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THE EXPRESSION OF INTERLEUKIN-1 RECEPTOR TYPE I AND II IN MONOCYTES AND MYELOCYTIC LEUKAEMIC CELLS

Angela Sally Flagg

A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg for the Degree of Master of Science

Johannesburg 1996

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other university.

A. Flagg.

A.S. Flagg

19 January, 1996.

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
ALL	Acute lymphocytic leukaemia
AML	Acute myelogenous leukaemia
ATL	Adult T cell leukaemia
B-CLL	B cell-type chronic lymphocytic leukaemia
Bp	Base pair
BSA	Bovine serum albumin
cAMP	Cyclic adenosine 3'5'-monophosphate
cDNA	Copy DNA
CML	Chronic myelogenous leukaemia
CNS	Central nervous system
Con A	Concanavalin A
CREB	cAMP response element binding protein
DAG	Diacylglycerol
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
EBV	Epstein Barr virus
EDTA	Ethylene diamine-tetra-acetic acid
EGTA	Ethylene bis(oxyethylenenitrilo) tetra-acetic acid
G protein	Guanine nucleotide-binding regulatory protein

GAP-DH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Glucocorticoid
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte macrophage-colony stimulating factor
GRE	Glucocorticoid response element
4-HC	4-Hydroperoxycyclophosphamide
HGFR	Haemopoietic growth factor receptor
F	Forward
ICE	Interleukin-1 converting enzyme
IFN	Interferon
IGF	Insulin-like growth factor
Ig-R	Immunoglobulin supergene receptor
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist
IL-1RtI	Interleukin-1 receptor type I
IL-1RtII	Interleukin-1 receptor type II
kDA	Kilodalton
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
LT	Leukotriene
M-CSF	Macrophage-colony stimulating factor

MOPS	3-[N-morpholino] propane sulphonic acid
MPS	Mononuclear phagocyte system
mRNA	Messenger ribonucleic acid
NF-kB	Nuclear factor-kappa B
PAGE	Polyacrylamide gel electrophoresis
PBMNC	Peripheral blood mononuclear cells
PDGF	Platelet derived growth factor
PEG	Polyethylene glycol
PG	Prostaglandin
PKA	Protein kinase A
PMA	Phorbol myristic acetate
R	Reverse
RIA	Radioimmunoassay
RNase	Ribonuclease
RT-PCR	Reverse transcription - polymerase chain reaction
SDS	Sodium dodecyl sulphate
SIRS	Systemic inflammatory response syndrome
TBS	Tris buffered saline
TBP	TATA binding protein
TCR	T cell receptor
TEMED	Tetramethylethylenediamine
TNF	Tumour necrosis factor

TNFR	Tumour necrosis factor receptor
UIS	Upstream induct. n sequence
UV	Ultraviolet

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ABSTRACT

Tbe antiinflammatory effects of interleukin-4 (IL-4) and the synthetic glucocorticoid dexamethasone were studied in adhered monocytes and the leukaemic cells HL-60 and THP-1, with respect to the expression of interleukin-1ß (IL-1ß), the (signalling) IL-1 receptor type I (IL-1RtI), and the (inhibitory) IL-1 receptor type II (IL-1RtII).

IL-4 (10 ng/ml) and dexamethasone (100 nM) decreased the expression of IL-1ß mRNA in monocytes as demonstrated using Northern blotting. The antiinflammatory effects of IL-4 were demonstrated by a RIA in monocytes at the protein level, where IL-4 inhibited the expression of IL-1ß, when this expression was induced by bacterial lipopolysaccharide (LPS) or IL-1ß.

IL-RtI was shown to be constitutively expressed in monocytes and HL-60 cells at the mRNA level as determined by reverse transcription - polymerase chain reaction (RT-PCR). The expression of IL-1RtI at the protein level was demonstrated in these cell types. IL-4 (10 ng/ml, 24 hours) upregulated the expression of IL-1RtI as determined by immunoprecipitation and Western blotting. Differentiation of the HL-60 cell line with tumour promoter, phorbol myristic acetate (PMA, 10 nM) for 24 hours induced an increase in the expression of IL-1RtI in these cells. This indicates that differentiation of this immature cell line towards a more macrophage - like state induces an increase in the IL-1RtI.

IL-4 and dexamethasone upregulated the expression of IL-1RtII at the mRNA level as demonstrated using RT-PCR. Thus IL-4 and dexamethasone act as antiinflammatory agents, at least in part, by down regulating the expression of IL-1ß and by upregulating the expression of the "decoy" IL-1RtII which acts as a molecular trap for IL-1ß. In addition IL-4 and dexamethasone upregulate the expression of IL-1RtI, suggesting that IL-4 and dexamethasone are not purely antiinflammatory agents.

SECTION 1

INTRODUCTION

IL-1 plays a vital role in the immune and inflammatory responses. In contrast, the over production of IL-1 can have many deleterious side effects (Dinarello 1994). It is therefore important to regulate the production and action of IL-1. IL-1 is inhibited by specific inhibitors (eg. the IL-1 receptor antagonist (IL-1ra) and by inhibitory cytokines (eg. IL-4) (Burger and Dayer 1995). The action of IL-1 can also be regulated at the level of receptor binding and signalling (see section 1.15.). The two IL-1 receptors play different roles in the regulation of IL-1 action. IL-1RtI is a signalling receptor and cells possessing this receptor are responsive to IL-1. IL-1RtII is a nonsignalling receptor. IL-1RtII acts as a molecular trap for IL-1, binding free IL-1 and thereby preventing IL-1 from binding to the signalling IL-1RtI. Thus IL-1RtII is possibly an unique negative regulator of IL-1 action (Colotta *et al.* 1993a). The cytokine IL-4 possesses some antiinflammatory properties, while the synthetic glucocorticoid dexamethasone is used as an antiinflammatory agent. It is proposed that IL-4 and dexamethasone not only inhibit IL-1 synthesis, but that they upregulate the expression of the IL-1RtII "decoy" receptor (Banchereau *et al.* 1994).

The major site of IL-1 production is monocytic cells, and IL-1 can act in an endocrine, paracrine, and autocrine manner on these cells. The production

and regulation of IL-1 and its receptors are discussed with the emphasis on cells involved in the immune and inflammatory responses. The role of IL-1 in the leukaemic disease process is discussed, as the leukaemic HL-60 and THP-1 cell lines are used as models for normal peripheral blood monocytes, as pure populations of monocytes are difficult to isolate. The role of the autocrine stimulation by IL-1 in the uncontrolled proliferation of leukaemic cells is also discussed. The effects of IL-4 and dexamethasone are investigated in these cell lines in order to ascertain whether the regulation of IL-1 receptors is altered in leukaemic cells.

1.1. THE MONONUCLEAR PHAGOCYTE SYSTEM

The mononuclear phagocyte system (MPS) of cells include macrophages, monocytes, and their precursor cells. The cells in the MPS play important roles in the normal functioning of the body, including, phagocytosis of dead or foreign material (including the destruction of bacteria and some malignant cells), antigen processing and presentation in immune responses, participation in the inflammatory response, and the regulation of various other cells also involved in the defences of the body by the production of cytokines (Weinberg and Athens 1993).

The precursor cells of the mononuclear phagocyte system reside in the bone marrow. Cells of the granulocyte lineage (neutrophils) share a common precursor with the mononuclear phagocytes. This common precursor

differentiates towards macrophages under the influence of the glycoprotein growth factor macrophage - colony stimulating factor (M-CSF) in the bone marrow. Granulocyte - colony stimulating factor (G-CSF) causes the precursor to differentiate into granulocytes, and granulocyte macrophage - colony stimulating factor (GM-CSF) results in the differentiation of both granulocytes and macrophages (Weinberg and Athens 1993).

The first cell to be recognised in the mononuclear phagocyte system is the monoblast, which resides solely in the bone marrow. The monoblast differentiates into the promonocyte and then the monocyte. Monocytes are found in the bone marrow and in blood (2 - 10 % of peripheral blood leukocytes). Monocyte nuclei, which are centrally placed, are oval, large and indented. Two or more nucleoli can be seen in the nucleus, and the cytoplasm is filled with small lysosomes. Monocytes are motile when they adhere to plastic and glass and are able to phagocytose, therefore pseudopodia are visible (Wheater *et al.* 1987).

Monocytes are young cells in transit from the bone marrow to the tissues of the body. Their half life in the vascular system is approximately 8,4 hours. After monocytes leave the blood they differentiate into tissue macrophages, where they remain. Nearly half of all monocytes from the blood migrate into the liver to become Kupffer cells. Tissue macrophages steadily die in order to accommodate the constant influx of monocytes from the blood (reviewed in Weinberg and Athens 1993).

1.2. INTERLEUKIN-1

Monocytic cells communicate with themselves and other cells in the body by means of a system of chemical messengers called cytokines. Cytokines regulate diverse functions in the body, from haemopoiesis to inflammation and the immune response. One of the key cytokines involved in these processes is IL-1. The IL-1 system consists of three polypeptides, namely the two agonists IL-1α and IL-1ß, and the antagonist, the IL-1ra (Carinci *et al.* 1992).

IL-1α and IL-1ß are two 17 kiloDalton (kDa) related polypeptide cytokines (26 % amino acid sequence homology) with a wide range of overlapping immunologic and non-immunologic functions (Carinci *et al.* 1992 and Furutani 1994). These two polypeptides represent separate gene products despite the fact that they have such similar properties, and that their crystal structures are similar (Carinci *et al.* 1992). It is thought that an ancestral IL-1 gene duplicated approximately 350 million years ago to produce these two forms of IL-1. IL-1ra possibly originates from this ancestral gene (Dinarello 1994). Both IL-1α and IL-1ß are synthesized as 31 kDa precursor molecules called proIL-1α and proIL-1ß respectively (Auron and Webb 1994).

It has been found that 90 % of IL-1α produced by mononuclear cells is cell associated and is membrane bound, while 80 % of IL-1ß produced by mononuclear cells is secreted into the extracellular medium (Lonnemann *et al.* 1989 and Carinci *et al.* 1992). Thus IL-1ß is found mainly in the blood, and hence it is the IL-1 with the more profound systemic effects.

1.3. INTERLEUKIN-1 GENE EXPRESSION

Resting peripheral blood monocytes are stimulated during the immune or inflammatory response to become "activated" monocytes by endogenous and exogenous stimuli. This activation is immediate, as a rapid response to infection or injury is required. Activation involves the decrease in transcription of certain genes, and the rapid (less than 15 minutes in some cases) increase in the transcription of others. Genes induced by the stimulation of monocytes by LPS include IL-1α, IL-1ß, IL-8, IL-6, and tumour necrosis factor α (TNFα). IL-1 produced by activated monocytes acts in an autocrine manner to maintain this activated state (reviewed in detail in Auron and Webb 1994).

1.3.1. INTERLEUKIN-1ß GENE EXPRESSION

The IL-1ß gene is an immediate early response gene, which is transiently expressed. The IL-1ß gene can be constitutively expressed in certain pathological states (see 1.7.). Induction of transcription causes an increase in the amount of IL-1ß mRNA in the cell (Auron and Webb 1994).

The production of IL-1ß is a tightly regulated affair, as IL-1ß plays an important role in many normal and disease processes. IL-1ß production is controlled at the pretranscriptional level ie. the signal for IL-1ß production is regulated. IL-1ß production is also controlled at the transcriptional level and at the posttranscriptional level, for example, the stability of IL-1ß mRNA is regulated. In addition IL-1ß production is regulated at the translational level, and at the post translational level due to postranslational modification and the

regulation of secretion (reviewed in (Fenton *et al.* 1988 and Auron and Webb 1994).

Various stimuli induce increased amounts of IL-1ß mRNA in the cell, although not all of these stimuli act in the same way. The tumour promoter PMA is a weaker inducer of IL-1ß transcription compared to LPS, but similar levels of mRNA are found in monocytes after stimulation with these agents because PMA increases the stability of IL-1ß mRNA. It is therefore possible that IL-1ß transcription can be induced by more than one mechanism (Auron and Webb 1994).

1.3.1.1. THE PROIL-1ß GENE'S UPSTREAM INDUCTION SEQUENCE

The transcription of the IL-1ß gene is regulated by an inducible enhancer element upstream from the transcriptional start site (position -3143 to -2729) called the upstream induction sequence (UIS) (see figure 1.1.) (Auron and Webb 1994).

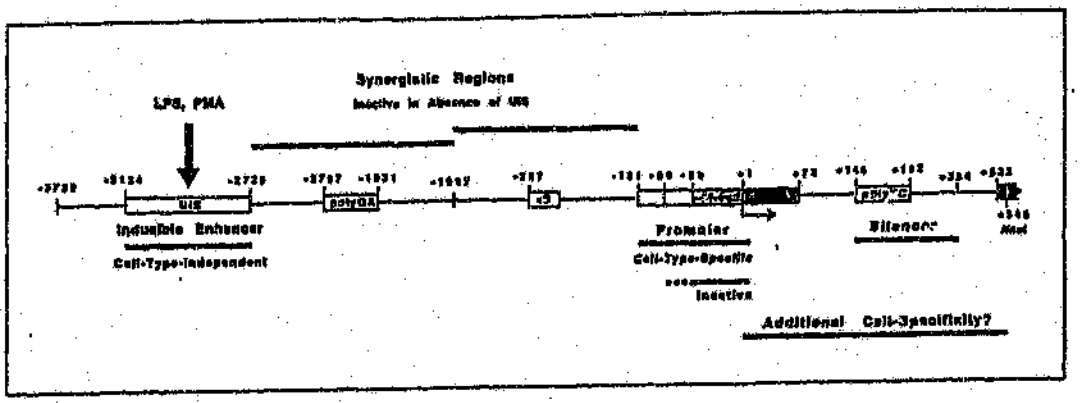


Figure 1.1. The proIL-1ß gene.
The proIL-1ß gene showing the different regions outlined in the text. Modified from Auron and Webb 1994.

The UIS is divided into 8 regions (B to I) (see figure 1.2.a.), which are either inhibitory or stimulatory with respect to transcription of the proIL-1ß gene. Region D to G is weakly transactivating, region I is strongly transactivating, while region H inhibits induction of the gene. Region B and C are not required for the induction of the proIL-1ß gene, but they synergise with the D to G regions (Auron and Webb 1994).

Region E and I bind the basic zipper transcription factors (nuclear factors), NF-IL6 and the cyclic adenosine 3'5'-monophosphate (cAMP) response element binding protein (CREB), as a heterodimer. CREB is activated upon phosphorylation of Ser_{133} (induced by cAMP). The active heterodimer is able to transactivate the proIL-1ß gene. The phosphorylation of NF-IL6 and CREB are essential for the activation of the heterodimer (see figure 1.2.a.) (Auron and Webb 1994).

The nuclear factor NF-kappaB (NF-kB) associates with NF-IL6, thus activating NF-IL6 by an unknown mechanism. In addition a weak NF-IL6 site may require the binding of NF-kB adjacent to it in order to transactivate the gene. NF-IL6 and NF-kB are not tissue specific factors, as NF-kB is present in many cell types. NF-kB pre-exists in the cell in an inactive form associated with the inhibitory molecule IkB. NF-IL6 is acitvated by phosphorylation. Phosphorylation of IkB causes the dissociation of this inhibitor from NF-kB thus allowing the translocation of NF-kB into the nucleus. Consequently, NF-IL6 and NF-kB take 15 minutes or less to activate the proIL-1ß gene (Auron and Webb 1994).

Other factors involved in the induction of the proIL-1ß gene are the proteins FOS and JUN, which make up the AP-1 factor (Muegge and Durum 1989). FOS and JUN have to be synthesized by *de novo* protein synthesis, as they do not exist in the cell as inactive factors prior to stimulation. Thus FOS and JUN take approximately 60 minutes to become active factors, and it is

therefore more likely that they are involved with the sustained expression of immediate response genes (Auron and Webb 1994).

Antiinflammatory glucocorticoids inhibit the induction of the proIL-1ß gene. The synthetic glucocorticoid dexamethasone, at concentrations of 5×10^{-7} M, inhibits the induction of the proIL-1ß gene by influencing the D to G and the I regions of the UIS. The antiinflammatory cytokine IL-4 has been shown not to exert its effects (repressing IL-1ß production) via these two regions (Auron and Webb 1994), but possibly affects other regions of the promoter (Donnelley *et al.* 1991).

1.3.1.2. THE PROIL-1ß GENE PROMOTER

The proIL-1ß promoter lies between position -131 and +12 in the gene. In the resting state the promoter is repressed, and needs to be de-repressed in order to initiate transcription. The transcription factor Spi-1 is essential in "relaxing" this repressed state of the promoter, as it is the factor that binds the TATA box binding protein (TBP) which is important in initiating transcription (see figure 1.2.b.). Since Spi-1 is a B cell and myeloid specific factor, it confers tissue specificity on the transcription of the proIL-1ß gene (Auron and Webb 1994).

The proIL-1ß promoter possesses a Spi-1 activation domain which contains a TBP binding site. NF-IL6 may be important in de-repressing the promoter. It is thought that the promoter is repressed by the binding of inhibitory

molecules to the TATA box, thus the TBP is unable to bind. Binding of Spi-1 and NF-IL6 may displace these inhibitory molecules and allow the TBP to join the transcription initiation complex. In addition, NF-kB may be able to bind to NF-IL6 and help to "recruit" the TBP (see figure 1.2.b.) (Auron and Webb 1994).

