with NSAIDs and also to look at the ability of NSAIDs to modulate IL-1 production *in vivo*.

CHAPTER 5

THE EFFECT OF SELECTIVE 5-LIPOXYGENASE INHIBITORS ON IL-1 PRODUCTION

5.1 INTRODUCTION

Lipoxygenase enzymes were first characterized in the plant kingdom; to date several lipoxygenase enzymes have been isolated from both animals and plants. The lipoxygenases catalyse the oxidation of Cis, Cis-1,4-pentadiene systems e.g. arachidonic, eicosatrienoic and eicosapentanoic acids by the addition of molecular oxygen. One such enzyme, 5-lipoxygenase (5-LO) catalyses the conversion of arachidonic acid (AA) into leukotrienes (LTs). The LTs were so named because they were isolated as products of AA metabolism from leucocytes and contained a conjugated triene in their structure. The discovery of LTs and the 5-LO pathway were closely associated with the biochemical and pharmacological investigation of "slow-reacting substances of anaphylaxis" (SRS-A) (Feldberg & Kellaway, 1938). A substantial body of work has now characterized SRS-A as a mixture of LTs C_4, D_4 and E_4. Like the prostaglandins, LTs are divided into groups (A-F) according to major structural differences and into sub-groups according to the number of double bonds in the side chains. LTs are formed *de novo* by the action of 5-LO on released AA through an intermediate, 5-hydroperoxy-eicosatetraenoic acid 5-HEPTE. That the formation of LTs is dependent upon the conversion of AA by a single enzyme was first demonstrated by using 5-LO isolated from potato tubers and later confirmed for mammalian 5-LO (Samuelson & Funk, 1989). Studies on the subcellular localization of 5-LO has demonstrated that the enzyme resides in the cytoplasm until the cell is appropriately stimulated whereupon the enzyme is translocated to the membrane. The membrane associated 5-LO requires the

presence of another membrane associated protein, Ca^{2+} and ATP for subsequent activation. The protein responsible for the activation of 5-LO has been characterized with the use of MK-886. MK-886 is a selective inhibitor of LT synthesis in intact, activated leukocytes but has little effect on isolated enzymes involved in LT synthesis, including 5-LO in cell-free systems. MK-886 has been shown to bind to and inactivate a membrane associated protein of MW 18kDa. Since the 18kDa protein appears to be involved in the activation of 5-LO, it has been termed five-lipoxygenase activating protein (FLAP) (Miller *et al.* 1990; Dixon *et al.*, 1990) Dixon *et al.* (1990) have shown that transfection of the genes encoding for FLAP and/or 5-LO into human osteosarcoma 143 cells, which express neither of the two proteins, results in significant LT production only when both FLAP and 5-LO are expressed by the cells. Whereas cyclo-oxygenase (CO) is widely distributed in mammalian cells, the 5-LO is restricted mainly to neutrophils, eosinophils, monocytes/macrophages and mast-cells. These cells originate in the bone-marrow and probably derive from the same stem-cell. As with prostaglandins, different LTs are formed in specific cell-types. For instance, human eosinophils and neutrophils synthesize predominantly LTC_4 or LTB_4 respectively (Weller *et al.* 1983) whereas monocytes/macrophages are able to synthesize both LTB_4 and the peptido-lipid leukotrienes.

LTs are believed to contribute to the pathophysiology of a number of disease states including asthma, psoriasis and inflammatory bowel disease (Zakrzewski *et al*, 1987; Ruzicka *et al.*, 1986, Wallace, 1990). Raised concentrations of LTs have been detected in the synovial fluid from patients suffering from RA and gout (Klickstein *et al.*, 1980, Davidson *et al.*, 1982 and Rae *et al.*, 1982). LTs have also been implicated in NSAID-induced gastrointestinal ulceration and bronchoconstriction in asthmatics. The NSAID, indomethacin has been shown to increase the production of LTB_4 in Ca^{2+} ionophore-stimulated neutrophils and rat peritoneal

macrophages (Ham *et al.*, 1983, Elliott *et al.*, 1989). The enhanced LT production from NSAID-treated cells may result from accumulated AA being shunted to the 5-LO pathway and/or the removal of the inhibitory effect of prostaglandins on the 5-LO enzyme. PGE_1 and PGE_2 have been shown to inhibit the production of LTs from neutrophils and rat peritoneal macrophages (Ham *et al.*, 1983; Elliott *et al.*, 1989).

The involvement of LTs in acute inflammation and anaphylactic bronchoconstriction has been clearly shown by the use of selective 5-LO inhibitors (Bhattacherjee *et al.*, 1988). However, their role in chronic inflammation remains to be investigated via the use of such selective 5-LO inhibitors. Since IL-1 has been implicated as a mediator of chronic inflammation, selective inhibitors of 5-LO could be employed to investigate the role for LTs in the modulation of IL-1 production. Data presented by others and in this present study (see Chapter 4) have shown that NSAIDs can potentiate the production of IL-1 from macrophages. Thus, two selective 5-LO inhibitors have been used to investigate:-

(i) Whether the NSAID-enhanced IL-1 production, from LPS-stimulated macrophages, is due not only to the inhibition of PGE_2 synthesis, but also the subsequent diversion of AA to the 5-LO pathway.

(ii) Whether LT synthesis is a mechanism by which LPS stimulates IL-1 production.

Selective inhibitors of 5-LO

There has been a great deal of research within the pharmaceutical industry to develop selective 5-LO inhibitors with a view to their use in treating inflammatory diseases such as asthma, inflammatory bowel disease and RA. Two chemically distinct, putatively selective, 5-LO inhibitors were made available for the purposes of the present study:-

(i) BWA4C

Corey *et al* (1984), working on the hypothesis that iron plays a key role in lipoxygenase catalysis, synthesized some amide analogues of AA in which strong co-ordination to iron was possible. They found that analogues containing a hydroxamic acid moiety were potent and selective inhibitors of LT biosynthesis. Wellcome Research laboratories have designed a number of novel acetohydroxamic acids (BWA4C, BWA137C, and BWA797C) that exhibit selective inhibition of 5-LO over a given concentration. BWA4C (N-(3-phenoxycinnamyl)-acetohydroxamic acid is a potent inhibitor of LTB_4 and [^{14}C] 5-HETE generation from AA. The synthesis of LTB_4 from 5-HPETE is also inhibited (Tateson *et al.,* 1988) thus implying inhibition of both 5-LO and LTA_4 synthase. The authors found the IC_{50} value for inhibition of 5-LO and cyclooxygenase in polymorphonuclear leukocytes to be $0.1 \pm 0.03 \mu M$ and $3.2 \pm 0.8 \mu M$ respectively.

(ii) L-651, 392

L-651,392 (4-bromo-2,7-dimethoxy-3H-phenothiazin-3-one) has been shown to be a potent and selective 5-LO inhibitor, with oral activity in animal models of allergy (Guindon *et al.*, 1987). Table 5.1 outlines the inhibiting profile of L-651,392 on AA metabolism.

Table 5.1 Inhibition of AA metabolites by L-651,392.

CELL TYPE	IC_{50} or % Inhibition (μM)
PMN (rat peritoneal	
LTB_4	0.06
PMN (human circulating)	
5-HETE	0.09
LTB_4	0.09
Rat Basophil Leukaemic Cells	
5-HETE	1.5 (74%)
LTB_4	1.5 (64%)
Macrophage (murine peritoneal)	
LTC_4	0.3
PGG_2	7 (26%)
PGI_2	3 (22%)
Platelet	
12-HETE	14 < (10%)

5.2 RESULTS

5.2.1 Effect of BWA4C and L-651,392 on LPS-stimulated IL-1 production

The selective 5-LO inhibitors (10^{-8}-10^{-5}M) had no significant effect in potentiating or inhibiting IL-1 secretion or cell-associated IL-1 accumulation stimulated with LPS $1\mu g.ml^{-1}$ (Fig. 5.1, 5.2). These experiments also show that the 5-LO inhibitors at the concentrations used, do not inhibit the assay system since LPS-induced IL-1 production is not affected.

5.2.2 Effect of BWA4C and L-651,392 on indomethacin-potentiated IL-1 production.

Both 5-LO inhibitors (10^{-8}-10^{-5}M) had no significant effect on the accumulation of cell-associated IL-1 potentiated by indomethacin (IND) 10^{-6}M (Fig. 5.3). The 5-LO inhibitors were also without significant effect on IND-potentiated IL-1 secretion, (Fig. 5.4).

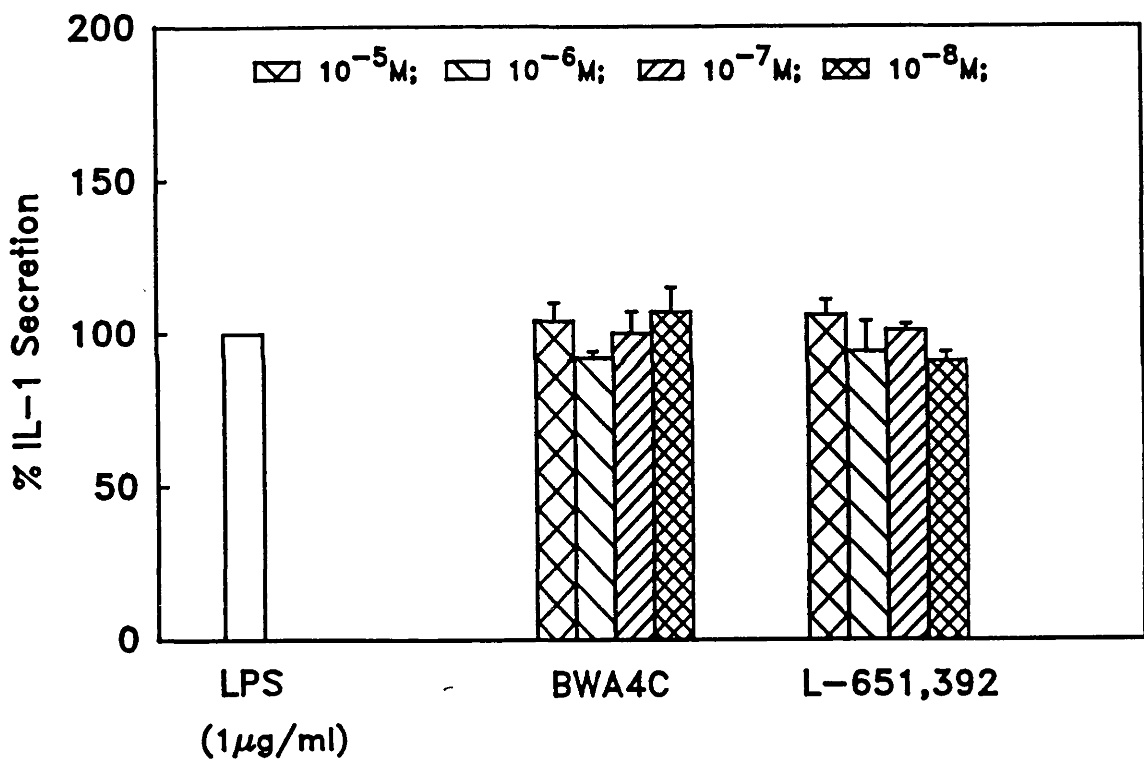


Figure 5.1 The effect of BWA4C and L-651,392 on LPS-stimulated IL-1 secretion. The 5-LO inhibitors were added 30 min prior to LPS. The macrophages were cultured for 20hr. Each bar represents the mean ± standard error (n=3).

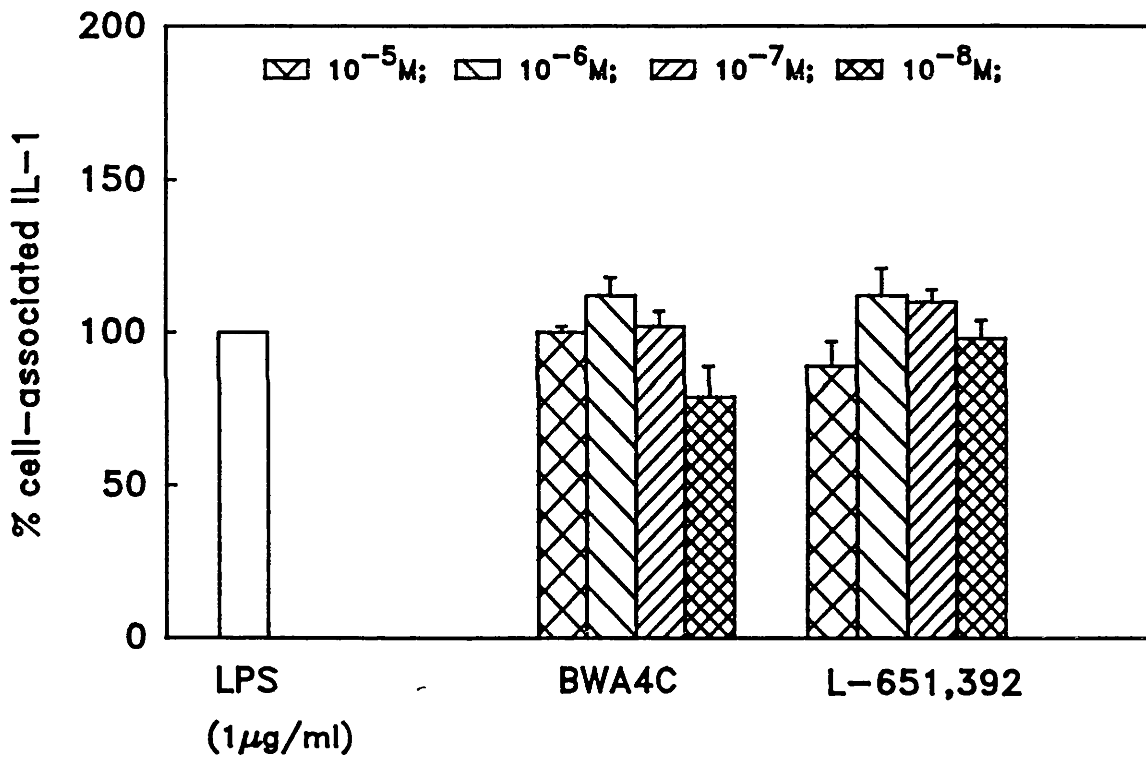


Figure 5.2 The effect of BWA4C and L-651,392 on LPS-stimulated accumulation of cell-associated IL-1. The 5-LO inhibitors were added 30 min prior to LPS. The macrophages were cultured for 20hr. Each bar represents the mean ± standard error (n=3).