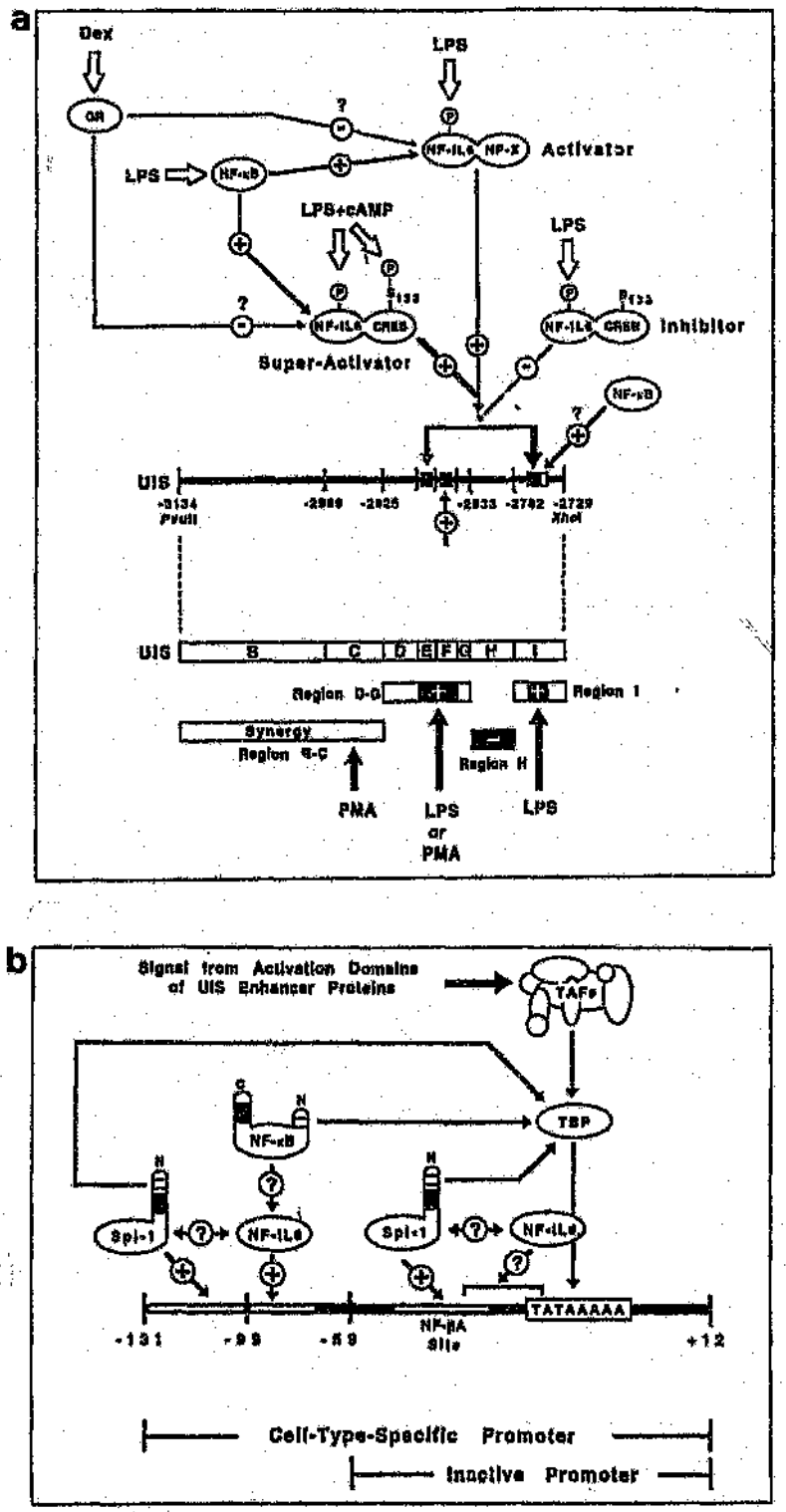


Figure 1.2. The proIL-1ß upstream induction sequence (UIS) (a.) and promoter (b.). From Auron and Webb 1994.

Not only does the induction of the proIL-1ß gene depend on the UIS and regions in its promoter, but also on a region within the first intron. The first intron contains a "strong silencer sequence" similar to region H in the UIS (Auron and Webb 1994).

1.3.2. INTERLEUKIN-1α GENE EXPRESSION

The approximately 12 kilobase (kb) IL-1α gene encodes for a 30,6 kDa (271 amino acids) precursor protein called proIL-1α (Furutani 1994).

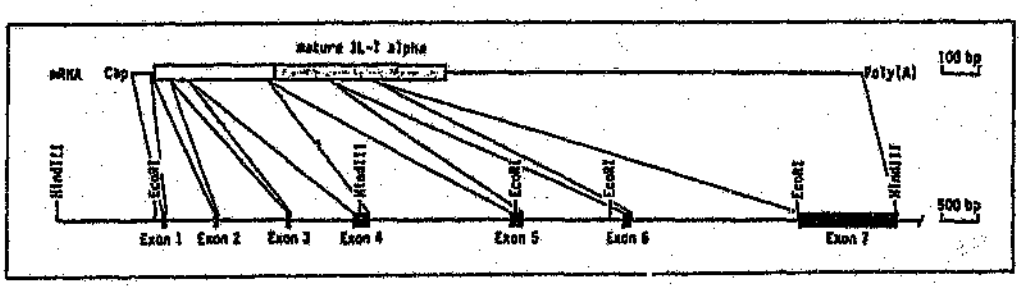


Figure 1.3. IL-1α mRNA and DNA coding regions.
From Furutani 1994.

The proIL-1α gene contains 7 exons and 6 introns (see figure 1.3.). Exon 1 is the 5' - untranslated region. Exons 2, 3, and 4 code for the precursor segment of proIL-1α. Exons 5, 6, and 7 code for the mature IL-1α protein. Exon 7 also contains a 3' - untranslated region (Furutani 1994)

The proIL-1α gene has upstream from the transcriptional start site regulatory elements which control the transcription of the gene. Positive regulatory elements reside between positions -47 and -70, and between -70 and -103. These two regions are thought to exert their influence through NF-kB, as they contain sequences homologous to NF-kB binding sites. Negative regulatory elements reside between positions -103 and -875, and between -875 and - 3600 upstream from the transcriptional start site (Furutani 1994).

The last intron in the proIL-1α gene contains a sequence similar to that which is able to bind the transcription factor Spi-1. In addition this intron contains a glucocorticoid response element (GRE) (Furutani 1994).

1.4. POSTTRANSCRIPTIONAL REGULATION OF INTERLEUKIN 1

A mechanism to control IL-1 production post transcriptionally, is to control the stability of the IL-1 mRNA produced. IL-1α and IL-1β mRNAs have different stabilities, yet they both have similar AU-rich 3' - untranslated regions. These regions cause the high turnover rate, and thus the short half-life, of these two mRNA species (Auron and Webb 1994). A short half-life for

an mRNA species coding for a "transiently" produced molecule is essential in order to effectively reduce the production of that molecule. Since IL-1α and IL-1ß mRNAs do have differing half-lives, it could be that additional factors control their stability. Dexamethasone and IL-4 have been shown to increase the breakdown of IL-1ß mRNA by destabilising it, while PMA increases IL-1ß mRNA stability (Auron and Webb 1994). Dexamethasone and IL-4 do not have the same effect on IL-1α mRNA (Lee *et al.* 1988 and Donnelly *et al.* 1991).

The transcription and the translation of IL-1 are controlled separately. This can be seen when monocytes are adhered to plastic. Adherence provides the stimulus for IL-1 transcription, which can be seen from the increase in IL-1 mRNA. The IL-1 mRNA will not be translated, however, unless the monocytes are stimulted to do so (Auron and Webb 1994).

1.5. PROCESSING AND SECRETION OF INTERLEUKIN 1

IL-1α and IL-1ß are synthesized as 31 kDa precursor polypeptides (proIL-1α and proIL-1ß) which have a 26 % amino acid sequence homology (Rubartelli *et al.* 1990). Whilst proIL-1α is a fully active molecule, proIL-1ß has almost no activity (Auron and Webb 1994).

The two precursor molecules have no hydrophobic amino - terminal signal peptide which allows the transfer of the protein across the rough endoplasmic

reticulum membrane (Rubartelli *et al.* 1990). Thus the mature forms are not secreted via the classical secretory pathway ie. they are not processed in the rough endoplasmic reticulum, and do not pass through the Golgi apparatus. IL-1 (mostly IL-1ß) is protected from protease digestion (Rubartelli *et al.* 1990) by trypsin - resistant vesicles which enclose the IL-1 in the cytosol. It is thought that processing may occur within these vesicles (Carinci *et al.* 1992). The compartmentalization of IL-1ß may function to separate IL-1ß from its receptor, thus preventing uncontrolled autostimulation within the cell (Rubartelli *et al.* 1990).

Processing of proIL-1α and proIL-1ß into the mature 17 kDa polypeptides involves the cleavage, by specific intracellular convertases, of the amino - terminal of the precursor molecules leaving the carboxy - terminal to form the mature peptide (Carinci *et al.* 1992). The processing of proIL-1ß is achieved by the interleukin 1 converting enzyme (ICE), which cleaves the 31 kDa precursor at Asp^{116}-Ala^{117} to produce the mature protein (Kostura *et al.* 1989). ICE is not responsible for the processing of proIL-1α (Auron and Webb 1994). The ICE holoenzyme consists of a p10 subunit associated with a p20 (or p22) catalytic subunit (Miller *et al.* 1993). ICE gene is located on chromosome 11q23 which is a band on the chromosome often associated with rearrangements resulting in many cancers such as leukaemia. It is conceivable that altered expression of ICE could contribute to the leukaemic disease process (Cerretti *et al.* 1992), especially as some leukaemias exhibit altered patterns of IL-1 expression, for example, IL-1 acts as an autocrine and

paracrine growth factor in some leukaemias (Dinarello 1993a). In some cells (for example neurons) overexpression of ICE leads to programmed cell death (Miura *et al.* 1993 and Barinaga 1994).

The degree of differentiation of a cell line influences the synthesis and processing of IL-1α and IL-1ß. Adherence of peripheral blood monocytes to plastic for 10 hours reduces the processing of IL-1, although the cells are still morphologically similar to monocytes at this stage. In addition, it has been shown that alveolar, breast, and inflammatory macrophages secrete less IL-1 compared to less differentiated peripheral blood monocytes. The IL-1ß mRNA levels are comparable in both cell types (Carinci *et al.* 1992).

The processing and secretion of IL-1α and IL-1 ß are independent events as IL-1α is secreted into the extracelluar medium up to 12 hours after IL-1ß (Carinci *et al.* 1992). In mononuclear cells IL-1α levels are at their highest 6 hours after stimulation with LPS, whilst IL-1ß levels peak at 12 to 16 hours post LPS stimulation. ProIL-1ß is mainly cytosolic, whereas proIL-1α is membrane associated and is able to bind the IL-1RtI (Lonnemann *et al.* 1989).

1.6. BIOLOGICAL PROPERTIES OF INTERLEUKIN-1

IL-1 plays a vital role in inflammation and the immune response. Over production of IL-1 can have deleterious effects. IL-1 was known by a le variety of names because of its many roles in inflammation and immunity (eg.

lymphocyte-activating factor, endogenous pyrogen, leucocyte endogenous mediator, osteoclast-activating factor, B cell-stimulatory factor, catabolin, haemopoietin-1, and proteolysis-inducing factor) before it became apparent that all these molecules were in fact IL-1 (Molloy *et al.* 1993). It is now known that IL-1 plays a vital role in many local and systemic events. Systemically IL-1 is involved in the acute phase response and fever. Locally IL-1 induces the expression of many genes such as chemokines (eg. IL-8) and enzymes involved in the synthesis of prostaglandins (PG), leukotrienes (LT) and nitric oxide (Dinarello and Wolff 1993). IL-1 also inhibits the expression of certain genes, for example the genes for albumin, cytochrome P450, and aromatase (Dinarello 1994).

IL-1 plays a vital role on the response to injury and infection (Molloy 1993). IL-1 has been implicated in the development of the systemic inflammatory response syndrome (SIRS) (Dinarello 1994a) in which the activation of cells in the mononuclear phagocyte system play an important role. IL-1 works in concert with tumour necrosis factor α (TNFα) in producing these symptoms in an autocrine, paracrine, or endocrine manner (Molloy *et al.* 1993). In animal models IL-1 induces fever, sleepiness, and anorexia. High concentrations of IL-1 (100 ng/kg) induces hypotension which is due to an increase in potent vasodilators such as platelet activating factor and cyclooxygenase products by increasing the expression of enzymes involved in PG, LT, and nitric oxide synthesis (Dinarello and Wolff 1993).

IL-1 increases the expression of endothelial cell adhesion molecules and chemotactic chemokines which induces the adhesion and migration of leucocytes to points of injury (Dinarello 1994). Endothelial cells also respond to IL-1 by increasing their procoagulant activity (Dinarello 1994).

IL-1 has many deleterious effects when overproduced, for example, IL-1 is catabolic and contributes (with TNFα) to a decrease in body mass and hypoglycaemia (Dinarello 1994). IL-1 has been implicated in joint diseases like rheumatoid arthritis (Dinarello and Wolff 1993). IL-1 is cytotoxic (possibly causing programmed cell death) to the insulin producing ß-cells of the islets of Langerhans (Dinarello 1994). IL-1 can lead to diseases such as scleroderma, where IL-1 increases collagen synthesis in fibroblasts (Kawaquchi *et al.* 1993).

IL-1 has potent effects on the central nervous system (CNS) either directly or indirectly by affecting the production of other factors. IL-1 acts as an endogenous pyrogen, induces slow wave sleep, and causes a decrease in rapid eye movement in animals. Other endogenous pyrogens include TNFα, IL-6, and interferon α (IFNα). IL-1 induces behavioral changes in rats (possibly mediated by IL-6), such as a decrease in interest in their environment and sleepiness (Dinarello 1994).

IL-1 has direct and indirect (mediated by other factors) effects on the hypothalamus, the pituitary, and the adrenal glands. IL-1 increases the

production of corticotrophin releasing factor, adrenocorticotrophic hormone (ACTH) (Molloy *et al.* 1993), endorphins, vasopressin, and somatostatin, but decreases the release of prolactin, and lutenizing hormone. The corticosteroids induced by IL-1 act in a negative feedback loop to inhibit the production of IL-1 (Dinarello 1994).

Hepatocytes respond to IL-1 by increasing the synthesis of acute phase reactants. IL-1 increases the hepatic synthesis of fibrinogen, complement components, and clotting factors, whilst decreasing the production of albumin, cytochromes, transferrin, and lipoprotein lipase (Dinarello 1994).

IL-1 acts as a growth factor for some cells, for example for fibroblasts and certain tumour cells. IL-1 may be acting as an autocrine growth factor in these cases. Other growth factors, such as platelet derived growth factor (PDGF) are required to produce proliferation in these cells but IL-1 initiates the process of proliferation. IL-1 acts in concert with various growth factors during haematopoiesis as IL-1 alone has little effect on haematopoiesis. Here IL-1 synergises with colony stimulating factors to regulate the cell cycle of early progenitor cells. IL-1 increases the synthesis of GM-CSF, G-CSF, M-CSF, and IL-3, and together they direct a progenitor cell towards a particular cell lineage. In addition IL-1 increases a cell's responsiveness to CSFs and increases the half-life of GM-CSF mRNA (Dinarello 1994a).

IL-1 is an activator of lymphocytes, where it acts as a helper cytokine. IL-2 and IL-4 are "true" T cell activators for T helper-1 and T helper-2 cells respectively. The more mature the T cell (after the acquisition of the T cell receptor (TCR) the less responsive these cells become to IL-1. Immature T cells in the thymus respond to IL-1 in the presence of IL-6. IL-1 and IL-6 synergise to produce IL-2 which then activates T cells. IL-1 on its own is capable of inducing the production of IL-4 by T cells. IL-4 then acts to induce proliferation. IL-1 acts as a helper cytokine, together with IL-4, in the activation of B cells. IL-1 also synergises with IL-6 in B cell activation (Dinarello 1994).

1.7. INTERLEUKIN-1 AND LEUKAEMIA

Leukaemias can be divided into two groups, namely myeloid and lymphoid leukaemias. These can be further divided into acute and chronic subgroups, depending on the course of the disease. A genetic event inducing a leukaemic state occurs in a haemopoietic progenitor cell in the bone marrow, resulting in progeny which have a block in differentiation, and thus are unable to mature fully. The leukaemogenic event usually occurs in a single cell, resulting in monoclonal progeny. These monoclonal cells have a growth advantage over the haemopoietic cells, eventually dominating the bone marrow and the blood. A growth advantage results from the autocrine stimulation of these cells by growth factors. Thus proliferation has been uncoupled from differentiation. The degree of differentiation correlates with the clinical course of the disease

as the leukaemic cell proliferates to a greater extent than its normal counterpart (reviewed in Sullivan 1993). It can happen that the genetic event heralding a leukaemic change in a progenitor cell occurs before the cell has been committed to the myeloid or lymphoid lineages. Thus the resulting leukaemic cells express some of the makers found on lymphoid and myeloid cells (Komada *et al.* 1993).

The development of leukaemia is a "multi-step" process. Carcinogenesis develops in 3 stages; initiation, promotion, and progression. Different carcinogens act at different stages in the development of cancer. Carcinogenesis usually starts with a genetic event, altering the DNA of the cells, allowing the cell to overcome the controls on replication. Additional stimuli are often required to promote the development of cancer. Protooncogenes, required for the normal control of replication and cell cycling in the cell, can be affected during carcinogenesis converting them into oncogenes. Oncogenes produce altered products, or the over- or underexpression of their products, for example promyelocytic HL-60 cells overexpress the oncogene *c-myc*, and lack p53 (Sullivan 1993).

The growth of the leukaemic clone is often induced by endogenously synthesised growth factors. One such growth factor is IL-1ß (Estrov 1993a), which is constitutively expressed in acute myelogenous leukaemia (AML) blast cells (Murohashi *et al.* 1989). IL-1 in turn induces the expression of GM-CSF in AML, which then acts as an autocrine growth factor. IL-1 also induces

accessory cells to secrete growth factors in AML, which then act in a paracrine manner (Oster *et al.* 1989). Here IL-1 mediates growth through other growth factors such as GM-CSF. Evidence of this is seen when IL-1 is added to cells in the presence of anti-GM-CSF, resulting in the inhibition of cellular proliferation (Carter *et al.* 1992).