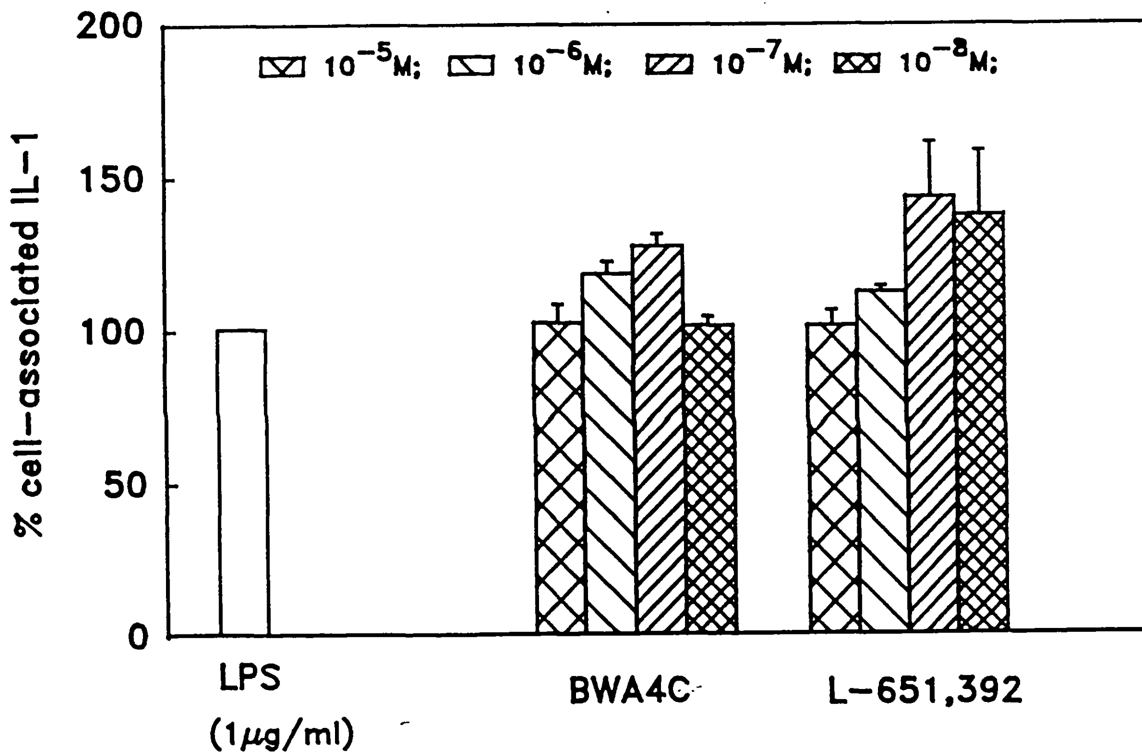


Figure 5.3 The effect of BWA4C and L-651,392 on indomethacin-potentiated accumulation of cell-associated IL-1 from LPS-stimulated cells. The concentration of indomethacin was 10^{-6}M. The 5-LO inhibitors were added 30 min prior to indomethacin and LPS. The macrophages were cultured for 20hr. Each bar represents the mean ± standard error (n=3).

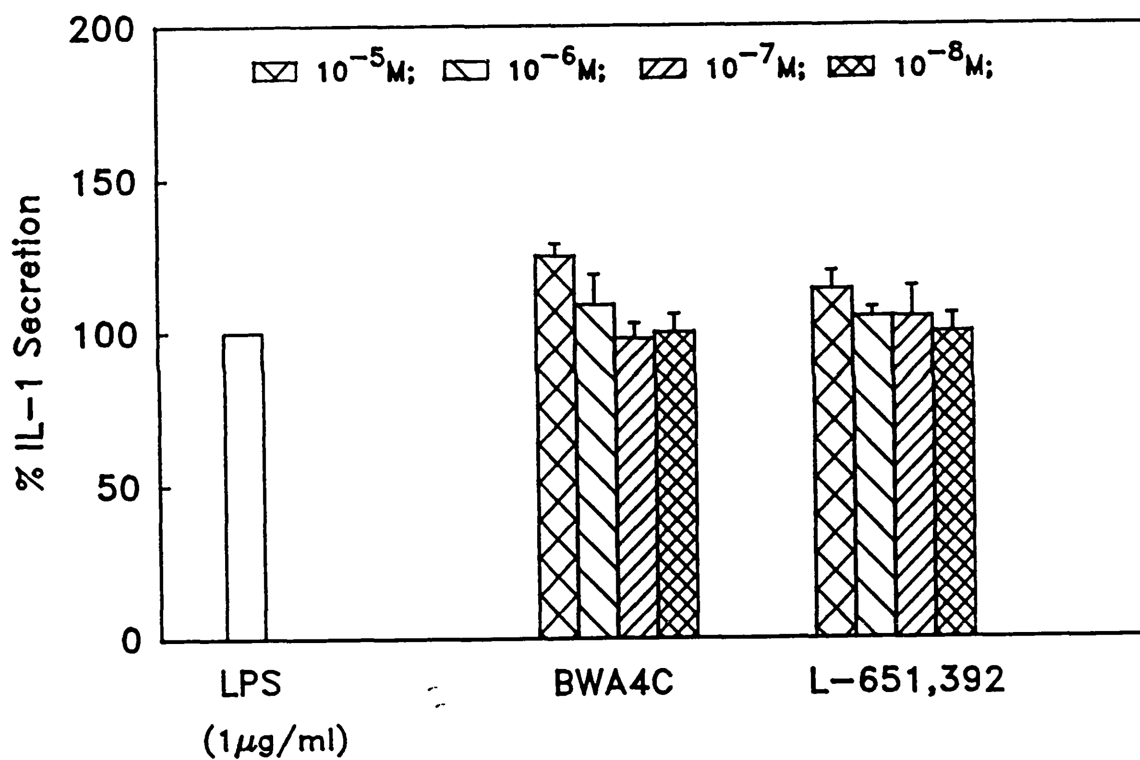


Figure 5.4 The effect of BWA4C and L-651,392 on indomethacin-potentiated IL-1 secretion from LPS-stimulated cells. The concentration of indomethacin was 10^{-4}M. The 5-LO inhibitors were added 30 min prior to indomethacin and LPS. The macrophages were cultured for 20hr. Each bar represents the mean ± standard error (n=3).

5.3 DISCUSSION

If LTs are involved in the production of IL-1, then compounds which inhibit their synthesis and/or action should affect IL-1 synthesis and secretion. Studies that have used dual inhibitors of cyclo-oxygenase and lipoxygenase pathways have shown that such compounds can inhibit IL-1 production from macrophages stimulated by zymosan (Dinarello *et al.*, 1984; Kunkel & Chensue, 1985; Lee *et al.*, 1988). Exogenously added LTs have been shown to enhance IL-1 production from human monocytes (Rola-Pleszczynski & Lemaire, 1985; Tatsuno *et al.*, 1990). Dinarello *et al.*, (1983) and Farra and Humes (1985) have suggested that LTs may also form a component of the transduction process stimulated by IL-1. Indeed, Censini *et al.*, (1989) have shown that IL-1 can dose-dependently stimulate LTC_4 formation by resident murine peritoneal macrophages. However, the evidence implicating LTs as modulators of IL-1 production has relied on non-selective inhibitors of LT synthesis e.g. BW755C, AA-861, ETYA and NDGA. Shultz and Altom (1988) have shown that such compounds inhibit thymocyte proliferation at non-cytotoxic concentrations, and that addition of exogenous LTs was unable to reverse their effects. Since all of the studies mentioned above have used the LAF assay to quantitate IL-1 production, it may well be that the non-specific effects of the compounds on the assay system have been overlooked by the authors. Furthermore, the LAF assay is non-selective for IL-1, therefore the possibility that IL-6, TNFα etc. production were being affected cannot be ruled out.