In chronic myelogenous leukaemia (CML) (Specchia *et al.* 1992), B-cell-type chronic lymphocytic leukaemia (B-CLL) (Dazzi *et al.* 1995), and adult T-cell leukaemia (ATL) (Mori *et al.* 1995) IL-1ß is constitutively produced. The growth of the CML blast cell is inhibited by the addition of neutralising antibodies directed against IL-1ß. In the CML blasts, IL-1ß upregulates CSF receptors (Hestdal *et al.* 1994) and in addition induces the release of these growth factors from accessory cells in the bone marrow stroma. Thus IL-1ß provides the growth advantage of the leukaemic cell by autocrine and paracrine stimulation. In order to inhibit the proliferation of these CML blasts, this growth loop needs to be interrupted. Thus a novel therapy for CML could involve the administering of IL-1ra, soluble IL-1 receptors, or of IL-4 which has been shown to inhibit the production in IL-1ß in CML cells (Estrov 1993a). IL-4 has also been shown to inhibit the production of IL-1ß in B-CLL (Dazzi *et al.* 1995) and in ALT (Mori *et al.* 1995).

Acute lymphocytic leukaemic (ALL) cells of the B cell lineage also proliferate in response to IL-1. Here IL-1α has been implicated. ALL cells express IL-1α mRNA constitutively. Neutralizing anti-IL-1α antibodies inhibited the

spontaneous proliferation of these cells thus indicating that IL-1α stimulated the cells in an autocrine fashion (Mori *et al.* 1994).

Spontaneous IL-1 and TNFα production has also been found in AML cells (Mori *et al.* 1994, Carter *et al.* 1992). Although IL-1α expression is lacking in these cells (Ferrari *et al.* 1993).

Since IL-1 has been shown to induce the proliferation of leukaemic cells, the inhibition or blocking of IL-1 might be useful in the treatment of these diseases. Various IL-1 inhibitory molecules have been identified, namely the IL-1ra, soluble IL-1 receptors, IL-4, and ICE inhibitors (Estrov *et al.* 1993b) (see section 1.15. for more on antagonizing IL-1). It must be noted that in the majority of cases IL-1 stimulated proliferation in leukaemic cells, although it has been found that some cell lines do not respond to IL-1 (Fukuda *et al.* 1993).

IL-1 often works in concert with TNFα to produce some of its biological effects in leukaemia. TNFα and IL-1 work in synergy to induce proliferation, by the induction of GM-CSF in those cells capable of synthesising GM-CSF. TNFα also increases the production of IL-1 by the cells (Carter *et al.* 1992).

IL-1 and TNFα have been shown to protect haemopoietic cells from the radiation and cytotoxic drugs used in conventional cancer therapy. As these modes of therapy can lead to lethal bone marrow suppression, agents must be

found to protect the normal cells in the bone marrow. IL-1 and TNFα protect normal haemopoietic precursors from the cytotoxic drug 4-hydroperoxycyclophosphamide (4-HC) but do not protect AML cells from the same drug. Unfortunately IL-1 and TNFα are not always selective in their protection of normal cells, as they have also been found to protect a human melanoma cell from lethal irradiation (Moreb and Zucali 1992).

1.8. CYTOKINE RECEPTORS

Cytokines act on a cell by inducing the transcription of various genes (Muegge and Durum 1989). Cytokines (extracellular signals) convey messages via highly specific membrane bound receptors. The receptors in turn activate second messengers within the cell, whose end result is to activate transcription factors which then alter the transcription of specific genes. Thus the receptors for these cytokines are the vital link between extracellular and intracellular communication systems. It is at this point that a high degree of specificity is conferred on the system, as well as an opportunity to regulate the effect of these potent extracellular messengers (Foxwell *et al.* 1992).

The cytokine receptors are a complex group of transmembrane receptors, consisting of extracellular ligand binding domains, transmembrane portions, and intracellular or cytosolic domains (reviewed by Foxwell *et al.* 1992). These cell surface receptors are usually expressed in low numbers depending on the cell type (from a few to a few thousand). Often, for the receptor (eg. the IL-6

receptor) to function, it requires a receptor-associated molecule (eg. GP130), which stabilizes the ligand-receptor binding (Taga and Kishimoto 1993). Their mechanisms of signal transduction are also complex, and in most cases have not been fully elucidated. It has been demonstrated that many cytokine receptors share signal transduction molecules, and that many cytoplasmic kinases are able to substitute for one another during signal transduction (Foxwell *et al.* 1992).

Cytokine receptors can be divided into 3 families, namely, the haemopoietic growth factor receptor (HGFR) family, the nerve growth factor family or the tumour necrosis factor receptor (TNFR) family, and the immunoglobulin supergene receptor (Ig-R) family. Not all the receptors found in these families are cytokine receptors and most of the ligands are not cytokines (Foxwell *et al.* 1992).

1.8.1 THE HAEMOPOIETIC GROWTH FACTOR RECEPTOR FAMILY

Cytokines such as IL-2, IL-3, IL-4, IL-5, IL-7, and GM-CSF have receptors which belong to the HGFR family of receptors. Characteristics of this family include: (1) conserved cysteine residues in their extracellular domains; (2) their intracellular domains lack kinase activity; (3) they generally bind only one ligand; (4) they usually have high and low affinity binding sites; and (5) many require receptor-associated molecules for high affinity binding of ligand (figure 1.4.) (Foxwell *et al.* 1992).

An example from the HGFR family is the receptor for IL-4, which is a 140 kDa cell surface receptor exhibiting single high affinity binding sites for its ligand, IL-4. A soluble IL-4 receptor has been detected in murine tissue fluids, but no soluble form has been detected in humans (Foxwell *et al.* 1992).

1.8.2. THE TUMOUR NECROSIS FACTOR RECEPTOR FAMILY

The members of this family have receptors which are similar to the two receptors for TNFα and TNFß. Binding of a ligand (or multiple ligands) results in the aggregation of multiple receptors chains on the cell surface. Multiple ligands can bind a receptor in this family, for example, TNFα binds its receptor as a trimer. This is unlike the ligands for the HGFR family, where only single ligands bind their receptors (Foxwell *et al.* 1992).

1.8.3. THE IMMUNOGLOBULIN SUPERGENE RECEPTOR FAMILY

The members of this family include the receptors for IL-1, insulin, and insulin-like growth factor (IGF), to name a few. Characteristic of these receptors are the immunoglobulin-like domains present in the extracellular domains (see figure 1.4.). Many of the receptors have intracellular domains which have tyrosine kinase activity except the IL-1 receptors (Foxwell *et al.* 1992).

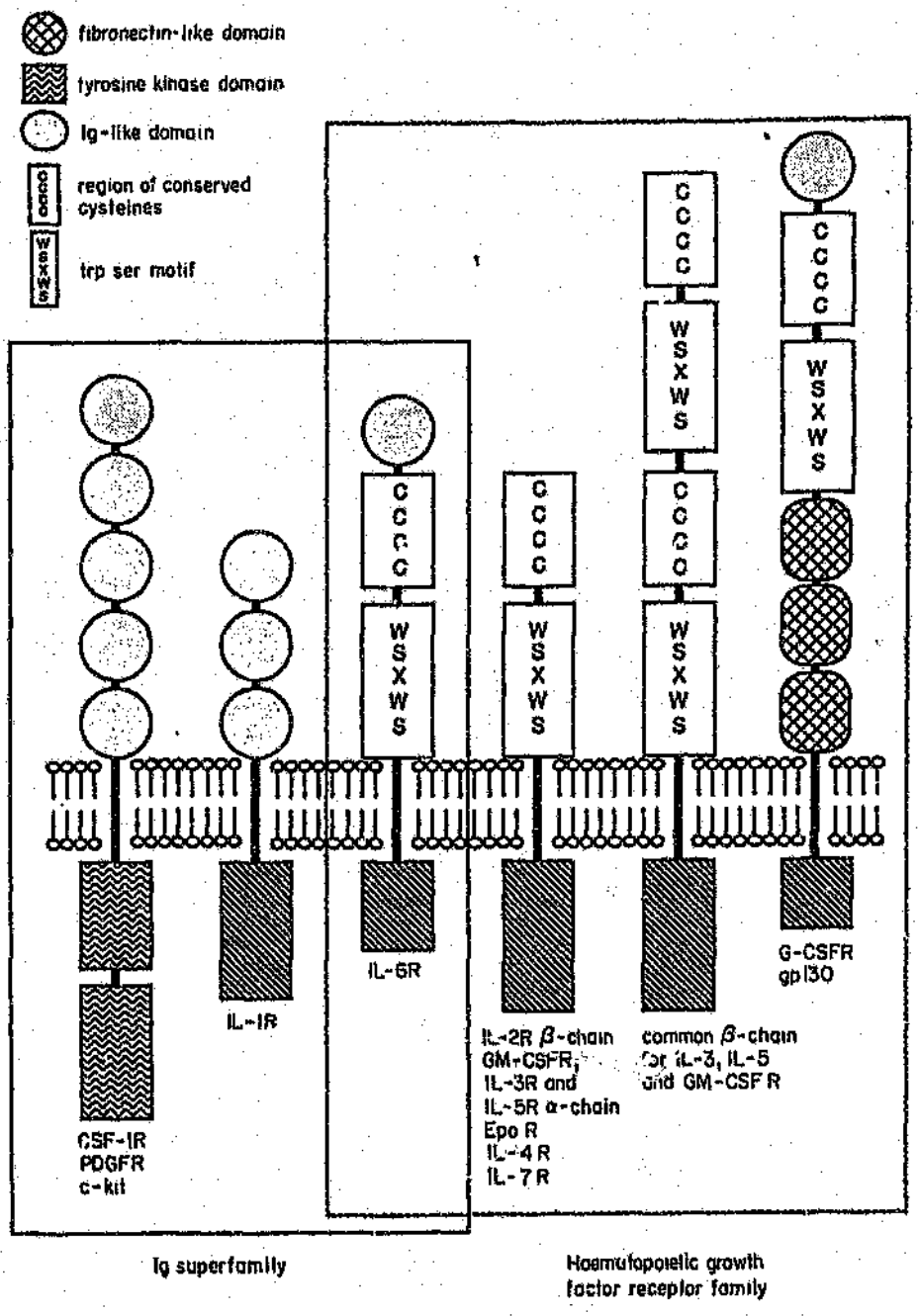


Figure 1.4. The immunoglobulin receptor superfamily and the haemopoietic growth factor receptor family.
IL-1R in the diagram refers to IL-1RtI (from Foxwell *et al.* 1992).

1.9. INTERLEUKIN-1 RECEPTORS

1.9.1. DISCOVERY OF THE RECEPTORS FOR INTERLEUKIN-1

It was found that IL-1α and IL-1ß bound to the same receptor, which was to be expected as these two polypeptides have similar biological activities. This receptor, which was found to occur on T lymphocytes and fibroblasts, consisted of a single 80 kDa transmembrane glycoprotein. By 1989, Sims *et al.* had cloned and sequenced this 80 kDa IL-1 receptor found on T cells. The cell line used to generate a cDNA probe for the human IL-1R was the mouse thymoma cell line, EL4 6.1, which expresses very high numbers (up to 10 000 receptors per cell) of the IL-1R. The murine and human T cell IL-1R share a high degree of homology (69 % amino acid identity) (Sims *et al.* 1989 and Solari 1990).

From the sequence generated, it was concluded that the T cell IL-1R (now known as the IL-1RtI) consisted of an extracellular domain (ligand binding segment), a transmembrane segment, and a cytoplasmic domain consisting of 215 amino acids. At this stage it was known that IL-1 binding to its receptor on T cells did not cause the breakdown of phosphatidylinositol, nor an increase in intracellular calcium concentrations. The only effect of ligand binding appeared to be the phosphorylation of certain proteins, but the significance of this was not known, as the receptor itself appeared not to function as a kinase. It was also noted that the ligand receptor complex was internalized, and was detected in the nucleus, but the functional importance

of this event is still to be determined (Sims *et al.* 1989).

It was later discovered that a smaller receptor for IL-1 was expressed on B lymphocytes (reviewed by Solari 1990). This 60 kDa receptor (now known as the IL-1RtII) was found to be antigenically distinct from the IL-1Rs found on T cells (Savage *et al.* 1989), and that it bound IL-1ß with a higher affinity than IL-1α. In addition it was noted that the IL-1RtII was incapable of receptor mediated endocytosis, unlike IL-1RtI.

It was still unclear at this stage, if the 80 kDa receptor and the 60 kDa receptor bound IL-1 as a heterodimer, if they were separate gene products, or if there was posttranslational modification of a single gene, and if the difference in molecular masses was simply due to differences in the degree of glycosylation.

1.9.2. IL-1RtI AND II DO NOT BIND INTERLEUKIN-1 AS A HETERODIMER

In 1993, Slack *et al* attempted to determine if a functional IL-1R unit was comprised of an IL-1RtI / IL-1RtII heterodimer in a similar fashion as the IL-2 receptor. The heterodimer theory was supported by the fact that both receptor types can be expressed on one cell, and that the polypeptide structures of the two receptors are similar to receptors which form multiple chains in order to bind their ligand. In addition it could not be shown that the IL-1RtII was able to signal across the cell membrane. The hypothesis was put

forward that the IL-1RtII was required to enhance the binding of IL-1, or that the type II receptor "delivered" IL-1 to the functional IL-1RtI.

Slack *et al.* concluded from competition and dissociation studies, that neither IL-1α nor IL-1ß could bind IL-1RtI and II simultaneously. The two receptors functioned independently of each other and did not form heterodimers. No ligand induced aggregation of the two IL-1Rs was found.

1.9.3. IL-1RtI AND II ARISE FROM TWO SEPARATE GENES

Monoclonal and polyclonal antibodies directed against IL-1R type I were able to block the binding of [^{125}I]-IL-1ß to the receptors on T cells, but were unable to block the binding of the labelled IL-1ß to the IL-1Rs found on bone marrow cells, pre-B cells, and on macrophage cell lines which expressed mainly IL-1RtII. The mRNA for IL-1RtI found in the T cells and fibroblasts were identical, but that the mRNA encoding the IL-1R in pre-B cells and macrophages was not the same species. From the above data they concluded that the IL-1RtI and II were the result of the expression of two separate genes (Chizzonite *et al.* 1989). It is thought that the two genes for IL-1RtI and II are derived from a common ancestor, as the genes have a 28 % amino acid sequence homology, and the introns in their extracellular domains are in identical positions (Sims *et al.* 1994b).

1.9.4. IL-1RtI AND II CAN BE EXPRESSED IN THE SAME CELL

It was thought that IL-1RtI was not expressed on B cells, where only the IL-1RtII was detected. In 1990, Benjamin and Dower showed that the human B lymphoblastoid cell line, CD23, which had been transformed by the Epstein Barr virus (EBV), expressed both IL-1R types. Benjamin and Dower described this as an "inappropriate" expression of the IL-1RtI due to the transformation of the cell line by the EBV.

In 1991, McMahan *et al.* cloned the 60 kDa IL-1RtII from the EBV transformed CB23 cell line, and found that it was expressed in many different cell types. The B lymphoblastiod cells were found to express both IL-1RtI and II. IL-1RtII was described as having an extracellular domain, which bound IL-1, with three immunoglobulin-like domains, a single transmembrane section, and a short cytoplasmic region of 29 amino acids.

IL-1RtII was found not to signal in haemopoietic bone marrow cells. Specific monoclonal antibodies against IL-1RtI and II were used to block either the IL-1RtI or II. It was shown that an antibody directed against the IL-1RtI blocked the action of IL-1 on haematopoietic progenitor cells. An antibody directed against the IL-1RtII, however, did not block the action of IL-1 on these cells (Hestdal *et al.* 1994).

1.10. BINDING PROPERTIES OF IL-1RtI AND II

The three polypeptides which make up the IL-1 family, namely, IL-1α, IL-1ß, and IL-1ra, bind the two IL-1Rs with different affinities. IL-1α and ß are agonists, while the IL-1ra antagonises IL-1, as binding of IL-1ra does not activate the cells. IL-1α and ß compete for receptor binding, but IL-1α binds to the IL-1RtI with a higher affinity than IL-1ß. IL-1ß binds to the IL-1RtII with a higher affinity than IL-1α (Stoppacciaro *et al.* 1991). IL-1Ra has a higher affinity for the IL-1RtI than for IL-1RtII (Sims and Dower 1994a).

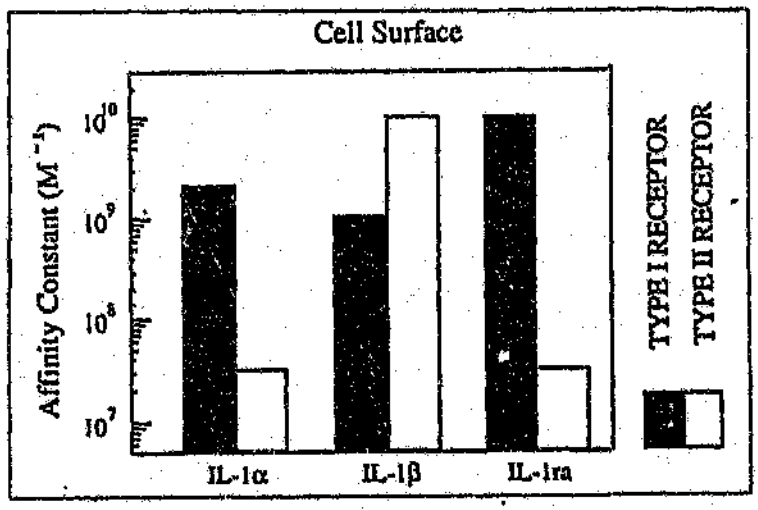


Figure 1.5. Relative binding affinities of IL-1α, IL-1ß, and IL-1ra to IL-1RtI and II. Modified from Sims and Dower 1994a.

The site on the human IL-1RtI which is needed to bind IL-1α and IL-1ß lies at amino acids 86 - 93 (Tanihara *et al.* 1992). A synthetic peptide corresponding to these amino acids was synthesized. This peptide blocked the action of IL-1 on the cells both *in vitro* and *in vivo*. Tanihara *et al.* (1992) propose this to be a new strategy to inhibit IL-1's action in disease.

1.11. CHROMOSOMAL LOCATION OF THE IL-1RtI AND IL-1RtII GENES

Both IL-1R genes have been conserved during evolution, as molecules similar to the IL-1RtI and II can be found in most eukaryotes. It has been suggested that, at the time of the development of mammals, the ancestral IL-1R gene duplicated (McMahan *et al.* 1991).

The IL-1RtI and II genes map to the same chromosomal location, on chromosome 2 (Copeland *et al.* 1991). The IL-1RtII gene is located on the long arm of chromosome 2, in the position 2q12 → 2q22 (McMahan *et al.* 1991), and the IL-1RtI gene maps to 2q12. Interestingly, the IL-1α and IL-1ß genes are located in the same region (2q13 → 2q21). It is unusual for a "hormone" and it's receptor to maintain their linkage. Another example of this phenomenon is the colony stimulating factor - 1 gene (CSF-1) which codes for macrophage - CSF, and its receptor, CSF-1R or *c-fms*. Both these genes, in humans, map to the long arm of chromosome 5 (Copeland *et al.* 1991).

1.12. IL-1RtI AND II TISSUE DISTRIBUTION

All IL-1 responsive cells express IL-1RtI (Sims and Dower 1994), and often IL-1RtI and II are coexpressed in a cell. Monocytes, B cells, neutrophils, and bone marrow cells express the IL-1RtII on their surfaces (McKean *et al.* 1993). Monocytes express both receptors, although the myelogenous leukaemia cell line THP-1 expresses both mRNA species, but only the IL-1RtI can be detected on the surface of the cell (Akeson *et al.* 1992). T cells and fibroblasts classically express IL-1RtI, although they also express IL-1RtII. Murine T helper-2 cells express both IL-1RtI and II constitutively at the mRNA and protein level (McKean *et al.* 1993). In this murine model it was found that the proliferation of the T helper-2 cells was mediated through the IL-1RtI and not through the IL-1RtII.

Certain cells, such as endothelial cells, smooth muscle cells, and mesothelial cells only express IL-1RtI (Colotta and Montovani 1994). Endothelial cells are an important target for circulating IL-1, and IL-1 has many effects on the activity of these cells (see section 1.6.), especially in relation to the extravasation of leucocytes to site of inflammation. Human umbilical cord endothelial cells (HUVEC) express 1000 - 3000 IL-1 type I receptors. IL-1RtII cannot be detected in HUVECs (Colotta *et al.* 1993b) and in human aortic endothelial cell (Akeson *et al.* 1992). IL-1RtII cannot be induced in HUVECs by IL-4. In contrast IL-4 increases IL-1RtII expression in neutrophils.

When the immune system is activated the neurological and endocrine systems

are also affected. Interestingly, Cunningham *et al.* 1993 found that only IL-1RtI mRNA and protein was expressed in certain parts of the brain and endocrine organs.

IL-1α stimulates haemopoietic cells through IL-1RtI, although both receptor types are expressed on haemopoietic bone marrow cells. Epithelial cells express low levels of IL-1RtI, and can be induced to express high levels of IL-1RtII (Groves *et al.* 1995).

1.13. INTERLEUKIN-1 SIGNAL TRANSDUCTION

There are many known biological effects of IL-1, but the signal transduction mechanisms underlying these effects are not well understood. The first step in the signal transduction of IL-1, is the binding of IL-1 to the 80 kDa IL-1RtI, as the IL-1RtII does not signal (Hestdal *et al.* 1994). Many second messengers have been implicated in the signal transduction of IL-1, although often different results have been obtained depending on the cell type investigated. Some of these second messengers include, cAMP, PGE_2, diacylglycerol (DAG), ceramide, and nitric oxide. Many enzymes, mainly protein kinases, have been implicated in IL-1 signal transduction, including, protein kinase A (PKA), PKCß, MAP kinase, MAP kinase kinase, beta casein kinase, tyrosine kinase (Ihle *et al.* 1994), protein phosphatases, nitric oxide synthase, and sphingomyelinase. The end point of these pathways are the transcription factors, such as, NF-kB, AP-1, and NF-IL6 (reviewed in Rosoff 1990 and Brooks and Mizel 1994).

Guanine nucleotide-binding regulatory proteins (G proteins) in the cell membrane activate adenylyl cyclase, and as IL-1 activates adenylyl cyclase, it is thought that IL-1 may act through a G protein (O'Neill 1992 and Brooks and Mizel 1994).

cAMP is a second messenger involved in the signal transduction of many hormones, and is implicated in the signal transduction of IL-1. Increases in cAMP lead to the activation of the cAMP dependant kinase PKA. In fibroblasts cAMP has been shown to be an important component of the signal transduction pathway for IL-1. On the other hand, the action of IL-1 on the erythroleukaemic cell line, EL4, is inhibited by the addition of cAMP. Thus the role of cAMP in the signal transduction of IL-1 is not clear (Brooks and Mizel 1994).

A major class of second messengers are the lipid second messengers. It has been shown that IL-1 induces PGE_2 and cyclooxygenase synthesis. IL-1 increases DAG levels in the cell (eg. monocytes), and this DAG could be derived from phosphatidylcholine or phosphatidylethanolamine (Rossoff 1990). IL-1 also increases phosphatidic acid, which could be converted to DAG. Ceramide is the breakdown product of sphingomyelin when acted on by sphingomyelinase. Ceramide could also be a source of DAG, as IL-1 stimulation induces an increase in ceramide levels in the cell. The DAG produced may then act on PKC (Brooks and Mizel 1994).

IL-1 has been shown to induce the production of nitric oxide in rat islets of Langerhans, and it is thought that this may also be an important signal transduction mechanism in macrophages (Brooks and Mizel 1994).

The IL-1 signal transduction pathway involves protein kinases and phosphatases. cAMP activates PKA and ceramide activates protein phosphatase 2A and MAP kinase. Both of these second messengers are involved in IL-1 signal transduction. Thus the end result of IL-1 signal transduction is a change in the phosphorylation patterns within a cell. Many transcription factors are activated or inactivated by phosphorylation, for example, NF-kB's inhibitory molecule, IkB dissociates from NF-kB upon phosphorylation. IL-1 has also shown increase the expression of Fos and Jun (Muegge and Durum 1989 and Brooks and Mizel 1994) which make up the transcription factor AP-1 (O'Neill 1992). NF-IL6 is thought to be phosphorylated in response to IL-1 by MAP kinases (Brooks and Mizel 1994). In addition, it has been found that the expression of protooncogenes *c-myb* and *c-myc* are increased by IL-1 stimulation (Munoz *et al.* 1991).

It is not known exactly what parts of the IL-1Rs are required for signalling. A large region in the intracellular portion of the mouse IL-1RtI is required for functioning of the receptor. The C terminal region of the IL-1Rt is required for the generation of active NF-kB (Leung *et al.* 1994). The cytoplasmic domain has no protein kinase activity itself, but it is phosphorylated at a threonine residue after IL-1 binding (Gallis *et al.* 1989, Bird *et al.* 1991 and

Kuno *et al.* 1993). Interestingly, IL-1RtI is internalised after IL-1 binds to it. The intracellular portion of the receptor has sequences similar to nuclear localisation sequences. Thus IL-1 internalisation may represent an additional signalling pathway (Kuno *et al.* 1993). Thus, the cytoplasmic domain of the IL-1RtI seems important in its ability to signal. The IL-1RtII has a much shorter cytoplasmic domain, and is unable to signal. A hybrid was made of the extracellular portion of the IL-1RtII and the intracellular portion of the IL-1RtI. This hybrid receptor was able to signal across the membrane and produce the activation of the same genes activated by IL-1RtI (Heguy *et al.* 19[illegible]).

1.14. THE TYPE I INTERLEUKIN-1 RECEPTOR

IL-1RtI is an 80 kDa (Bird *et al.* 1991) type I transmembrane protein, which possess an extracellular segment comprising the ligand (IL-1) binding domain, a single transmembrane portion, and an intracellular domain (Sims and Dower 1994). The entire IL-1RtI consists of 557 amino acids. The extracellular segment's most striking feature is that it consists of 3 immunoglobulin - like domains (Sims *et al.* 1989 and Sims and Dower 1990) consisting of approximately 319 amino acids. The intracellular (cytoplasmic domain) consists of 213 amino acids (the IL-1RtII cytoplasmic domain only has 29 amino acids), and the short membrane spanning region consists or 20 amino acids (Colotta *et al.* 1994b).

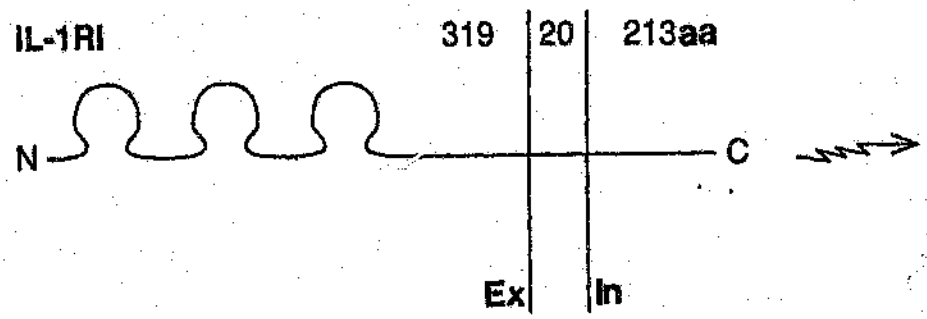


Figure 1.6. The structure of the IL-1RtI cell surface receptor.
The diagram shows that the extracellular (Ex) domain has 319 amino acids, the transmembrane domain has 20 amino acids, and the intracellular (In) domain has 213 amino acids. N: amino terminus; C: carboxy terminus; the arrow indicates an intracellular signal. Modified from Colotta *et al.* 1994b.

The IL-1RtI gene contains 3 promoters, indicating a high level of transcriptional regulation. The mRNA for IL-1RtI is approximately 5 kb long, although 3,3 kb of this is the 3'-untranslated region. The mature protein contains a N-terminus signal peptide (Sims *et al.* 1994b).

1.14.1. IL-1 ONLY SIGNALS USING IL-1RtI

There is evidence that IL-1RtII is not a functional signalling receptor (Colotta *et al.* 1994), and that IL-1 only signals using the IL-1RtI. It has been suggested that all IL-1 responsive cells express IL-1RtI, despite the fact that very low numbers of the type I have been found on some cell types. The low number of IL-1RtI on some cells points to the possibility that a very low receptor occupancy rate is required for a full biological response. It is difficult to modify the levels of IL-1RtI (Sims *et al.* 1994). On the other hand IL-1RtII levels are easily modified (Sims *et al.* 1994), indicating that regulation of IL-1 action at the receptor level is controlled by IL-1RtII.

1.15. IL-1 RECEPTOR TYPE II - THE "DECOY" RECEPTOR

The IL-1RtII was cloned from B cells. This new receptor had a molecular mass of 60 kDa, and was shown to be similar to the IL-1RtI (see figure 1.7.) in that it had an extracellular domain (332 amino acids), a transmembrane domain (26 amino acids), and a truncated cytoplasmic domain (29 amino acids) (Colotta *et al.* 1994).

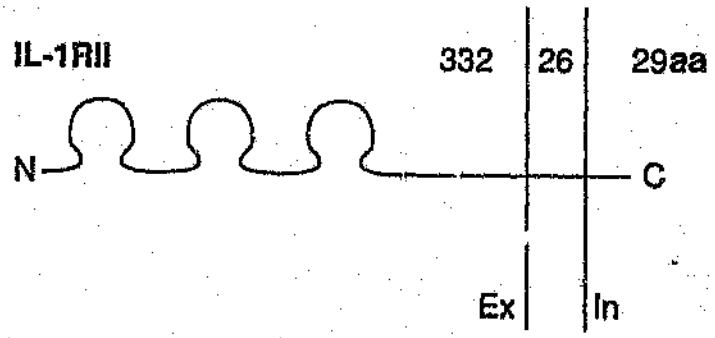


Figure 1.7. The structure of the IL-1RtII cell surface receptor.
N: amino terminus; C: carboxy terminus; Ex: extracellular; In: intracellular. Modified from Colotta *et al.* 1994.

Evidence that some cells only expressed the IL-1RtI, and these cells were fully responsive to IL-1 indicated that the IL-1RtII may not be required for signalling. In addition in cells expressing both IL-1Rs, it was found that antibodies directed against IL-1RtI inhibited the cells response to IL-1, but that anti-IL-1RtII had no effect on the responsiveness of the cells to IL-1. This indicated that the IL-1RtII was not a signalling receptor. Colotta *et al.* (1994) suggest that IL-1RtII functions as a "decoy" receptor (a deceptor), acting as a molecular trap for IL-1 and thus making less IL-1 available to bind to the signalling IL-1RtI. Since there is no evidence that IL-1RtII is able to signal, but that it is able to bind to IL-1 (circulating IL-1ß in particular), it is

feasible that the IL-1RtII is a negative regulator of IL-1 activity (Colotta *et al.* 1994).

Gene transfer experiments have been used to examine the hypothesis that IL-1RtII is a "decoy" receptor. IL-1RtII expression was induced in cells only expressing IL-1RtI. The increased expression of IL-1RtII decreased the ability of the cells to respond to IL-1, while it did not affect TNFα action on the cells. The inhibition of IL-1's action in these cells occurs at lower concentrations of IL-1. At higher concentrations this inhibition disappears, indicating that the IL-1RtII is mopping up IL-1 as opposed to inducing a negative second messenger signal in the cell (Colotta *et al.* 1994).

The IL-1RtII acts as a "decoy" receptor by acting as a molecular trap for IL-1. The IL-1RtII binds IL-1ß making less IL-1 available to bind to the signalling IL-1RtI, thus inhibiting the action of IL-1 on the cell. IL-1RtII binds the circulating cytokine IL-1ß with a higher affinity than IL-1RtI. IL-1ra binds preferentially to IL-1RtI, with such a slow dissociation rate that the IL-1RtI becomes functionally blocked. Thus the regulation of IL-1RtII plays an important role in the regulation of the action of IL-1 on cells. Antagonising IL-1 is important in IL-1's regulation in the body. Some agents which antagonise IL-1 do so by increasing the expression of the IL-1RtII "decoy" receptor (Colotta *et al.* 1994).

1.15.1 IL-4 AND THE IL-1RtII "DECOY" RECEPTOR

IL-4 is an antiinflammatory cytokine of between 15 and 19 kDa. IL-4 has many effects on many cells (see figure 1.8. for a summary of these effects). The mature IL-4 polypeptide consists of 129 amino acids, although it is first synthesised as a 153 amino acid precursor (reviewed in Banchereau *et al.* 1994).

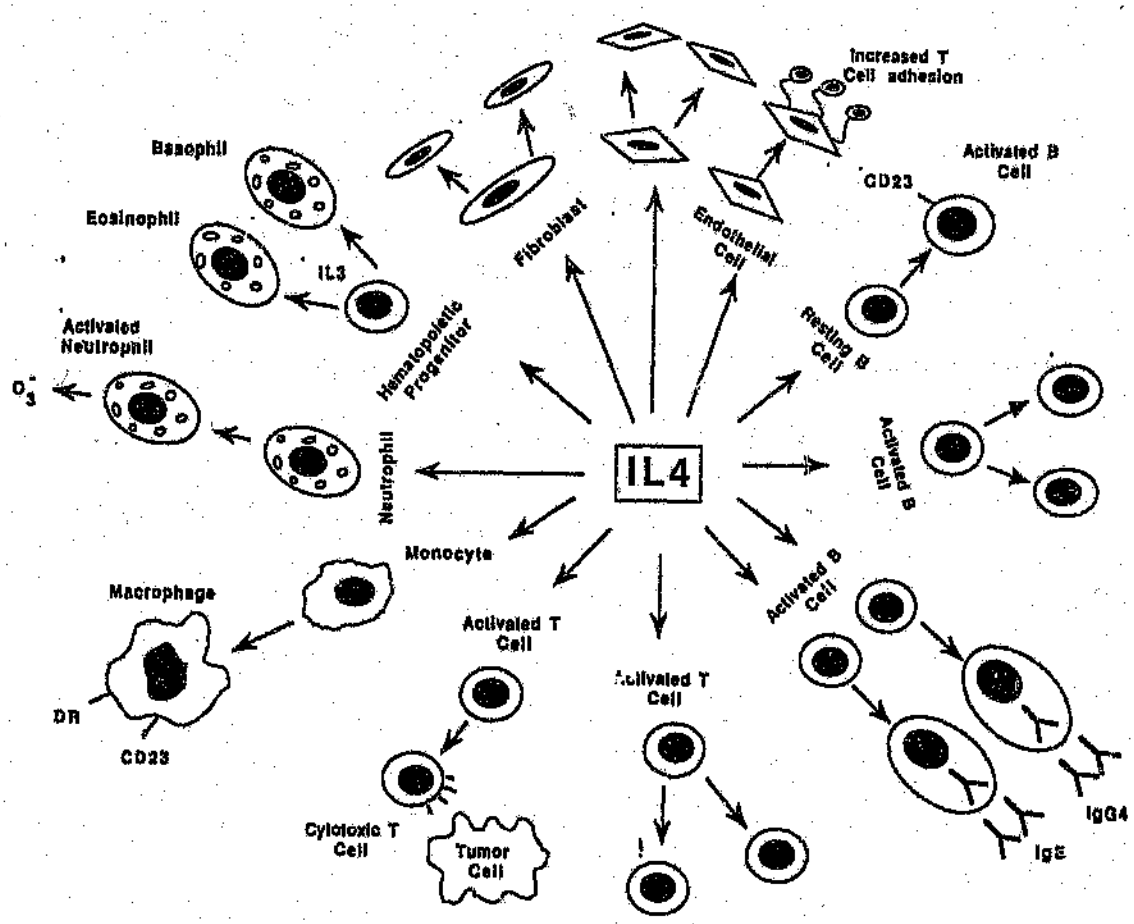


Figure 1.8. The stimulatory effects of IL-4.
From Banchereau *et al.* 1994.

IL-4 inhibits the proliferation of myelomonocytic cell lines, when that proliferation is induced by IL-3, GM-CSF, or M-CSF. It has also been shown that IL-4 suppresses progenitor colony growth in CML and AML. Evidence suggests that IL-4 decreases the growth of these leukaemic cells by inhibiting the production of IL-1ß (Estrov *et al.* 1993).

IL-1ß increases the time of survival of neutrophils *in vitro*, but IL-4 inhibits this increase in survival, by inhibiting the action of IL-1ß. It was found that IL-4 upregulates the mRNA for IL-1RtII and the IL-RtII protein in these cells. IL-4 also induces the release of a 45 kDa soluble IL-1R from the neutrophils, which represents a soluble form of IL-1RtII (Colotta *et al.* 1993a).