Two chemically distinct, putatively selective 5-LO inhibitors, BWA4C and L651-392 failed significantly to affect IL-1 production from LPS-stimulated resident murine peritoneal macrophages when the EL4 NOB1 bioassay was used to quantitate IL-1. Although LT production was never measured during the course of the experiments, the inhibitors at the maximum concentration used (10^{-5}M) represent approximately 20-50 times

their IC_{50} concentration in murine macrophages cultured in serum-containing media. Serum proteins can reduce the activity of BWA4C and L651-392, but as serum-free conditions were used in the experimental protocol, the IC_{50} for the two compounds should be greatly enhanced. The results presented alone imply that LTs are not involved in the stimulation of IL-1 production . However, it may be that LTs are not produced by peritoneal macrophages stimulated by LPS and this was not measured. A report by Humes *et al.* (1982) suggested that LPS is a poor stimulant of LTC_4 production from murine peritoneal macrophages. However, in a more recent detailed analysis of the effects of LPS-type molecules on AA metabolism in resident murine peritoneal macrophages, Luderitz *et al* (1989) showed that LPS $0.5\text{-}60\mu g.ml^{-1}$ from deep-rough mutants of *Salmonella minnesota* and *Escherichia Coli* (Re - Rc chemotypes)stimulate significant amounts of LTC_4 and PGE_2 from the peritoneal macrophages. LPS from *S. abortus, S. friedenau* and isolated lipid A did not stimulate LT synthesis. Throughout the studies presented in this thesis a single batch of *S. minnestoa* LPS (Chemotype Re) was used, thus, LT synthesis would have been expected from Luderitz's observations. As mentioned above, the ability of NSAIDs to enhance LT synthesis from rat macrophages and human neutrophils has been reported. Therefore, the ability of indomethacin to enhance LT synthesis from LPS-stimulated peritoneal macrophages may represent a possible mechanism by which the NSAID enhances IL-1 accumulation and secretion (see Chapter 4). However, BWA4C and L651-392 again, were without effect at inhibiting the indomethacin-potentiated IL-1 production. With the use of more selective 5-LO inhibitors and a more selective assay for IL-1 it can be concluded that in an *in vitro* environment LTs do not affect IL-1 production from resident murine peritoneal macrophages. Whether the same holds true for other cell-types or *in vivo* remains to be investigated.

CHAPTER 6

THE EFFECT OF INDOMETHACIN AND INHIBITORS OF DIACYLGLYCEROL METABOLISM ON IL-1 PRODUCTION AND STIMULATION OF EL4 NOB1 CELLS

6.1 INTRODUCTION

Protein kinase C (PKC) is a ubiquitous serine and threonine specific protein kinase found in many cell-types (Nishizuka, 1984). The activation of PKC has been implicated in the synthesis and secretion of IL-1 (see Chapter 3). *In vivo* PKC is activated by diacylglycerol (DAG) generated from the stimulated hydrolysis of phospholipids by specific phosphodiesterases (Phospholipase C (PLC) for phosphotidylinositol and PLC and PLD for phosphotidylcholine). In addition to physiologically generated DAG, synthetic DAGs such as 1-oleoyl 2-acetyl glycerol (OAG), 1,2-diocanaoylglycerol (diC_8) and 1,2-didecanoyl glycerol (diC_{10}) are able to activate PKC in intact cells (Go et al. 1987).

If activation of PKC by DAG has a role in signal transduction mechanisms, it is to be expected that there are processes for terminating the activation by DAG of PKC. Two main mechanisms for the metabolism of DAG have been elucidated to date:-

(i) phosphorylation of DAG by diacylglycerol kinase (DK) to give phosphatidic acid.
(ii) deacylation of DAG by diacylglycerol lipase (DL).

In neutrophils, it has been suggested that DAG produced by receptor stimulation is metabolized by the kinase rather than the lipase pathway (Muid *et al.*, 1987, Mege *et al.*, 1988). Dale and Penfield (1985,1987) showed that the synthetic DAG, oleoylacetyl glycerol (OAG) can be metabolized by both the DK and DL pathways in neutrophils. This observation was based on the use of two enzyme inhibitors; R59022 a DK inhibitor and

RHC80267 a DL inhibitor. Both R59022 and RHC80267 have been shown to potentiate PKC activation in platelets stimulated by OAG, both have an IC_{50} of 4μm and R59022 has an IC_{80} of 10μM at their respective enzyme (Sutherland & Amin, 1982; De Chaffoy de Courcelles *et al.*, 1985).

In Chapter 3 it was shown that diC_8 is a poor stimulator of IL-1 production and one explanation given for its lack of potency was metabolic instability. The following study has used indomethacin and putatively selective DAG kinase and lipase inhibitors to investigate the role of DAG turnover in IL-1 production from peritoneal macrophages and IL-1 activation of the T-cell EL4 NOB1.

6.2 RESULTS

6.2.1 The effect of R59022 on diC_8-stimulated IL-1 production

In Chapter 3 (Fig.3.15) it was shown that diC_8 is a poor stimulator of IL-1 production. When diC_8, 100μM was incubated with increasing concentrations of R59022 it was found that at 10^{-5}M R59022 there was a dramatic increase in the production of both secreted and cell-associated IL-1 (Fig. 6.1). Concentrations above 10^{-5}M were cytotoxic to the peritoneal macrophages.

6.2.2 The effect of indomethacin on diC_8-stimulated IL-1 production

Indomethacin (IND) 10^{-4}M was unable to enhance the ability of diC_8 (12.5-200μM) to stimulate IL-1 production (Fig. 6.2).

6.2.3 The effect of RHC 80267 on diC_8-Stimulated IL-1 production

RHC80267 (10^{-8}-10^{-5}M) was unable to enhance the ability of 100μM diC_8 to stimulate IL-1 production (Fig. 6.3)

6.2.4 The effect of RHC80267 and R59022 on diC$_8$-stimulated IL-1 production

A combination of RHC80267 and R59022, both at 10^{-5}M, produced no additional increase in IL-1 production over that observed with R59022 10^{-5}M alone (Fig. 6.4).

6.2.5 The effect of R59022, RHC8067 and indomethacin on rhIL-1α-stimulated EL4 NOB1 cells

Both R59022 and indomethacin produced a left-shift in the dose-response curve for rhIL-1α on EL4 NOB1 cells (Fig 6.5). The left-shift was only observed at concentrations greater than 10^{-6}M for both R59022 and indomethacin. RHC8029 had no effect up to 10^{-5}M. Concentrations of R59022 above 10^{-5}M were found to be cytotoxic as judged by trypan-blue exclusion and optical examination. Figure 6.6 represents typical dose-response curves for the cells in the presence and absence of the above compounds.