IL-4 not only inhibits the production of IL-1ß (by decreasing IL-1ß transcription and by destabilising proIL-1ß mRNA) (Fenton *et al.* 1992) and upregulates the expression of the inhibitory IL-1RtII "decoy" receptor, but it also upregulates the expression and synthesis of IL-1ra in LPS stimulated peripheral blood mononuclear cells. IL-4 synergistically increased the expression of IL-1ra with LPS (Vannier *et al.* 1992). This increase in IL-1ra expression was at the level of gene transcription, as the half-life of IL-1ra mRNA was the same in IL-4 and non-IL-4 stimulated monocytes (Orino *et al.* 1992). Thus, IL-4 increases the expression of IL-1ra and at the same time increases IL-1RtII. IL-1ra preferentially binds to IL-1RtI, thus blocking these signalling receptors, and leaving the non-signaling "decoy" receptors to mop up IL-1ß in the extracellular medium. Thus IL-4 and IL-1ra work in concert

to regulate the action of IL-1.

IL-4 cannot be simply labelled an anti-inflammatory cytokine, as it increases the proliferation of T cells stimulated with IL-1. IL-4 acts in an autocrine fashion in T helper-2 cells, and it has been shown that IL-4 increases the expression of IL-1RtI in murine T helper-2 cells (Clark 1992). Thus IL-4 has both pro-inflammatory (by upregulating IL-1RtI) and anti-inflammatory properties (by upregulating IL-1RtII).

The inhibitory activity of IL-4 in cells such as monocytes, may be due to its effect of NF-kB. IL-4 inhibits the production of IL-6 in monocytes, and at the same time IL-4 decreases the amount of active NF-kB in the nucleus. This effect is, however, tissue specific as IL-4 does not inhibit the action of IL-1 on fibroblasts (Donnelly *et al.* 1993).

1.15.2. DEXAMETHASONE AND THE IL-1RtII "DECOY" RECEPTOR

The anti-inflammatory agent dexamethasone is a synthetic glucocorticoid (GC). GCs bind to specific cytoplasmic steroid receptors. The GC receptor - ligand complex translocates into the nucleus, where it binds to specific sequences of DNA called GC response elements (GREs). Binding to a GRE alters the transcription of the attached gene (Scheinman *et al.* 1995). GCs modulate inflammatory and immune responses, affecting many different signalling pathways. For example, GCs decreased IL-2 receptor expression and increased $ß_2$-adrenoceptor expression (Barnes and Adcock 1994). In general

GCs act by decreasing the *de novo* expression of certain cytokine genes (eg. IL-1, IL-2, IL-6, GM-CSF, TNFα, and gamma interferon) (Scheinman *et al.* 1995) and lipid mediator enzymes (Colotta *et al.* 1994). GCs inhibit lymphocyte proliferation (directly or indirectly) and their ability to secrete cytokines, thus aiding in immunosuppression (Scheinman *et al.* 1995).

GCs inhibit the activity of IL-1 by decreasing IL-1 release from peripheral blood mononuclear cells (PBMNCs) and neutrophils (Re *et al.* 1994). Interestingly GCs do not inhibit the expression of IL-1ra (Santos *et al.* 1993). Dexamethasone has been found to increase the expression of IL-1RtII (at the mRNA and protein level) in human neutrophils (Shieh *et al.* 1993a and Re *et al.* 1994). Dexamethasone induces the release of a soluble IL-1RtII "decoy" (Colotta *et al.* 1993a). Dexamethasone increased the expression of IL-1RtI transcripts and protein, but to a lesser extent than for IL-1RtII (Re *et al.* 1994). Thus, dexamethasone increases the number of nonsignalling binding sites for IL-1. The net effect is the inhibition of the action of IL-1 on the cell. Although this action of dexamethasone is important in its anti-inflammatory activity, it must be noted that GC activity affects many other proteins. Thus the modulation of the IL-1RtII decoy receptor must be added to a list of GCs anti-inflammatory activities (Barnes and Adcock 1994). This provides evidence that IL-1RtII is a unique negative regulator of IL-1s activity (Colotta *et al.* 1993a).

In human lung fibroblasts Monick *et al.* (1994), dexamethasone does not

affect IL-1 receptors. Thus the hypothesis that dexamethasone increases IL-1RtII thus inhibiting the action of IL-1 is not true for all cell types.

GCs inhibit the activity of the transcription factor NF-kB. NF-kB binding sites have been found in a large number of cyt· kine promoters (including the UIS for IL-1). Inhibiting NF-kB decreases the expression of genes activated by this transcription factor. Dexamethasone may act by preventing the dephosphorylation of IkB, and thus the dissociation of IkB from NF-kB (Scheinman *et al.* 1995).

1.15.3. IL-1 RECEPTOR ANTAGONIST AND THE IL-1RtII "DECOY" RECEPTOR

IL-1ra is a naturally occurring 23 kDa molecule which is first synthesised as a precursor molecule. It has a leader sequence, and is thus secreted via the classical secretory pathway. There is a secreted and an intracellular form of the IL-1ra, but the two forms appear to have the same activities (Dinarello 1994). IL-1ra preferentially binds the IL-1RtI, thus blocking the binding of IL-1α and IL-1ß to the signalling receptor (Dayer and Burger 1994). Not only does the IL-1ra block IL-1 binding to its receptor, but it does not elicit a signal from the receptor. Thus it is a true and specific receptor antagonist (reviewed by Dinarello and Thompson 1991), inhibiting the effects of IL-1 on cells (Hong *et al.* 1993). IL-1ra is able to bind to the IL-1RtII (Granowitz *et al.* 1992), but it has a lower affinity for this receptor than for the IL-1RtI.

Interestingly, some AML cells do not produce IL-1ra, but exogenous IL-1ra inhibits the proliferation of CML progenitor cells. IL-1ra has also been found to decrease the production of GM-CSF by AML blasts (Estrov *et al.* 1992), and the addition of IL-1ß reverses this effect (Estrov *et al.* 1993b). Thus one possible explanation of the increased proliferation of leukaemic blast cells could be that they do not produce IL-1ra, and thus the signalling IL-1RtI is not blocked.

1.15.4. IL-13 AND THE IL-1RtII "DECOY" RECEPTOR

IL-13 is an anti-inflammatory cytokine similar to IL-4 and IL-10. IL-4 shares a 25 % nucleic acid sequence homology with IL-13. The gene for IL-13 is on chromosome 5 (q23-31), located next to the IL-4 gene. IL-13 acts on mononuclear phagocytes, B cells, large granular lymphocytes, and endothelial cells. It inhibits the production of cytokines by stimulated mononuclear phagocytes, in a similar fashion to IL-4 and IL-10 (Colotta *et al.* 1994b).

IL-13 increased the expression of the IL-1RtII on neutrophils, and that there was an increase in the soluble IL-1RtII in the extracellular medium. Thus one of the anti-inflammatory properties of IL-13 includes the mopping up of IL-1 by increasing the expression of the non-signalling IL-1RtII "decoy" (Colotta *et al.* 1994b).

1.15.5. THE SOLUBLE IL-1RtII "DECOY" RECEPTOR

Another mode of inhibiting the action of IL-1 is to release into the extracellular environment a soluble IL-1RtII "decoy" receptor (sIL-1RtII). Many cytokines and growth factors modulate their responses by the production of non-signalling soluble receptors (reviewed in Rose-John and Heinrich 1994). The sIL-1RtII is a 45 kDa truncated form of the membrane bound 60 kDa IL-1RtII. It resides in the extracellular medium as monomer which is able to bind IL-1 (Estrov *et al.* 1993b). The sIL-1RtII is produced at the surface of the cell by proteolytic action (as opposed to alternate splicing of the IL-1RtII mRNA), which causes the shedding of the now truncated receptor. In neutrophils shedding of the sIL-1RtII is achieved by TNFα, LPS, dexamethasone, PMA, and IL-4 (Sims *et al.* 1994).

The production of sIL-1RtII has therapeutic relevance, as sIL-1RtII inhibits the proliferation of AML and CML blasts. This activity of sIL-1RtII can be reversed by the addition of IL-1ß thus implicating IL-1ß in AML and CML (Estrov *et al.* 1993b and Carter *et al.* 1994). Patients with sepsis express high levels of IL-1RtII mRNA, therefore (Giri *et al.* 1994) it has been suggested that this increase in IL-1RtII expression leads to higher levels of sIL-1RtII in the extracellular medium. This may represent an attempt by the cells to modulate the action of the IL-1 released during sepsis (Giri *et al.* 1994). It has also been shown that the cutaneous allergic late-phase response in humans is partially inhibited by a soluble recombinant form of IL-1RtI (Mullarkey *et al.* 1994).

The sIL-1RtII has binding affinities similar to that of the membrane bound receptor. sIL-1RtII binds IL-1ß with a higher affinity than IL-1α and IL-1ra (Arend *et al.* and Re *et al.* 1994) (see figure 1.9.). Membrane bound IL-1RtII exhibits similar binding affinities to those of the sIl-1RtII. Interestingly when the sIL-1RtII is shed, its affinity for Il-1ra (which is already low in the membrane bound form) decreases 2000 times. Thus sIL-1RtII does not bind and therefore inactivate the IL-1ra (Symons *et al.* 1995). The cell surface IL-1RtII cannot bind proIL-1ß, but the sIL-1RtII is able to bind this molecule in the extracellular medium. This effect of sIL-1RtII may have biological significance as the secretion (or release from necrotic cells) of proIL-1ß has been reported. Thus bound proIL-1ß is then unable to be processed into mature IL-1ß by extracellular proteases (Symons *et al.* 1995).

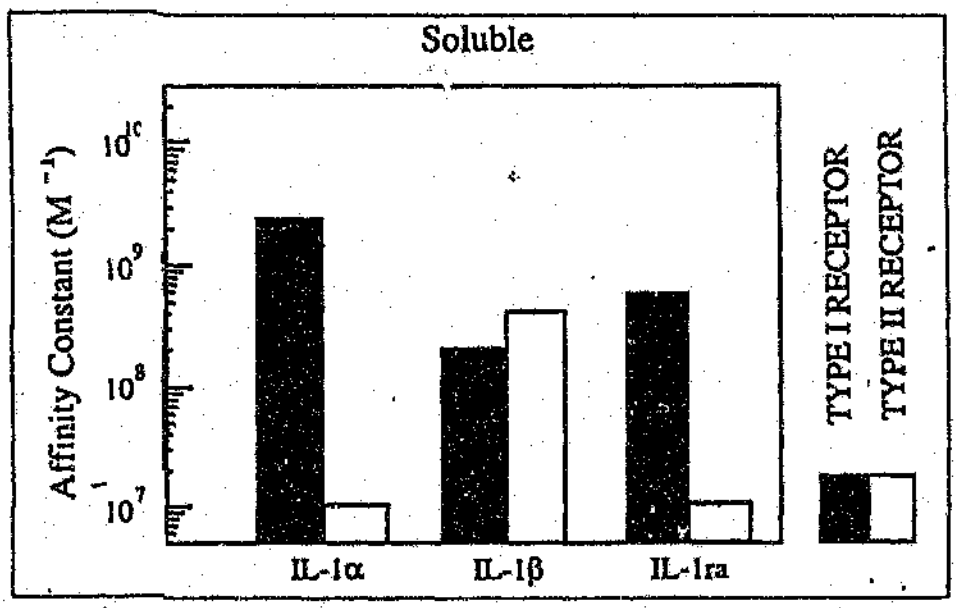


Figure 1.9. The relative affinities of IL-1α, IL-1β, and IL-1ra for soluble IL-1RtI and II. Modified from Sims and Dower 1994.

1.15.6. IL-1RtII EXPRESSION IS CELL CYCLE DEPENDENT

The level of expression of IL-1R type II within a cell depends on the stage of the cell cycle. Within a population of B cell blasts, there will be groups of cells at different stages in the cell cycle, and thus there will be differing levels of IL-1R type II between the groups. Generally, there will only be a small group of B cell blasts expressing significant amounts of IL-1R type II (Stoppacciaro *et al.* 1991).

Increased expression of IL-1R type II is found in quiescent or non-cycling cells, and cells which are beginning to cycle and those stopping to cycle, i.e. in transitional cells. IL-1ß binding to IL-1R type II is high in cells that are in the G_0 - G_1 phase (non-cycling), indicating an increase in IL-1R type II. IL-1ß binding decreases in cells in the S phase (transitional), and is undetectable in cells in the G_2 - M phase (cycling). The data suggests a possible role for IL-1 in preparing the cells to enter the cell cycle (Stoppacciaro *et al.* 1991).

1.16. OBJECTIVES OF THIS STUDY

This report discusses the effects of IL-4, dexamethasone, IL-1ß, and PMA on the expression of IL-1ß, IL-1RtI, and IL-1RtII in peripheral blood monocytes and the leukaemic cell lines, HL-60 and THP-1. IL-1RtI is the functional, signalling IL-1R. IL-1RtII is reported to be a nonsignalling "decoy" IL-1R which acts as a molecular trap for IL-1ß, hence inhibiting its action on a cell (Sims *et al.* 1994).

The objective of this project is to establish whether the expression of IL-1RtI and II is regulated in the same way in the HL-60 and THP-1 cells, compared to normal peripheral blood monocytes. The reasons for this are threefold. **Firstly**, to confirm findings in the literature (Colotta *et al.* 1993a) relating to the antiinflamatory effects of IL-4 and dexamethasone on monocytic cells with respect to IL-1ß, IL-1RtI and IL-1RtII expression. The effects of these agents have been extensively tested in neutrophils (Colotta *et al.* 1993a and Re *et al.*

1994). **Secondly,** if it is found that IL-1RtI and II are regulated (by IL-4 and dexamethasone) in a similar manner in HL-60 and THP-1 cells compared to normal monocytes, then these two cell lines could be used in future as a model for studying anti-inflammatory agents on monocytes. **Thirdly,** if a difference is found in the regulation of IL-1RtI and II in the HL-60 and THP-1 cells in relation to normal monocytes, then these differences could be investigated further. An altered regulation of expression of IL-1Rs in leukaemic cells indicates the altered regulation of the action of IL-1 on these cells, since IL-1RtII acts as an inhibitor of IL-1 action. IL-1 contributes to the proliferation of some leukaemic cells and may be involved in the leukaemic disease process. It has been shown that IL-1 is constitutively produced by many monocytic leukaemias and that IL-1 may act in an autocrine, paracrine, and endocrine manner to induce proliferation in these cells (Dinarello and Wolff 1993). An alteration in the regulation of IL-1's action on leukaemic cells (possibly caused by an altered IL-1RtI and II expression) would affect the proliferative activity of IL-1 on these cells. It is the degree of differentiation, and hence the degree of proliferation of leukaemic cells, which correlates with the clinical course of the disease (Sullivan 1993).

The effect of PMA on HL-60 and THP-1 cells was analysed to assess the role of differentiation on the expression of IL-1RtI, II, and IL-1ß.

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The effect of PMA on HL-60 and THP-1 cells was analysed to assess the role of differentiation on the expression of IL-1RtI, II, and IL-1ß.

SECTION 2

MATERIALS AND METHODS

2.1. FRESH AND CULTURED CELLS

2.1.1. CULTURED CELLS

Three cell types were used in this project, namely, the human myelogenous leukaemia cell lines, HL-60 and THP-1 cells, and freshly isolated peripheral blood mononuclear cells (PBMNCs). The leukaemic cell lines were cultured in sealed culture flasks (Nunclon, Denmark) at 37°C in RPMI-1640 medium (Highveld Biological, RSA) containing 10 % heat inactivated foetal calf serum (FCS) (Delta Bioproducts, RSA). The RPMI contained HEPES (0,05 M), sodium bicarbonate (1,85 g/l), and the antibiotics streptomycin (80 000 U/l) and penicillin G (100 000 U/l) both from Sigma, USA. The cultured cells were passaged every four to five days.

2.1.2. PBMNC ISOLATION

Peripheral blood monocytes were isolated either by adherence to plastic petri dishes, or by density gradient centrifugation using Percoll (Sigma, USA). In both cases peripheral venous blood was obtained from consenting healthy volunteers (ethics clearance number: 6/3/86). Whole blood was collected by venepuncture into Vac-U-Test tubes containing lithium heparin (14.3 U.S.P./ml blood). Buffy coats (leukocytes) were obtained after centrifugation at 200 x g for 10 minutes on a Beckman TJ-6 centrifuge. The PBMNCs were separated from the other leukocytes by density gradient centrifugation on Ficoll gradients (Histopaque, *p* 1.007, Sigma, USA). The PBMNCs were washed 3 x in sterile

saline at 4°C.

2.1.3. MONOCYTE ISOLATION BY ADHERENCE

In order to separate adherent cells (mainly monocytes and some B lymphocytes) from nonadherent cells (mainly T lymphocytes), PBMNCs were incubated in plastic petri dishes (Nunclon, Denmark) at concentrations of approximately 2 x 10 cells/ml. Cells were counted using a haemocytometer (Weber Scientific International, England) in the presence of the dye trypan blue (0,1 % w/v), which is excluded from living cells, thus only viable cells were counted. After incubating the cells at 37°C for 2 hours nonadherent cells were aspirated from the adherent fraction of cells and replaced by fresh medium containing 2 % heat inactivated FCS. The nonadherent fraction of cells was washed with saline and also resuspended in fresh medium containing 2 % heat inactivated FCS. Both fractions were then stimulated with various agents, and incubated at 37°C in a humidified incubator in the presence of 5 % CO_2. Cells were harvested from the petri dishes by scraping the adherent cells using a disposable cell scraper (Costar, USA).

2.1.4. MONOCYTE ISOLATION BY PERCOLL DENSITY GRADIENT CENTRIFUGATION

Monocytes were isolated using a modified method described by Denholm and Wolber (1991).

2.1.4.1. ISOLATION OF PBMNCS

PBMNCs obtained after the centrifugation of peripheral venous blood through a Ficoll gradient, were resuspended in RPMI medium containing 10 % heat inactivated FCS.

2.1.4.2. PRIMARY CELL SEPARATION

The 50 % Percoll (Sigma, USA) gradient was prepared by mixing 22 ml of isotonic Percoll with 1 ml FCS and 14,7 ml of 10 x Hanks' balanced salt solution (Freshney 1994). A gradient was generated by centrifugation of the Percoll at 18 000 rpm at 4°C for 25 minutes using a Sorvall RC-5B SS 34 rotor.

PBMNCs (2 ml) were loaded onto this preformed gradient. Cell separation was achieved by centrifugation at 3000 rpm at 4°C for 30 minutes using a RT 6000 Sorvall centrifuge.

The monocytes appear in a band in the top 5 mm of the gradient after centrifugation. This layer was collected and washed 3 x in 1 x phosphate - buffered saline (PBS) at pH 7,4 (0,14 M NaCl, 2,7 mM KCl, 8 mM disodium hydrogen orthophosphate, 1,4 mM potassium dihydrogen orthophosphate). The yield of cells were checked at this point using the trypan blue exclusion method.

2.1.4.3. SHEEP ERYTHROCYTE ROSETTING

In order to remove contaminating T lymphocytes from the monocyte fraction after centrifugation on the Percoll gradient, the T lymphocytes were rosetted with

neuraminidase-treated sheep red blood cells (N-SRBC). The neuraminidase (type III from *V. cholerae* from Sigma, USA) at a concentration of 0,23 g/ml was incubated with 0,6 ml packed SRBC (washed 3 x in 1 x PBS) and 10 ml 1 x PBS for 30 minutes at 37°C. The N-SRBCs (kindly prepared by Brenda Killroe of the National Institute of Virology) were washed 2 x with 1 x PBS and resuspended in 30 ml PBS.

Equal volumes of the monocyte fraction was mixed with the N-SRBCs. The mixture was centrifuged at 200 x g at 20°C for 5 minutes. The pellet was gently resuspended sc as not to dislodge the erythrocytes from the T lymphocytes. This mixture (4 ml) was loaded onto a Ficoll gradient and centrifuged at 2000 rpm at room temperature for 30 minutes in a Sorval RT 6000 centrifuge. The rosetted T lymphoctes pelleted, while the now purer monocyte fraction remained as a buffy coat on the Ficoll gradient. The cell viability and yield were checked using trypan blue exclusion.

2.2. CYTOKINES AND OTHER REAGENTS

The following cytokines were used to stimulate the cells. IL-1α (10 ng/nl) (Boehringer Mannheim, Germany), IL-1ß (10 - 100 ng/nl) (gift from C.A. Dinarello, Tufts University, Boston, USA), and IL-4 (10 ng/ml) (Genzyme, USA). Other stimuli used included bacterial lipopolysaccharide (LPS, 0,01 - 1 μg/ml) (Sigma, USA), phorbol myristic acetate (PMA, 10 nM) (Sigma, USA), dexamethasone (100 nM) (Sigma, USA) and concanavalin A (Con A, 1 μg/ml) (Sigma, USA).

2.3. LYMPHOCYTE ACTIVATING FACTOR (LAF) ASSAY

In order to determine if the IL-1ß is biologically active a LAF assay was performed (Gery *et al.* 1972). In this assay proliferation of mouse thymocytes (T lymphocytes) induced by IL-1ß is measured by tritiated thymidine ([^{3}H]-thymidine) incorporation into the DNA of the cells.

Thymocytes were removed from 3 endotoxin resistant mice (c3H/HeJ mice, ethics clearance number: 89/137/1), washed and resuspended on RPMI medium containing 10 % heat inactivated FCS and 2- mercaptoethanol (0,5 μM). A submitogenic dose of Con A (1 μg/ml) was added to the thymocytes. Con A synergizes with IL-1ß to induce proliferation in thymocytes (Gery *et al.* 1972). A submitogenic dose therefore "primes" the thymocytes before the addition of IL-1ß. Various concentrations of IL-1ß were added to the primed thymocytes (200 000 cells per well), ranging from 10 ng/ml to 156 pg/ml. No IL-1ß was added to control wells. The experiment was done in triplicate and any proliferation was noted after 24, 48, and 72 hour incubations at 37°C in a humidified incubator in the presence of 5 % CO_2. The thymocytes were pulsed with [^{3}H]-thymidine (0,5 μCi/well with a specific activity of 29 Ci/mmol) (Amersham, England) 6 hours before the end of each incubation period. The cells were harvested using a cell harvester (Titertek) which traps the DNA from the lysed cells on glass filter paper. The individual pieces of filter paper were placed in 2 ml of aquagel scintillation fluid and counted for 1 minute in a Packard Tri-carb liqiud scintillation counter. Proliferation was indicated by an increased incorporation of [^{3}H]-thymidine.

2.4. FLOW CYTOMETRY: CD14 AND CD11b SURFACE ANTIGEN EXPRESSION

The CD14 surface antigen is expressed on myelomoncytic cells, including monocytes and is the cell surface receptor for the LPS and LPS - binding protein (LBP) complex (Wright, 1990). As T lymphocytes do not express CD14 it is a useful marker in determining the percentage monocytes in a given population of cells, for example in the adherent fraction of cells after the nonadherent fraction has been removed, or the percentage monocytes in the monocyte fraction after Percoll density gradient centrifugation.

Cells (500 000) to be tested for CD14 expression were resuspended in 90 μl of BSA - PBS [1 x PBS containing 0,2 % bovine serum albumin (BSA, Boehringer Mannheim, Germany) and 0,2 % sodium azide] plus 10 μl of anti-CD14 (kind gift from Dr Clive Gray) (Coulter). After a 30 minute incubation on ice, the cells were washed twice with BSA - PBS and resuspended in BSA - PBS containing fluoresing secondary antibody (Coulter). After a 30 minute incubation on ice the cells were washed twice in BSA - PBS. The cells were then fixed in 200 μl of a formalin fix solution (1 x PBS containing 1,5 % BSA and 1,5 % formalin). As a control for each experiment, the non-specific binding of the secondary antibody was measured on cells in the absence of the primary anti-CD14 antibody. The percentage CD14 positive cells was measured on a Epics-Profile flow cytometer, with an increase in fluorescence indicating a higher expression of CD14 on the cells present.

In addition, the CD11b (Coulter, USA, gift of Brenda Smith) surface antigen expression was measured using the method described above. CD11b is the Mo1 or C3bi receptor

expressed mainly on peripheral blood monocytes (Horejsi 1991).

2.5. RNA ISOLATION, QUANTIFICATION AND ANALYSIS

2.5.1. RNA ISOLATION

Total cellular RNA was isolated using an acid guanidinium thiocyanate - phenol - chloroform extraction method (Chomczynski and Sacchi 1987). Care was taken at all times not to contaminate the RNA with ribonucleases (RNases). Gloves were worn and the Milli Q water used to make up the solutions was kept RNase free by treating it with 0,2 % (v/v) diethyl pyrocarbonate (DEPC) overnight. DEPC was removed by autoclaving.

After an experiment between 2 x 10^6 and 9 x 10^6 cells were pelleted and mixed with 0,5 ml of a denaturing guanidinium isothiocyanate solution [4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7,0 ;prepared in the laborotory and autoclaved), 0,5 % sarcosyl and 0,1 M 2-mercaptoethanol. A stock denaturing solution without mercaptoethanol was made which could be kept for 3 months at 4°C. The working solution containing mercaptoethanol was no longer used after one month.

Once the cells were lysed, 50 µl 2 M sodium acetate (pH 7,0), 0,5 ml watersaturated phenol (distilled), and 150 µl chloroform : isoamylalcohol (49:1) were added respectively. After vortexing for 10 seconds this mixture was incubated on ice for 20 minutes, and then centrifuged at 10 000 x g for 15 minutes at 4°C. The resultant upper

aqueous phase, which contained the RNA, was aspirated off carefully so as not to contaminate the RNA with any DNA or protein from the interphase.

The RNA was precipitated (out of the aqueous phase) by adding 0,5 ml of ice cold isopropanol, and incubating at -20°C for 2 to 24 hours. The RNA was pelleted by centrifugation at 10 000 x g for 15 minutes at 4°C. The RNA was reprecipitated by adding 0,3 ml of RNase free water and 0,3 ml of ice cold isopropanol to the pellet, and incubating at -20°C for 1 to 24 hours. The precipitated RNA was pelleted by centrifugation as above and washed twice for 20 minutes on ice in 0,2 ml 75 % ethanol in order to remove any denaturing solution contaminating the RNA. It was suspected that the presence of denaturing solution might inhibit the conversion of RNA to copy DNA (cDNA) by the enzyme reverse transcriptase. The RNA was pelleted and allowed to air dry so as to remove any ethanol which might also be inhibitory to reverse transcriptase. The RNA was then dissolved at 65°C for 10 minutes in 20-50 µl RNase free water.

2.5.2. TOTAL CELLULAR RNA QUANTIFICATION

The total cellular RNA concentration was measured spectrophotometrically using a Beckman spectrophotometer. RNA from each sample was diluted 100 x in RNase free water and the absorbance (A) measured at 260 nm. The concentration of RNA was calculated using the following equation:

$$[\text{RNA}] \text{ in } \mu g/ml = 40 \times A_{260\ nm} \times \text{dilution factor}$$

where 40 is the average extinction coefficient for ribonucleic acids, and A_{260nm} is the wavelength at which ribonucleic acids absorb maximally. In order to determine the degree of contamination of the RNA sample with DNA and proteins, the ratio of $A_{260\ nm}$: $A_{280\ nm}$ was calculated. Samples exhibiting a ratio of 1.5-2 : 1 were considered adequately pure (Glasel, 1995).

2.5.3. DETERMINING THE INTEGRITY OF ISOLATED RNA

During the isolation of total cellular RNA, the RNA is susceptible to degradation by RNases, which can be of an endogenous or exogenous origin (see precautionary measures above). It is important therefore to estimate the integrity of the isolated RNA before proceeding with further experiments. The integrity of the RNA was estimated by denaturing agarose gel electrophoresis.

2.5.3.1. DENATURING AGAROSE GEL ELECTROPHORESIS

Electrophoresis of intact total cellular RNA on a denaturing agarose gel resulted in the separation of distinct bands of the major RNA species produced by the cells, namely, the 28S, 18S, and 5S ribosomal RNA (rRNA). Denatured RNA appeared as a smear on the gel.

The denaturing agarose gels were prepared as follows. Agarose (50 ml of a 1 % solution) (Low melting point agarose, Sigma, USA) solution was prepared in a 1 x MOPS buffer (pH 5,5 - 7) containing 0,02 M 3 - [N-morpholino] propane sulfonic acid (MOPS), 0,005 M sodium acetate, and 0,001 M ethylenediamine-tetra-acetic acid (EDTA). This solution was microwaved to melt the agarose, and allowed to cool down to below 42°C before

adding 2,7 ml 37 % formaldehyde, (the denaturant). In order to visualize the RNA on the gel 0,5 μg/ml of ethidium bromide was added. Ethidium bromide intercalates with nucleic acids and fluoresces under ultra violet (UV) light. The gel set after 15 - 30 minutes.

Total cellular RNA (10 μg) was dissolved in 15 μl of RNA sample buffer containing 480 μl/ml formamide, 107 μl/ml 10 x MOPS, 173 μl/ml 37 % formaldehyde, 180 μl/ml RNase free water, 53 μl/ml glycerol, and 7 μl/ml of a saturated solution of the dye bromophenol blue. This mixture was denatured by heating the samples to 65°C for 10 minutes before loading the samples onto the gel. The electrode buffer used was a 1 x MOPS buffer containing 0,5 μg/ml ethidium bromide. Electrophoresis was performed at 60 volts (constant current) for 1 - 2 hours. The 28S, 18S, and 5S rRNA bands were then visualized under UV light (UVP, USA).

2.5.4. METHYLENE BLUE STAINING OF NORTHERN BLOTTED RNA

Northern blots were stained with methylene blue in order to determine the efficiency of transfer of RNA during Northern blotting, and to check the integrity of the RNA. Once the nylon was stained with methylene blue the blot was no longer used in the hybridization of specific radiolabelled cDNA probes.

The dried blot was soaked in 5 % acetic acid for 15 minutes before being transfered to stain for 5 - 10 minutes. The stain contained 0,5M sodium acetate (pH 5,2) and 0,04 % methylene blue. The blot was then destained in 70 % ethanol to clear the backround.

The stained RNA was photographed immediatley, as the stain tended to fade over time.

2.6. NORTHERN BLOT ANALYSIS OF IL-1β RNA

2.6.1. NORTHERN BLOTTING

Northern blotting involves the transfer by capillary action of total celluar RNA onto a nylon membrane (Hybond-N, Amersham International, England). The RNA is then on a more suitable matrix for further analysis.

Total cellular RNA (10 μg) was loaded onto a denaturing agarose gel (see section 2.5.3.1.) with or without ethidium bromide. After electrophoresis at 60 volts for 1 - 2 hours, the gel was washed for 15 minutes in 10 x SSC (1,5 M sodium chloride and 0,15 M sodium citrate) in order to wash out the formaldehyde from the gel, and to bring the pH of the gel to pH 7. The gel was then inverted onto a parafilm covered piece of glass. The nylon membrane plus two pieces of Whatman no. 3 filter paper where then layered on to the gel, making sure no bubbles were trapped between the gel and the nylon. Both the nylon and the filter paper was cut to be 1 mm bigger than the gel. Wads of tissue paper and another piece of glass were placed on top of the gel/nylon/filter paper. The glass was weighted down in order to increase the rate of transfer. Thus both capillary action and pressure were used to transfer the RNA to the nylon. The transfer was allowed to proceed for 18 hours, after which the nylon was inverted and soaked in 2 x SSC, and allowed to air dry thoroughly. The RNA was crosslinked to the blot by exposing the blot to short wavelength UV light for 4 minutes. The nylon membrane was then either prepared for hybridization, or it was stained with methylene blue in order to check

the efficiency of transfer, and as another method to detemine the integrity of the RNA.

2.6.2. PREHYBRIDIZATION OF NORTHERN BLOTS

In order to block non-specific sites of hybridization for specific DNA probes, the blots were prehybridized with herring sperm DNA. Approximately 25 ml of prehybridization solution was used to block 3 blots. The prehybridization solution consisted of 5 x SSPE (3 M sodium chloride, 0,2 M sodium phosphate, and 0,02 M EDTA) at pH 7,4, 49 % (v/v) formamide (deionized using amberlite granules), 5,4 % (v/v) of a 100 x Denhardts solution, and 0,8 mg/ml of single stranded herring sperm DNA. The Denhardts solution was filtered before use and contained 20 mg/ml Ficoll, 20 mg/ml polyvinylpyrollidine, 20 mg/ml BSA, and 0,5 % (w/v) sodium dodecyl sulphate (SDS). A stock of herring sperm DNA (10 mg/ml) was prepared by aspirating the DNA through an 18 gauge needle, and sonicating it at 16 - 20Hz. The herring sperm DNA was then denatured to a single stranded form by heating it to 96°C for 3 minutes and then cooling it immediately on ice. The blots were prehybridized overnight in a shaking waterbath at 42°C.

2.6.3. RANDOM PRIMER LABELLING OF cDNA PROBES WITH ^{32}P

Specific cDNA probes are labelled with [α-^{32}P]-dCTP using the method of random primer labelling. The Klenow fragment of DNA polymerase I generates a radiolabelled cDNA probe from a template probe by incorporating [α-^{32}P]dCTP randomly into the newly synthesised probe.

The components of a Promega random primer labelling kit were allowed to thaw on ice. The specific DNA templates were denatured to single strands by heating to 96°C for 3 minutes and then cooling immediately on ice. The reaction mixture, which was then assembled in a separate Eppendorff tube, consisted of the following components: 1 x labelling buffer; unlabelled dTTP, dATP, and dGTP at 20 μM each, 0,83 μg/ml denatured DNA template, 10 mg/ml nuclease-free acetylated BSA, 5 μCi [α-^{32}P]dCTP, and 5 U of Klenow enzyme. The reaction mixture was made up to 50 μl with nuclease free water, and incubated at room temperature for 1 hour. The labelling reaction was stopped by heating the mixture to 96°C for 2 minutes and subsequent chilling on ice.

2.6.3.1. REMOVAL OF UNINCORPORATED LABEL

A Quiqick Nucleotide Removal Kit (Quiagen, Germany) was used to remove unincorporated radioactive (and non - radioactive) nucleotides from the radiolabelled probe. This addtitional step results in cleaner Northern blots. The manufacturer's protocol was followed.

2.6.4. HYBRIDIZATION OF NORTHERN BLOTS

The prehybridization solution was replaced by a hybridization solution containing 5 x SSPE, 50 % (v/v) formamide, 5,4 % (v/v) of 100 x Denhardts solution, 0,4 mg/ml single stranded herring sperm DNA, 4 % (v/v) of polyethylene glycol (PEG), and the denatured ^{32}P-labelled probe (25 ng). This hybridization solution (25 ml) was enough for 4 blots. Hybridization proceeded overnight at 42°C in a shaking waterbath.

2.6.5. STRINGENCY WASHES

The stringency washes function to remove any radiolabelled probe which is weakly bound to the blot, thus removing probe which may not be specifically bound to its corresponding mRNA species. The washing procedure consists of a series of washes, with each successive wash containing a lower concentration of the SSPE buffer and thus having a higher stringency. The washes consisted of:

1. 15 minutes at 42°C in 2 x SSPE plus 0,1 % SDS (repeated).
2. 30 minutes at 42°C in 1 x SSPE plus 0,1 % SDS.
3. 15 minutes at room temperature in 0,1 x SSPE plus 0,1 % SDS.

After each wash the activity of the blot was monitored using a Geiger-Muller counter. The washes would be stopped if the acivity dropped significantly.

2.6.6. AUTORADIOGRAPHY

Once the stringency washes were completed, the blots were placed on filter paper and allowed to dry briefly before wrapping the blots in cling wrap. The blots were then exposed to X-ray film (Fugi, Japan) in a cassette with enhancing screens (Kodak, USA) for 1 to 3 days, depending on the strength of the radioactive signal.

2.6.7. DENSITOMETRIC SCANNING

The bands that appeared on the autoradiograph were compared with each other using scanning densiometry (Biomed Instruments), where the relative heights and areas under a peak of absorbance were measured.