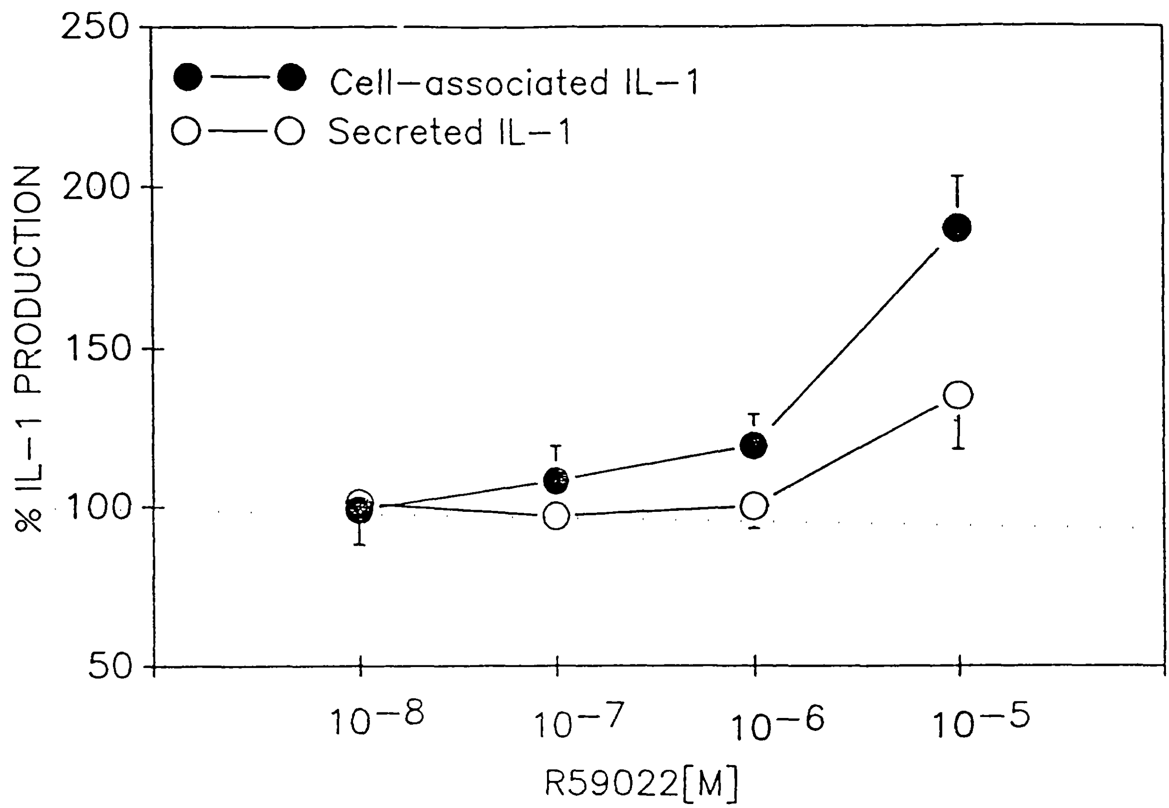


Figure 6.1 The effect of R59022 on diC_8-stimulated IL-1 production. Macrophages were incubated with 100μM diC_8 and increasing concentrations of R59022. Each bar represents the mean ± standard error (n=3).

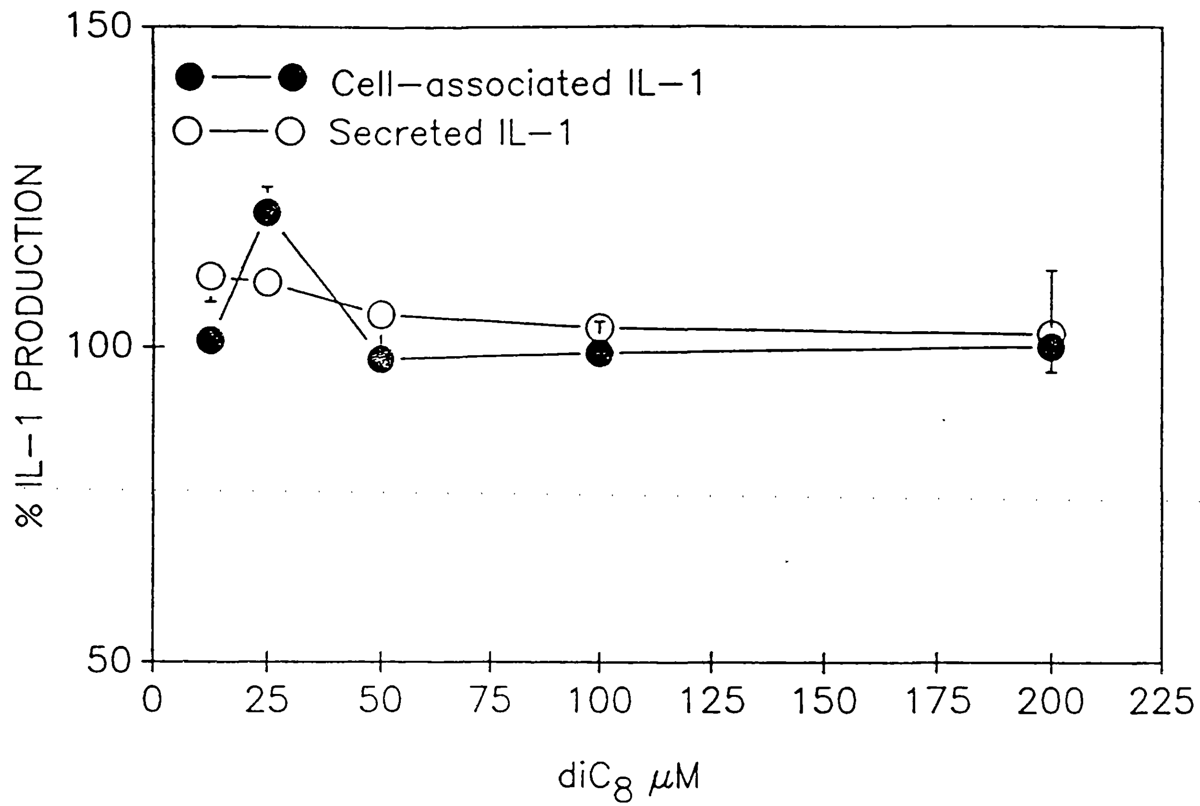


Figure 6.2 The effect of indomethacin on diC_8-stimulated IL-1 production. Each point represents the mean ± standard error (n=3).

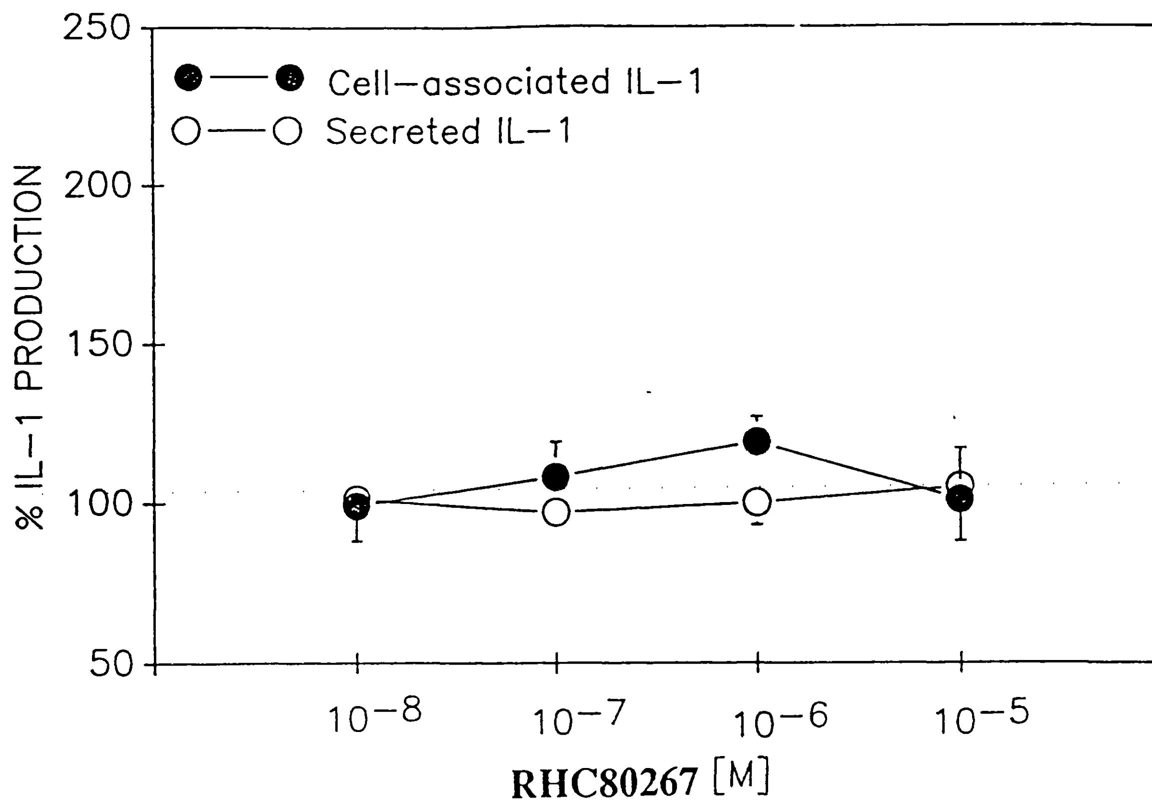


Figure 6.3 The effect of RHC80267 on diC_8-stimulated IL-1 production. Macrophages were incubated with 100μM diC_8 and increasing concentrations of RHC80267. Each point represents the mean ± standard error (n=3).