2.6.8. STRIPPING OF NORTHERN BLOTS

Often the same Northern blot would be stripped of its radioactivity and reprobed with a different probe. The nylon membrane was incubated at 65°C in a shaking waterbath with 200 ml of 50 % fromamide and 2 x SSPE. The membrane was rinsed in 0,1 x SSPE. The membrane was then prehybridized as described above.

2.7. REVERSE TRANSCRIPTION - POLYMERASE CHAIN REACTION ANALYSIS OF IL-1RtI, IL-1RtII, AND IL-1ß RNA

Reverse transcription (RT) of total cellular RNA into cDNA is followed by the amplification of specific cDNA species to detectable levels during the polymerase chain reaction (PCR). The RT-PCR technique allows the semi-quantitative analysis of gene expression (the transcription of specific mRNA species) within the cell.

2.7.1. REVERSE TRANSCRIPTION

The synthesis of cDNA from total cellular RNA is achieved by the use of the retroviral enzyme reverse transcriptase. To initiate synthesis, 0,25 μg of oligo dTs were used as primers to bind to the poly A tails of 0,5 μg of RNA. This was achieved by heating them together to 60°C for 5 minutes and then chilling on ice. The rest of the reaction mix was then added to the primed RNA. The mix consisted of 20 U of RNase inhibitor (Amersham International), 1 mM of each of the 4 dNTPs, 1 x RT buffer (Promega) (50 mM Tris-HCl, pH 8,3; 50 mM KCl; 10 mM $MgCl_2$; 10 mM DTT; and 0,5 mM spermidine), 10 U of AMV reverse transcriptase (Promega), and Baxter water (Sabax, R.S.A.) making a final volume of 20 μl. The reaction was allowed to proceed at 37°C for

1 hour and was terminated by heating to 70°C for 5 minutes and cooling immediatly on ice.

2.7.2 THE POLYMERASE CHAIN REACTION

During RT total cellular RNA was converted to cDNA. During PCR a specific cDNA strand is amplified enzymatically to a detectable level by a thermostable DNA polymerase (Saiki, 1988). The DNA polymerase is directed by oligonucleotide primers synthetically synthesized to be complementary to sequences in a specific cDNA copy of an mRNA species. One primer is directed against the 5' strand, whilst the other is directed against the 3' strand, to sequences of a known distance apart. The polymerase would then extend in a 5' to 3' direction using the cDNA as template. This process would result in a fragment of DNA of known length. The amplification is exponential, as each new fragment synthesized would then act as a template for another.

2.7.2.1 PCR PRIMER SELECTION

The primers pairs were selected so as to produce PCR fragments of differing sizes, so that they could be distinguished from one another when electrophoresed on a non-denaturing agarose gel. The sequences for the primer pairs follow. The length of the PCR products formed in base pairs (bp) appear in brackets. Table 1.0 gives the primer sequences. GAP-DH referred to in the table stands for glyceraldehyde-3-phosphate - dehydrogenase.

Table 1.0. Oligonucleotide primer sequences.

mRNA	Product size	Primer sequence
IL-1RtI	119bp	F primer 5'-CTGGACCCCTTGGTAAAAGAC-3' R primer 3'-CCGACTATTTACGTTCCTTGC-5' (Ferrari *et al.* 1993)
IL-1RtII	387bp	F primer 5'-AATGTTGCGCTTGTACGTGT-3' R primer 3'-CAGGTAACTCGAGTCTCAAA-5'
IL-1RtII (alt.)	1211bp	F primer 5'-GGAGCAATGTTGCGCTTGTACG-3' R primer 3'-GTTAGGATAGGGTTCACTTTATTTAC-5' (Akeson *et al.* 1992)
IL-1ß	811bp	F primer 5'-ATGGCAGAAGTACCTAAGCTCGC-3' R primer 3'-TTGACTGAAGTGGTACGTTAAACACA-5' (Larrick *et al.* 1989)
GAP-DH	587bp	F primer 5'-CCCTTCATTGACCTCAACTACATG-3' R primer 3'-GACTTGCCCTTCGAGTGACCGTAC-5'

Note the following abbrieviations in table 1.0. Forward (F), reverse (R), and gylceraldehyde-3-phosphate - dehydrogenase (GAP-DH).

2.7.2.2. THE PCR CYCLING PROGRAMME

The PCR reaction mixture contained 10 μl of cDNA from the RT reaction which supplied 0,2 mM of each dNTP and 2 mM $MgCl_2$ to the PCR mix (the final volume was 50 μl). In addition to this the PCR mix contained a 1 x $MgCl_2$-free Thermophilic buffer (Promega) (50 mM KCl; 10 mM Tris-HCl, pH 9,0; and 0,1 % Triton X-100), primer pairs at concentration ranging from 0,01 μM to 0,25 μM, and Baxter water. The DNA within this mixture was denatured at 96°C for 5 minutes and cooled on ice before 0,5 - 1,0 U of thermostable DNA polymerase (Dynazyme, Finnzymes, Finland) was added. To prevent evaporation 50 μl of nuclease-free mineral oil (Sigma, USA) was layered on top of the PCR mixture.

The reaction mix was then heated to an intermediate 72°C for 2 minutes, before the temperature was cycled between the denaturing temperature of 93°C for 30 seconds, the primer annealing temperature of 51-57°C for 30 seconds, and the DNA polymerase extension temperature of 70°C for 1 minute. A total of 30 - 40 cycles were completed (using a Hybaid Omnigene PCR machine) followed by a final extention at 72°C for 5 minutes.

2.7.2.3. VISUALIZATION OF PCR PRODUCTS

The PCR products were then analysed on a non-denaturing agarose gel for 1 - 2 hours at 60 volts. The 2 - 3 % agarose gel was made up with a 1 x TAE buffer (pH 7,2) containing 25 mM Tris base, 20 mM sodium acetate, and 1 mM EDTA. Ethidium bromide (1 μg/ml) was added to the gel to allow visualization of the PCR products under UV light. The samples were diluted with DNA loading buffer containing 1 x TAE,

50 % glycerol, and bromophenol blue. The tank buffer was 1 x TAE which also containied 1 μg/ml ethidium bromide.

The resulting bands of cDNA representing either IL-1ß, IL-1RtI, IL-1RtII, or GAP-DH, made visible under UV light were photographed, and the negatives were scanned using a densitometer (see section 2.7.8.).

2.7.2.4. ENDONUCLEASE RESTRICTION OF PCR PRODUCTS

In order to confirm that the PCR products generated are indeed the correct products, specific restriction sites for endonucleases were identified in their sequences. Thus the sizes of the resultant restriction fragments were compared to the theoretical values calculated. The PCR products from primers previously used in the laborotory were not restricted.

The alternate IL-1RtII PCR product (1211 bp) was digested with Eco RI (GAATTC), which cleaves at position 475, generating fragments of 475 and 736 base pairs. The PCR products were incubated at 37°C for 1 hour 30 minutes with 20 U of restriction endonuclease.

2.7.2.5. SEMI-QUANTIFICATION USING RT-PCR

It is possible using RT-PCR to analyse (semi-quantitatively) the expression of specific genes after the addition of various stimuli to the cells. This is achieved by adding into the reaction mixture an internal standard in the form of a set of primers directed against the cDNA of a constituitivley expressed gene whose expression is not affected by the agents used to stimulate the cells. These "standard" primers are added in addition to the

primers for the gene whose expression is being analysed. Thus changes in the concentration of the PCR product of interest, relative to the standard, can be observed. The expression of the GAP-DH gene was used as the standard in this study. GAP-DH is a house keeping gene which is a good standard to use for the study of gene expression, as GAP-DH mRNA levels do not vary to a great extent during cell growth (Dukas *et al.* 1993). By adding a standard to the same reaction mix, there are no tube-to-tube variations as the both reactions are subjected to exactly the same conditions. Variation in the amount of internal standard amplified between tubes is therefore a indication of the unequal addition of RNA to the RT.

2.8. RADIOIMMUMOASSAY (RIA) OF IL-1ß PROTEIN

The concentrations of IL-1ß secreted into the cell supernantants were measured by RIA. The medium in which the cells were maintained was harvested and tested for IL-1ß using an IL-1ß [^{125}I] assay system (Amersham, England). This assay relies on the competition between the IL-1ß in the supernatants of the cells and a fixed amount of [^{125}I] IL-1ß for the binding of an IL-1ß specific antibody. Thus low binding of [^{125}I] IL-1ß indicates a high concentration of IL-1ß secreted from the cells.

A standard curve was constructed using known concentrations of IL-1ß ranging from 0,0775 fmol/tube to 10 fmol/tube. A standard containing no IL-1ß was added, as well as a tube containing no anti-IL-1ß antiserum to determine non-specific binding of IL-1ß to the tube. Supernatants were diluted so that their IL-1ß concentrations would fall on the standard curve.

Unknown sample (25 µl) was incubated with 25 µl of antiserum (directed against IL-1ß) for 4 hours at room temperature. [^{125}I] IL-1ß (25 µl) was added, vortexed and incubated for 24 hours at room temperature. The [^{125}I] IL-1ß - antiserum complex was removed from the reaction mixture by the addition of 62,5 µl of an Amerlex-M secondary antibody reagent for 10 minutes at room temperature. This reagent contained a secondary antibody which was magnetically labelled, allowing the easy removal of the magnetized complex using a magnet after centrifugation. The levels of radioactivity in each tube were then measured using a Gamma-scintillation counter.

2.9. THE ISOLATION AND ANALYSIS OF IL-1RtI AND IL-1RtII PROTEIN

2.9.1. ISOLATION OF TOTAL CELLULAR PROTEIN

Between 4 x 10^6 and 9 x 10^6 cells were washed in saline to remove contaminating proteins from the FCS supplemented RPMI medium in which the cells were maintained. The cells were lysed by resuspending the pellet of cells in 200 - 300 µl lysing buffer (pH 7,2) which contained 50 mM Tris, 5 mM EDTA, 5 mM ethylene bis (oxyethylenenitrilo) tetra-acetic acid (EGTA), and 0,1 % Triton X-100 (a non-ionic detergent). Just before the lyzing buffer was used, 2 mM of the protease inhibitor phenylmethylsulphonyl fluoride (PMSF) was added. PMSF, which denatures quickly at room temperature, was stored in 100 mM aliquots in isopropanol at -20°C. One part of 100 mM PMSF was diluted with 49 parts of lyzing buffer just before use.

The cells were incubated for 30 minutes at 4°C in the above lysing buffer, and then homogenized by drawing the cells 25 - 50 x through a 21 gauge needle. The efficiency of lysis was checked by staining the cell with 0,1 % trypan blue, a dye which is excluded from living cells. The proteins were separated from other cellular debris by centrifugation at 10 000 rpm (in an Heraeus microfuge) for 10 minutes at 4°C. The supernantant, containing the proteins was then placed in a fresh Eppendorff tube and frozen at -70°C until required.

2.9.2. PROTEIN QUANTIFICATION

The total protein concentration in each sample was determined using the method by Bradford (Bradford 1976), using BSA as a standard.

A BSA standard curve was constucted by measuring the absorbance at 595 nm (Beckman spectrophotometer) of standard solutions of BSA, ranging from 0,25 μg/μl to 0,0158 μg/μl, which had reacted to Bradford reagent for 10 minutes (100 μl of standard plus 1 ml of Bradford reagent prepared by myself). The Bradford reagent contained 0,01 % (w/v) Coomassie brilliant blue G250, 5 % (v/v) absolute alcohol, and 8,5 % (w/v) phosphoric acid. The reagent was filtered using Whatman No. 1 filter paper and stored in the dark at 4°C. Generally, the protein samples had to be diluted 50 - 100 x in order for their concentrations to fall on the standard curve. As for the BSA standard curve, the protein samples were reacted with Bradford reagent, and the resultant absorbance was measured at 595 nm.

2.9.3. PROTEIN SEPARATION BY DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

Protein samples were separated by electrophoresis on denaturing polyacrylamide gels. The gels were made denaturing by the addition of 0,1 % (w/v) sodium dodecyl sulphate (SDS). The gel was assembled so that the protein samples first ran through a 4 % stacking gel to improve the resolution of the proteins when they next ran through the 10 % resolving gel.

A 30 % (v/v) stock acrylamide / methylene bis-acrylamide solution was prepared which contained 29,1 % (w/v) acrylamide and 0,9 % (w/v) methylene bis-acrylamide, and was stored in the dark at 4°C for up to 4 weeks.

The 10 % resolving gel was prepared first and poured into the PAGE casting unit (Hoefer, San Fransisco, USA), followed by the 4 % stacking gel. The resolving gel contained 10 % acrylamide / bis-acrylamide, and 1 x SDS resolving gel buffer (1,5 M Tris pH 8,8 and 0,1 % (w/v) SDS). Polymerization was initiated by the addition of 0,033 % (v/v) tetramethylethylenediamine (TEMED), and 0,1 % (w/v) fresh ammonium persulphate.

The stacking gel contained 4 % acrylamide / bis-acrylamide, 1 x SDS stacking gel buffer (0,5 M Tris pH 6,8 and 0,1 % (w/v) SDS), 0,033 % (v/v) TEMED, and 0,1 % (w/v) fresh ammonium persulphate.

Both gel solutions were degassed for 5 minutes before the addition of the TEMED and the ammonium persulphate, as oxygen inhibits polymerization.

The protein samples were diluted in a Tris buffer (50 mM Tris pH 7,4). Between 50 and 100 μg of protein was loaded per lane. The samples were loaded onto the gel in a 1 x protein loading buffer (0,125 M Tris-HCl pH 6,8; 2 % (w/v) SDS; 5 % (v/v) 2-mercaptoethanol; 11,6 % (v/v) glycerol (using an 87 % glycerol solution); and 0,01 % bromophenol blue). The proteins were electrophoresed at a constant current of 10 mA per gel for stacking, and then at 15 mA per gel in the resolving gel. The PAGE electrode buffer contained 0,025 M Tris pH 8,3, 0,192 M glycine, and 0,1 % (w/v) SDS.

2.10. ANALYSIS OF IL-1RtI PROTEIN BY WESTERN BLOTTING

2.10.1. WESTERN BLOTTING

After the protein samples were separated, they were transfered to a nitrocellulose membrane (Hybond-C extra, Amersham, England) by a process known as Western blotting. Here the proteins were transblotted onto the nitrocellulose electrophoretically. The negaively charged proteins were attracted to the positive electrode, and thus moved from the gel onto the membrane, as the membrane was placed between the gel and the positive electrode. The transblotting system (Hoefer, San Fransisco, USA) consisted of a container (with a positive and negative electrode) into which 4 supporting cassettes could be placed. Each cassette contained a piece of Whatman No.3 paper, a nitocellulose membrane, the gel, and another piece of Whatman No. 3 paper.

The transblot buffer contained 25 mM Tris, 0,192 M glycine, 20 % (v/v) methanol, and 0,1 % (w/v) SDS. The proteins were transblotted for 3 hours at a constant current of 200 mA. The blots were allowed to air dry before proceeding with the immunodetection of the IL-1RtI protein.

2.10.2. THE IMMUNODETECTION OF IL-1RtI PROTEIN

Non-specific binding sites on the membrane were blocked using 3 % BSA in 1 x Tris buffered saline pH 7,5 (TBS) (20 mM Tris and 0,5 M NaCl). The membranes were blocked by gently agitating for 1 hour at room temperature in the blocking solution. The membrane was then washed gently in 1 x TBS for 15 minutes.

The primary antibody (diluted 1000 x) was then added to the membrane in 5 ml of antibody buffer (1 % (w/v) BSA in 1 x TBS buffer containing 0,05 % Tween-20 (TTBS). The primary antibody was a rabbit anti-IL-1RtI polyclonal antibody (gift of C.A. Dinarello). The membrane was incubated with the primary antibody overnight (gently agitating) at 4°C. The membrane was then washed in 1 x TTBS for 15 minutes.

The membrane was then incubated with a 1:200 diluted biotinylated secondary antibody (anti-rabbit Ig, from donkey, Amersham, England) in 5 ml of antibody buffer for 1 hour at room temperature. Unbound secondary antibody was washed off, after the incubation period, with 1 x TTBS for 15 minutes.

The bound secondary antibody was detected by horseradish peroxidase labelled streptavidin (Amersham, England) as streptavidin binds strongly to the biotin bound to

the secondary antibody. A 1:200 dilution of the streptavidin- peroxidase was made in the antibody buffer. The membrane was incubated for 1 hour at room temperature. Unbound Streptavidin-peroxidase was washed off in 1 x TTBS for 15 minutes.

The substrate for horseradish peroxidase (4 chloro-1-naphthol) was then added in order to stain for peroxidase activity (purple bands) , which would then indicate the presence of IL-1RtI protein bound to the membrane. The substrate was added as part of a colour development solution which contained 0,05 % (w/v) 4 chloro-1-naphthol, 16,7 % (v/v) absolute alcohol, and 83,3 % (v/v) 1 x TBS. The chloro-1-naphthol was first dissolved in the ethanol which was cooled to -20°C. Immediately prior to use 0,07 % hydrogen peroxide was added to the colour development solution. The membrane was immersed in this solution and gently agitated until bands appeared (5 - 30 minutes). The membrane was then washed with water to stop the colour reaction. The bands were then scanned by a densiometric scanner as previously described (section 2.6.8).

2.11. ANALYSIS OF IL-1RtI and IL-1RtII PROTEIN BY IMMUNOPRECIPITATION

2.11.1. IMMUNOPRECIPITATION

Immunoprecipitation of a specific protein involves the detection of that protein using a specific antibody. The protein - primary antibody is precipitated by a secondary antibody (unbiotinylated) bound to protein G (Sigma, USA). Protein G are group C streptococcus cells in suspention.

The secondary antibody was complexed with protein G in the following way. An aliquot of protein G was washed 3 x in saline. The protein G (150 μl) was incubated with 100 μg of secondary antibody at room temperature for 2 hours. The complex was washed 3 x in 1 x PBS, and resusupended in 250 μl of 1 x PBS.