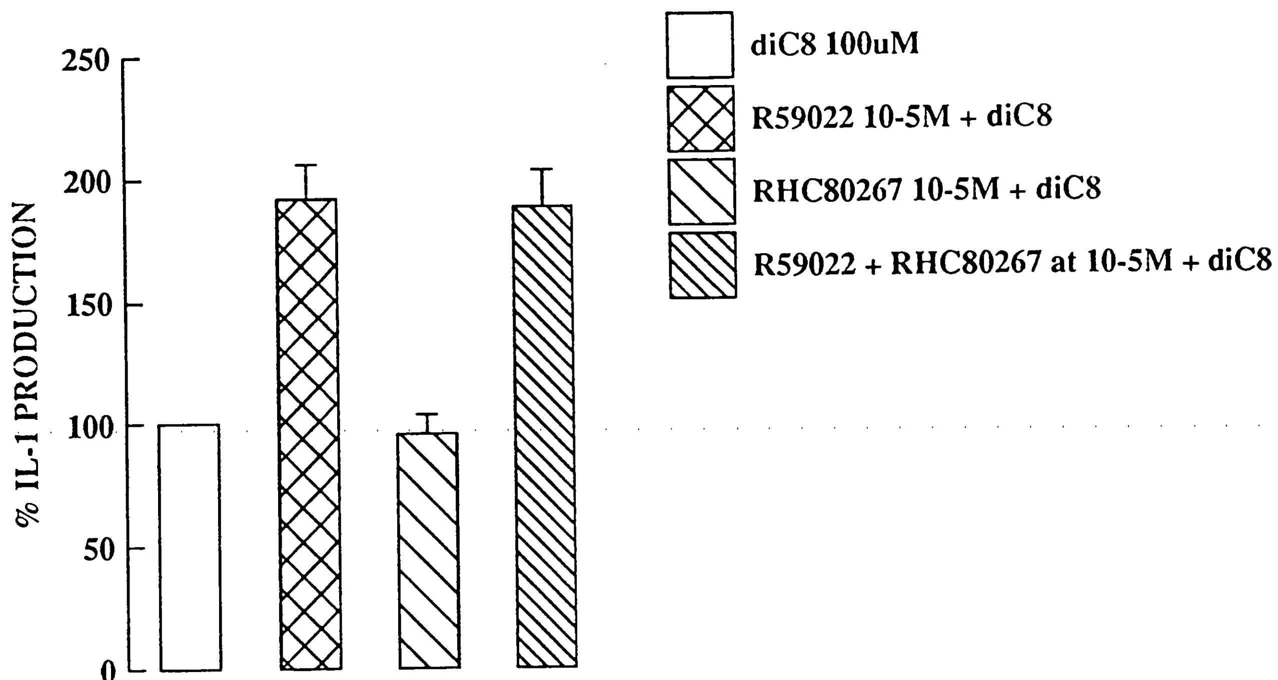


Figure 6.4 The effect of RHC80267 and R59022 on diC_8-stimulated IL-1 production. Macrophages were incubated with 100μM diC_8. A combination of the two inhibitors, both at 10^{-5}M , was compared with R59022 at 10^{-5}M. Each point represents the mean ± standard error (n=3).

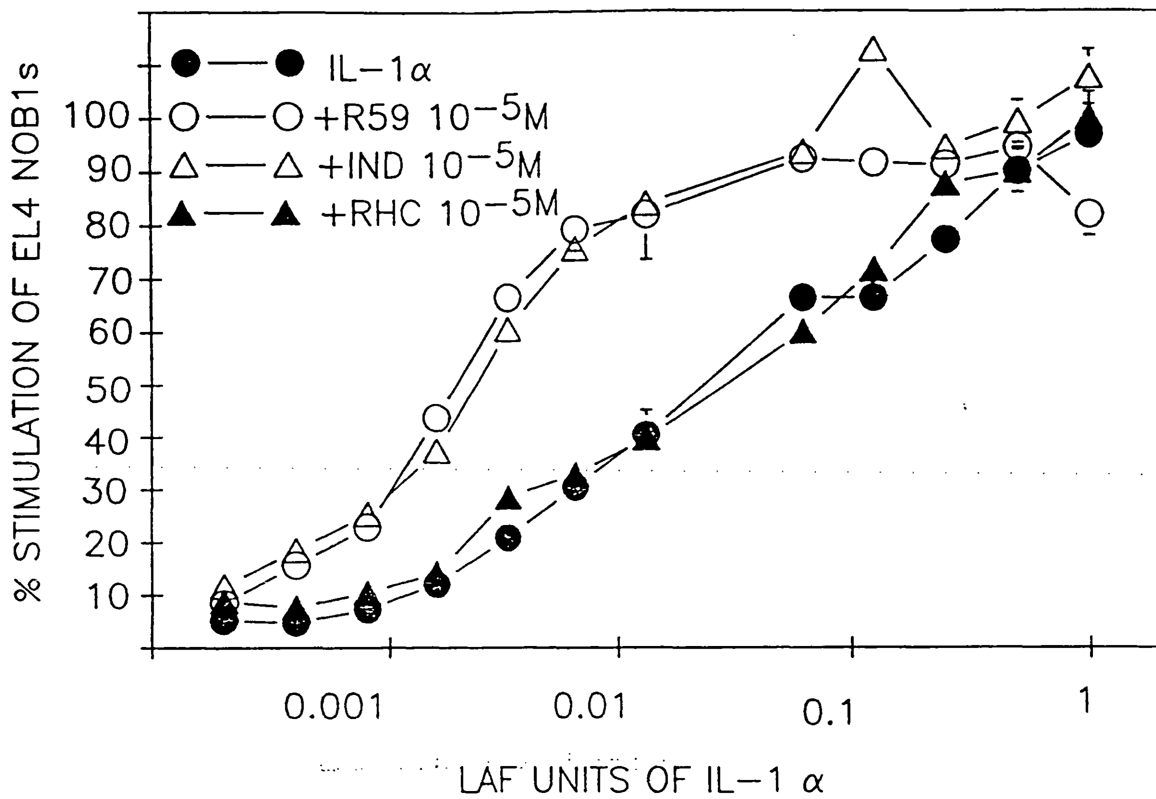


Figure 6.5 The effect of R59022, RHC80267 and indomethacin on rhIL-1α-stimulation of EL4 NOB1 cells. Each point represents the mean ± standard error (n=6).

6.3 DISCUSSION

A number of reports have suggested that indomethacin may lead to an accumulation of DAG within stimulated cells via the inhibition of DAG metabolism:-

- In 1980 Rittenhouse-Simmons reported that indomethacin treatment of thrombin-stimulated platelets lead to an increase in the accumulation of DAG within platelets. The author reported that indomethacin inhibited DL in broken platelet preparations from which he concluded that inhibition of DL was responsible for the indomethacin-induced accumulation of DAG in whole platelets.

- Dale & Penfield (1985,1987) showed that indomethacin at high concentrations (> 100μM) and R59022 could potentiate A23187-stimulated superoxide production from human neutrophils in a qualitatively similar manner (although a Ca^{2+} ionophore, A23187 can lead to a low level of DAG turnover in membranes by increasing intracellular Ca^{2+}). The DL inhibitor RHC80267 was without effect. The authors proposed that indomethacin could be acting as a DK inhibitor.

- Twomey (1990) showed that both indomethacin and R59022 could potentiate the production of superoxide from neutrophils stimulated with diC_8. RHC80267 was without effect.

The results presented in this Chapter show that whilst R59022 can potentiate diC_8-stimulated IL-1 production from resident peritoneal macrophages, indomethacin up to 10^{-4}M has no enhancing effect. Futhermore, RHC80267 a DAG-lipase inhibitor had no effect on diC_8-stimulated IL-1 production. This suggests that diC_8 metabolism is handled primarily by the DK-pathway in resident peritoneal macrophages. Combinations of RHC80267 and R59022 had no additional effect over R59022 alone, thus suggesting that redirection of diC_8 metabolism to the DL-pathway is not limiting its stimulatory effect.

When the effect of R59022, RHC80267 and indomethacin was examined on EL4 NOB1 cells stimulated with rhIL-α it was found that:-

1) RHC80267 had no effect
2) R59022 at concentrations greater than 10^{-6}M produced a left-shift in the IL-1 dose-response curve with no effect on the maximum.
3) Indomethacin 10^{-5}-10^{-4}M produced a left-shift of the dose-response curve similar to that observed with R59022.
4) Addition of RHC 80267 to R59022 treated EL4 NOB1s produced no additional left-shift in the IL-1 dose response curve.

The observations regarding the ability of IL-1 to stimulate EL4-NOB1 cells and the enhancement by R59022 and indomethacin parallels the findings of Dale & Penfield. They showed that indomethacin and R59022 enhance the production of superoxide from neutrophils by stimuli that lead to the formation of DAG or an analogue of DAG itself.

The only consistent observation regarding signal transduction pathways for IL-1 has been the inability to detect increases in Ca^{2+} in cells responding to IL-1. Mizel et al (1990) proposed that IL-1 leads to increases in cAMP by the activation of adenylate cyclase via a G-protein. Arguments against such a mechanism have been put forward by O'Neil et al (1990a). The basis for the arguments being:-

1. Increases in cAMP result in suppression of gene expression in lymphocytes, whereas IL-1 generally enhances gene expression.

2. The single transmembrane segment of the IL-1 receptor is inconsistent with known adenylate cyclase-coupled receptors which have seven transmembrane segments.

The activation of the IL-1 receptor appears to be rapid and

consistent with G-protein coupling and activation of a phospholipase. Activation of the IL-1 receptor on T-cell membranes causes the binding of the GTP-analogue GTPγs to a G-protein within 1 minute. There is also an increase in GTPase activity O'Neil et al (1990b). One working hypothesis is that the IL-1 receptor is coupled to a G-protein that activates phospholipase C which, in turn, hydrolyses DAG from membrane phospholipids. The DAG so produced triggers a protein kinase distinct from PKA or PKC. The IL-1-induced increase in DAG is not associated with the hydrolysis of phosphotidylinositol 4,5 bisphosphate into inositol trisphosphate. This suggests the involvement of other phospholipids in the generation of DAG such as phophotidylcholine or phophotidylethanolamine. The observation that R59022 can potentiate the stimulation of EL4 NOB1 cells by IL-1α adds support to the hypothesis that DAG generation is involved in the IL-1 signal transduction pathway in T cells. Furthermore, it suggests DAG generated by an IL-1 stimulus is metabolized by the DK-pathway.

In conclusion, direct measurements of membrane phospholipid turnover, and studies with labelled diC_8 are required to support the observations that:-

(i) R59022 potentiates diC_8-stimulated IL-1 production by inhibiting its metabolism.

(ii) IL-1 stimulates DAG turnover in EL4 NOB1 cells and that R59022 and indomethacin can inhibit the metabolism.

(iii) Indomethacin does not inhibit the breakdown of diC_8 in peritoneal macrophage which could explaine the potentiation of IL-1 production observed with R59022 but not with indomethacin.

CHAPTER 7.

GENERAL DISCUSSION.

The aims of this chapter are threefold. Firstly, to summarize the main experimental findings of this project, secondly to place such findings in context with the current state of research on IL-1 and lastly to outline avenues through which the above work may be extended.

7.1 SUMMARY OF FINDINGS IN CONTEXT

The work undertaken during this project was initiated in the light of observations made by Dale & Penfield (1987). They showed that certain NSAIDs could potentiate the generation of superoxide from stimulated neutrophils and that this effect could be dissociated from the activity of the drugs on cyclooxygenase. Since toxic oxygen metabolites are known to produce tissue damage and have been implicated in self perpetuating mechanisms underlying joint damage in RA, interest was raised in other pro-inflammatory effects of NSAIDs. In the 1980s the role of IL-1 as a key mediator in the pathophysiology of RA began to emerge (see Introduction). Thus, to examine the effect of NSAIDs, which are so widely used in the palliative treatment of RA, on IL-1 production became of prime importance. In 1986 Kunkel et al. showed that NSAIDs could potentiate the secretion of IL-1 from LPS-stimulated peritoneal macrophages. As more cytokines began to be characterized it became apparent that the lymphocyte activating factor (LAF) bioassay used by Kunkel et al. was very non-selective and insensitive (see Introduction). Furthermore, it was shown that the LAF bioassay was strongly inhibited by PGE_2 (Otterness et al. 1989). Thus, a false positive could be produced by NSAIDs in the LAF assay by virtue of their ability to inhibit prostaglandin synthesis from stimulated macrophages. In 1987 Gearing et al reported the development of a selective, sensitive and reproducible bioassay for IL-1.

The achievements of this project were :

1) Establishing Gearings bioassay and the associated expertise in cell culture required for the purposes of maintaining the reproducibility of the cell-lines involved (see Methods).

2) Repeating the experiments of Kunkel et al. using the above bioassay and a range of NSAIDs.

3) Extending the observations of Kunkel et al. and others to look at IL-1 accumulation and secretion by stimulated macrophages. This was in light of information that IL-1 associated with cells may well be an important biologically active form of IL-1 (see Chapter 1).

4) Assessing the role of PGE_2 as an inhibitor of IL-1; revealing dose and time-dependent effects.

5) Investigating the mechanisms involved in NSAID-potentiated IL-1 production.

Table 7.1 summarises the observations made. The effect of NSAIDs on LPS-induced IL-1 production was initially examined using four chemically distinct NSAIDs, Aspirin (ASP), Indomethacin (IND), Piroxicam (PIR) and sodium mecolofenamate (NaM). All were shown dose-dependently to potentiate the accumulation of cell-associated IL-1. This effect was shown to parallel the ability of the drugs to inhibit cyclo-oxygenase (as measured by PGE_2 production) and was dose-dependently reversed by exogenous PGE_2 at concentrations likely to be produced by the stimulated macrophages. At concentrations of 10^{-5}M and greater for IND, PIR and NaM a decrease in cell-associated IL-1 production was observed with a concomitant increase in IL-1 secretion. The ability of NSAIDs to enhance IL-1 secretion was not due to a cytotoxic effect. An effect via inhibition of PGE_2 synthesis was

excluded since doses in excess of those required to inhibit cyclooxygenase were required to potentiate IL-1 secretion. Thus, these findings were in agreement with previous observations that PGE_2 serves as a down-regulator of IL-1 production. In contrast to reports by Kunkels group, the ability of NSAIDs to potentiate IL-1 secretion could be dissociated from their ability to inhibit PGE_2 production. However, by extending the observations to look at cell-associated IL-1, a clear relationship between NSAID, PGE_2 inhibition and potentiation of IL-1 accumulation emerged. The mechanism(s) by which NSAIDs potentiate IL-1 secretion was investigated using IND as a representative NSAID that consistently enhanced IL-1 secretion. It was shown that IND could not trigger the secretion of cell-associated IL-1 stimulated by h-TNFα and ionomycin. This ruled out a non-specific effect such as a general increase of membrane permeability to intracellular IL-1. The observation also suggests that IND does not directly stimulate the secretion of cell-associated IL-1 but enhances a secretory signal. Whether IND is enhancing an LPS-specific secretory mechanism or can generally enhance the activity of any stimulus capable of inducing IL-1 secretion remains to be examined. The only other stimulus tested which resulted in IL-1 secretion was diC_8 and IND was unable to affect its ability to stimulate IL-1 secretion.

The possibility that redirection of arachidonic acid to the 5-lipoxygenase (5-LO) pathway contributes to NSAID-potentiated IL-1 production was investigated using two selective 5-LO inhibitors, BWA4C and L-651,392. Concentrations of BWA4C and L-651359 up to 10μM were unable to affect either NSAID-enhanced IL-1 production or basal LPS-stimulated IL-1 production.

The observations of Dale and Penfield (1987) and Twomey (1990) that indomethacin could enhance diC_8-stimulated superoxide production could not be paralleled with regard to IL-1

production from peritoneal macrophages. DiC_8 proved to be a poor stimulator of IL-1 production. However, its stimulatory effect was enhanced by R59022, a selective diacylglycerol kinase (DK) inhibitor but not by RHC86022, a diacylglyceol lipase inhibitor. This suggests that diC_8 was being metabolized via the DK pathway and that this was rapid enough to curtail its activity as a stimulator of IL-1 production. Indomethacin and R59022 (10^{-5}-10^{-4}M) were found to potentiate the stimulation by rhIL-1α of EL4 NOB1 cells. The mechanisms behind this have not been investigated in any great depth.

TABLE 7.1 **Summary of stimuli used to induce IL-1 production**

STIMULUS	STIMULATION OF CELL-ASSOCIATED IL-1	STIMULATION OF IL-1 SECRETION
LPS	++++	+++
TNF	+++	NE
IONOMYCIN	+++	NE
A23187	+++	NE
diC_8	+	(+) ?
PAF	NE	NE
SUBSTANCE-P	NE	NE
NEUROKININ A/B	NE	NE
C-GRP	NE	NE

KEY:- NE *no effect;* ++++ *indicative of activity*

7.2 FUTURE WORK

More elementary questions remain to be answered . For example:-

1) How is IL-1 secreted ?

2) What transduction mechanisms are required to initiate IL-1 transcription and translation ?

3) How does LPS activate cells ?

4) To what extent are different forms of IL-1 (α and β) differentially regulated ?

5) The role of arachidonic acid metabolites in the regulation of IL-1 production

Some avenues for more research with regard to NSAID-potentiated IL-1 production could include:-

1) ***Differential measurement of IL-1α and IL-1β.***

Since the completion of the present work, monoclonal antibodies against both forms of IL-1 have become commercially available. Such monoclonals that inhibit the biological activity of IL-1 could be used to investigate whether NSAIDs differentially potentiate IL-1α or IL-1β production. Furthermore, the monoclonals would also be of use in validating the specificity of the bioassay.

2) ***Differential effects of NSAID on macrophage populations.***

The above work concentrated exclusively on resident murine peritoneal macrophages. Drawing an analogy with mast-cells, the pharmacological activity of cromoglycate has been shown to be dependent upon the species and population of mast-cells (skin, lung, peritoneal, mucosal) under study. It is quite

conceivable that the present observations regarding NSAID made above may only apply to resident murine peritoneal macrophages. The extension of the above work to human macrophages would be highly desirably especially if arguments against the widespread use of NSAID in RA were to be put forward.

3) ***Role of arachidonic acid (AA) and its metabolites.***

AA ,15-HETES and lipoxins etc. have been implicated as second messengers in cell activation. NSAIDs could be enhancing the production of such metabolites and in turn enhancing IL-1 production. The use of selective phospholipase inhibitors may help address the role of AA metabolites in IL-1 production.

4) ***In vivo model of IL-production.***

It would be interesting to see if animals pretreated with NSAIDs produced increased quantities of IL-1 in vivo. Systemic administration of LPS or other bacterial agents to rodents, or chronic models of inflammation known to result in increased IL-1 production, may serve as models for such a study.

5) ***Circulating levels of IL-1.***

Although filled with logistical problems, it would be interesting to compare circulating levels of IL-1 in RA patients (especially since it is related to disease activity see Chapter 1) prior to and after NSAIDs. Similarly a comparison of NSAID-treated and untreated RA patients would also be of interest. Obvious problems with patient compliance, fluctuations in disease activity etc. would make this a complicated task.

6) ***Effect on mRNA.***

The production of IL-1 relies on the transcription and translation of IL-1 mRNA. It would be of interest to see if NSAIDs could effect total IL-1 mRNA production and/or stability of mRNA within the cell.

7) ***Protein phosphorylation.***

Many stimuli lead to cell-activation via the activation of various kinases, which in turn phosphorylate cellular proteins that act as enzymes/catalysts in the modulation of cell function. As was mentioned previously, the mechanism by which LPS activates IL-1 production is not known. However, it would be interesting to discover whether NSAIDs could enhance the phosphorylation of proteins by LPS and to identify such proteins. It is also conceivable that NSAIDs act at a post-phosphorylation event.

It was mentioned that indomethacin and R59022 could potentiate the activation of EL4 NOB1 cells stimulated with rhIL-1 (as measured by IL-2 production). The ability of NSAIDs to enhance IL-1- induced activation of cells is obviously of great importance. Indeed there are isolated reports that NSAIDs can potentiate IL-1-induced loss of proteoglycan from cartilage, in culture and *in vivo* (Dingle et al 1990). This could represent a further arm to the pro-inflammatory profile of NSAIDs: that is, sensitising cells to activation by IL-1.
The mechanism behind such an effect may form the basis of a study in itself.

7.3 CONCLUSION

NSAIDs are very commonly prescribed. This reflects the high prevalence of rheumatic diseases approximately 8% world-wide. Since many different NSAIDs are now available for prescription, patients, physicians, regulatory authorities and pharmaceutical companies all have considerable interest in the risk/benefit ratio of NSAIDs. In chapter 1 it was mentioned that there are a growing number of reports which describe pro-inflammatory activities of NSAIDs. Newman & Ling (1983) reported that certain NSAIDs could actually be detrimental to patients with osteoarthritis. It may well transpire that NSAIDs represent a " Jekyll and Hyde " medication : relieving pain and swelling at the expense of exacerbating chronic destructive inflammation.

The pro-inflammatory effects of NSAIDs may be intrinsically linked to the inhibition of prostaglandin synthesis or a consequence of some distinct activity. The dissection of the two would prove of value in the design of new NSAIDs. Prostaglandins themselves are emerging as potent anti-inflammatory agents especially with regard to chronic inflammatory events such as cytokine production, MHC/antigen expression and regulation of adhesion molecules. Perhaps the design of anti-inflammatory agents that do not inhibit prostaglandin synthesis represents the way forward.

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