The secondary antibody - protein G bound nonspecifically to certain proteins. The protein sample was therefore incubated (for 1 hour at 37°C) with the complex before starting on the primary antibody step. The secondary antibody - protein G complex was then spun down in a microfuge (Eppendorff) (2500 rpm for 15 minutes at 4°C), and the proteins remaining in the supernatant were used for the immunoprecipitation.

Isolated protein (75 - 150 μg) was incubated with 1 μg of primary antybody for 1 hour at room temperature. The secondary antibody - protein G complex was added, and the mixture was incubated for another 2 hours at room temperature. The protein G, bound via antibodies to the target protein, was removed from solution by centrifugation at 2500 rpm for 15 minutes at 4°C in a microfuge, and washed 3 x in 1 x PBS. The pellet was resuspended in 40 μl of 1 x denaturing protein sample buffer (see 2.10.3.). The samples were boiled for 3 minutes. This process resulted in the dissassociation of the protein G. The protein G was removed from the solution by centrifugation in the microfuge. The sample of protein containing the target protein and antibodies was then separated on a 10 % SDS PAGE gel.

2.11.2. COOMASIE BLUE STAINING

PAGE gels were stained with Coomasie Blue in order to visualise the protein bands. The gels were stained for 1 to 24 hours. The Coomasie blue stain consisted of the following: 10 % acetic acid; 30 % methanol; and 0,1 % Coomasie blue (R 250). The gels were destained with a solution of 45 % methanol and 10 acetic acid until the blue background stain cleared.

2.11.3. SILVER STAINING

PAGE gels were stained with a silver stain in order to detect faint protein bands. The gel was washed thoroughly in distilled water to remove any acetic acid. The gel was incubated in an oxidising solution (3,2 mM nitric acid and 3,4 mM potassium dichromate) for 5 minutes, and then washed twice for 5 minutes in distilled water. The gel was incubated for 30 minutes in 0,2 % (w/v) silver nitrate, rinsed for 1 minute in distilled water, and then immersed in developer (0,28 M sodium carbonate and 0,037 % formaldehyde) for 30 seconds. The development was terminated, when the protein bands could be visualised, with 5 % (v/v) acetic acid. The gel was then washed in distilled water.

SECTION 3

RESULTS

The aim of this project was to investigate the effects of PMA, IL-4, dexamethasone, and IL-1ß, on the expression of IL-1ß, IL-1RtI, and IL-1RtII in normal monocytes and on the leukaemic cell lines, HL-60 and THP-1. This was done with the view of possibly using HL-60 and THP-1 cells as monocyte models for the analysis of the effects of anti-inflammatory agents on IL-1Rs.

The results obtained from this study are summarised below. The effects of IL-1α, PMA, IL-4, dexamethasone and IL-1ß on IL-1ß mRNA levels were analysed in PBMNCs and adhered monocytes. IL-1ß protein levels were analysed in order to determine if IL-4 inhibited the induction of IL-1ß by IL-1α.

As no data could be generated from the IL-1RtI and II Northern blots, IL-1RtI and II mRNA levels were measured using RT-PCR. The effects of PMA, IL-4, dexamethasone, and IL-1ß were tested. The effect of the stimuli on IL-1ß mRNA levels were also analysed, in order that the techniques of RT-PCR and Northern blotting could be compared.

The RT-PCR data generated for IL-1RtI was inconclusive. It was possible that the stimuli used did not affect IL-1 receptor expression at the mRNA level.

Therefore the regulation of IL-1RtI by PMA, IL-4, dexamethasone, and IL-1ß was analysed at the protein level. When producing an adherent fraction of monocytes, the yield of cells was low (25 % of the PBMNC cells remain). As a result, the isolated protein samples from adherent monocytes contained low concentrations of protein, making it difficult to detect IL-1RtI by conventional Western blotting and immunodetection. Therefore, IL-1RtI was immunoprecipitated from the protein samples. The advantage of immunoprecipitation was that it concentrated the protein, which was then Western blotted and IL-1RtI was immunodetected. A Western blot of immunoprecipitated IL-1RtI generated intense IL-1RtI protein bands on the immunoblot, which made scanning and quantification of the bands easier.

The IL-1RtII monoclonal antibody was not suitable for Western blotting, as it bound weakly to the blot and was removed during the washes in between incubation with the primary and secondary antibodies during immunodetection. No IL-1RTII protein could be detected using this antibody for Western blotting and immunodetection. The antibody was, however suitable, for the immunoprecipitation of IL-1RtII. IL-1RtII was therefore immunoprecipitated from adhered monocytes and HL-60 cells. Using Coomasie blue staining, no IL-1RtII protein could be detected. Silver staining, a more sensitive technique, revealed a ± 63 kDa protein band. Since IL-1RtII has been reported to be a 60 - 68 kDa protein, this ± 63 kDa protein was possibly IL-1RtII. In order to confirm that the ± 63 kDa protein was in fact

IL-1RtII, the immunoprecipitated protein was Western blotted and it was attempted to detect the IL-1RtII on the blot using the conventional immunodetection technique. It was thought that the IL-1RtII antibody would be able to detect IL-1RtII protein, if that protein was present on the blot in a high enough concentration. Unfortunately the ± 63 kDa protein band could not be detected on the blot. Either it was not IL-1RtII protein, or the antibody failed to remain bound to the IL-1RtII on the blot.

3.1. LYMPHOCYTE ACTIVATING FACTOR ASSAY

A LAF assay was performed to test for IL-1ß biological activity. Mouse thymocytes were isolated and treated with submitogenic concentrations (1 µg/ml) of Con A. Con A at these concentrations acts synergistically with IL-1ß (10 ng/ml to 156 pg/ml) to induce proliferation in the immature thymocytes (Gery *et al.* 1972). Proliferation was measured by the amount of [^{3}H]-thymidine incorporated into the DNA.

Maximal proliferation was noted at 72 hours after IL-1ß and Con A co-stimulation. The highest concentration of IL-1ß (10 ng/ml) induced maximal proliferation of the thymocytes, indicating that the IL-1ß used was biologically active (see figure 3.1.).

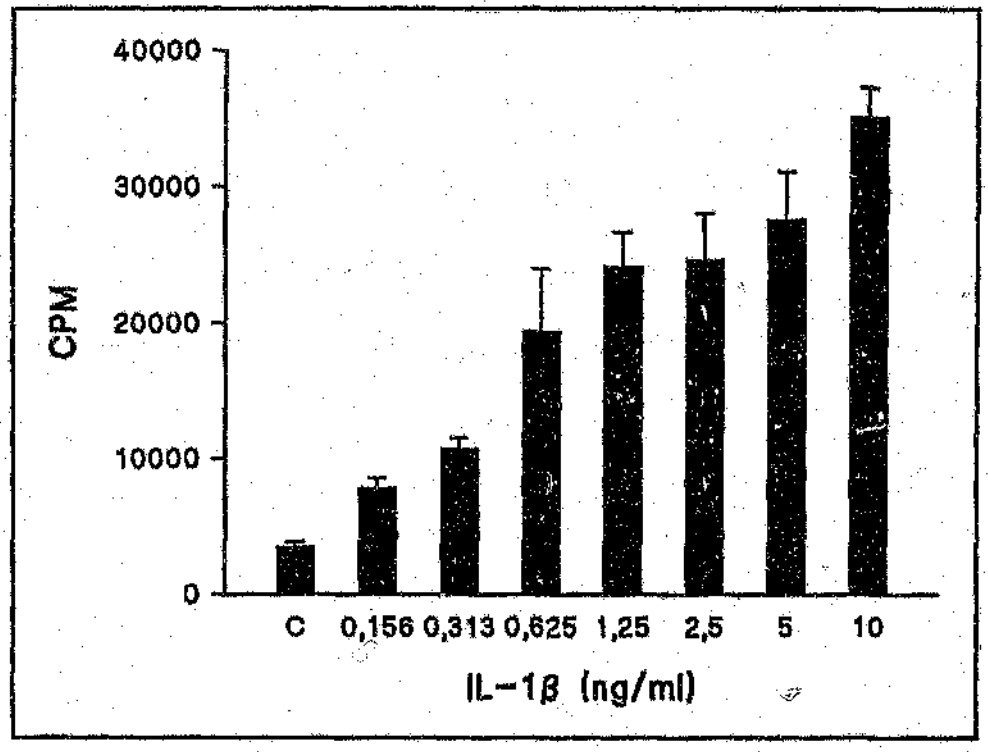


Figure 3.1. Determining the biological activity of IL-1ß using a LAF assay.
The incorporation of [^{3}H]-thymidine was measured after 72 hours of stimulation with Con A (1 µg/ml) and various concentrations of IL-1ß (n=3; mean ± SEM).

3.2. CD14 AND CD11b SURFACE ANTIGEN EXPRESSION

CD14 and CD11b surface antigen expression was determined by flow cytometry. The CD14 surface antigen is the receptor for the complex of LPS and LPS binding protein (LBP). It is expressed on myelomonocytic cells, and not on lymphoid cells. Thus determining the percentage CD14 positive cells in a mixed population of cells such as PBMNCs, gives an indication of the number of monocytes in the population. It is important to define what cell types make up a population of cells, as the receptors for interleukin-1 are expressed on cells other than monocytes, for example, on T cells, B cells, and neutrophils. After separating neutrophils from the other leukocytes on a Ficoll gradient, a mixed population of PBMNCs was obtained. Of this latter population 18,2 % (n=1) was CD14 positive. As the majority of monocytes express CD14 and as other cells (eg. neutrophils) also express CD14 one can therefore only say that approximately 18,2 % of the cells were monocytes.

Flow cytometry revealed two groups of cells within the mixed PBMNC population. The larger group were small cells possibly representing lymphocytes. These small cells were only 1 % CD14 positive. The other group consisted of fewer cells, but these cells were larger and were 73,6 % CD14 positive (n=1). Thus these larger cells may represent the monocytes in the mixed population.

A monocyte enriched fraction of cells was made by adhering PBMNCs to plastic petri dishes for 2 hours and aspirating off the non-adherent fraction

(rich in lymphocytes). The percentage CD14 positive cells in the adherent fraction consisted of two groups of cells. The one subset consisted of larger more granulocytic cells, as determined by forward and side scatter. Of these cells 82,8 % - 90,0 % were CD14 positive (n=3), indicating the presence of monocytic cells. The other group of cells were smaller and were mainly CD14 negative.

Percoll density gradient centrifugation (see 2.1.4.) of PBMNCs resulted in the formation of a monocyte rich fraction of cells. Although the recovery of cells after Percoll fractionation was low (9,1 % of the PBMNCs), the cells recovered were 75,5 % CD14 positive (n=1), indicating that the fraction of cells were mostly monocytes. Unfortunately the low cell recovery after Percoll fractionation made it impossible to collect enough cells in order to conduct experiments. Thus experiments were conducted on monocyte fractions enriched by the method of adherence (see 2.1.3.).

In addition to analysing the expression of CD14 on the cells, the expression of the myeloid antigen CD11b (Mo1) was also determined. A mixed population of neutrophil depleted PBMNCs (see 2.1.2.) consisted of 15,8 % CD11b positive cells (n=1).

The increased expression of CD11b on the promyelocytic leukaemic cell line, HL-60, is a model for analysing the differentiation of that cell line. Phorbol esters like PMA induce the differentiation of HL-60 cells towards

macrophages, including the induction of adherence to plastic (Collins 1987). Despite this, in the present study HL-60 cells expressed low CD11b before (0,5 %, n=1) and after (0,3 %, n=1) stimulation with the PMA for 24 hours. After 24 hours of PMA stimulation, the HL-60 cells only partially adhered to the petri dish, indicating that they had not yet sufficiently differentiated towards a more macrophage - like state. This lack of differentiation could explain the low CD11b expression. A longer exposure to PMA was required to induce differentiation (and hence an increase in adherence) in HL-60 cells. PMA stimulation for 48 hours induced adherence in these cells.

The acute myelogenous leukaemic cell line, THP-1 (Tsuchiya *et al.* 1980), expressed higher CD11b levels than HL-60 cells (8,8 - 11,6 %, n=2). PMA induced adherence of the THP-1 cells to the plastic petri dishes after 24 hours. PMA (10 nM) stimulation for 24 hours resulted in a slight increase in this expression (6,1 - 16,3 %, n=2). This lack of response to PMA by HL-60 cells raised the possibility that the PMA was not functional. This seemed unlikely as it was shown that PMA (10 nM, 24 hours) induced an increase in the expression of IL-1RtI in HL-60 cells.

3.3. ANALYSIS OF RNA INTEGRITY

During the isolation of total cellular RNA, the RNA is exposed to both endogenous and exogenous RNases, which will degrade the RNA. Although the experiments were performed under conditions that inhibit RNases (eg. the guanidium inhibits RNases), it was still important to check for RNA integrity. Here, the RNA was separated according to size by denaturing agarose gel electrophoresis. The gels contained the intercalating agent ethidium bromide, with fluoresces under UV light. The major bands visible on the gel after separation were the 28S rRNA, 18S rRNA, and 5S rRNA, as these species are the most numerous. When the RNA was intact, distinct 28S, 18S, and 5S bands were seen, with the 28S band appearing twice as intense as the 18S band (see figure 3.2.). Degraded RNA appeared as a smear on the gel. Partial degradation of the RNA sample resulted in a diminished 28S rRNA band.

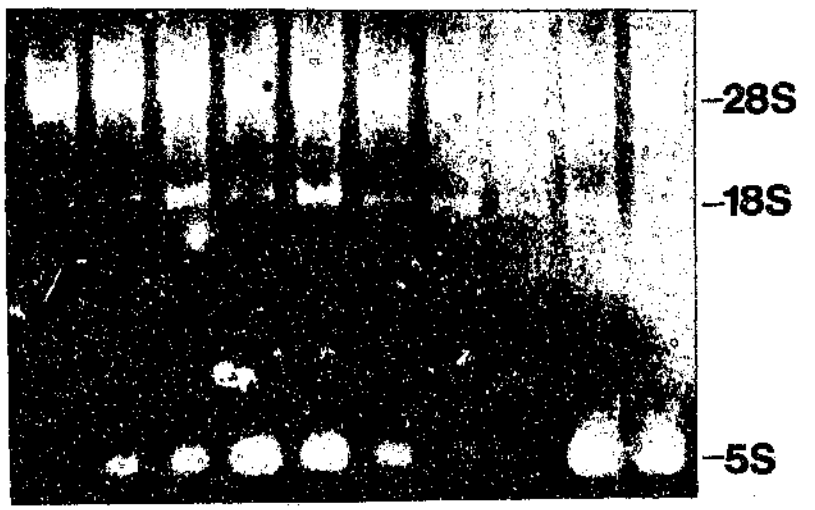


Figure 3.2. An RNA integrity gel.
Total cellular RNA was electrophoretically separated on a 1 % denaturing agarose gel. The labels 28S, 18S, and 5S represent the 3 major rRNA bands of intact RNA.

3.4. METHYLENE BLUE OF NORTHERN BLOTS

The Northern blots, consisting of a nylon membrane, were stained with methylene blue to check the efficiency of transfer of RNA during blotting. In addition the integrity of the RNA and equal loading of the blot could be verified (see figure 3.3.) (see section 2.5.4.).

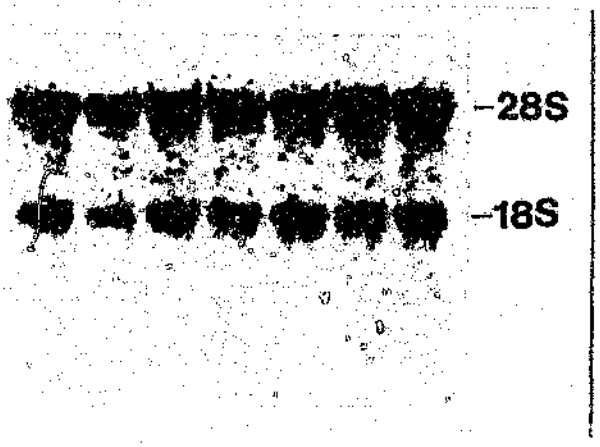


Figure 3.3. Methylene blue staining of total cellular RNA on a northern blot.

3.5. NORTHERN BLOT ANALYSIS OF IL-1ß mRNA EXPRESSION

The aim here was to analyse the expression of IL-1ß mRNA in PBMNCs, to determine if the cells responded to the IL-1 used in the experiments. The following questions were asked. Is IL-1α or IL-1ß a better inducer of IL-1ß mRNA expression, and what effect does IL-4 and dexamethasone have on the expression of IL-1ß at the mRNA level?

Total cellular RNA was isolated from various cells (see 2.5.1.) and 0,5 - 10 μg RNA was separated on a 1% denaturing agarose gel and Northern blotted. IL-1ß mRNA was detected using a radiolabelled cDNA probe (see 2.6.).

Unstimulated PBMNCs expressed undetectable amounts of IL-1ß (see figure 3.4.a.). IL-1α and IL-1ß stimulate the expression of IL-1ß RNA after 1 hour 15 minutes, 2 hours, and 5 hours 30 minutes in PBMNCs. IL-1ß (10 ng/ml) induced more IL-1ß expression than IL-1α (10 ng/ml) (see figure 3.4.b. for densitometric scanning data). Equal loading of the agarose gel with the RNA samples was ensured by determining the concentration of the RNA spectrophotometricaly. As the house-keeping gene GAP-DH was not probed for on this blot, the results can only be used as an indication of the effects IL-1α and IL-1ß have on IL-1ß production. Two rows of bands can be seen on the blot in figure 3.4. The presence of an additional band and trailing of the bands may indicate that the RNA was partially degraded. The IL-1ß probe could have bound nonspecifically to the RNA on the blot, although this seems unlikely as only two distinct bands were detected.

From the information in figure 3.4. one cannot ascertain whether the increase in IL-1ß mRNA due to IL-1α and IL-1ß stimulation is due to an increase in the transcription of the IL-1ß gene, or due to an increase in the half-life of IL-1ß mRNA species (see discussion, section 4.3.).

It is difficult to interpret the results in figure 3.4. for the following reasons: (1) no internal standard was probed for (eg. GAP-DH); (2) densitometric scanning of large diffuse bands was difficult; (3) the RNA appears partially degraded in the autoradiograph (although blots were stained with methylene blue to detect degraded RNA); and (4) the experiment was only performed once.

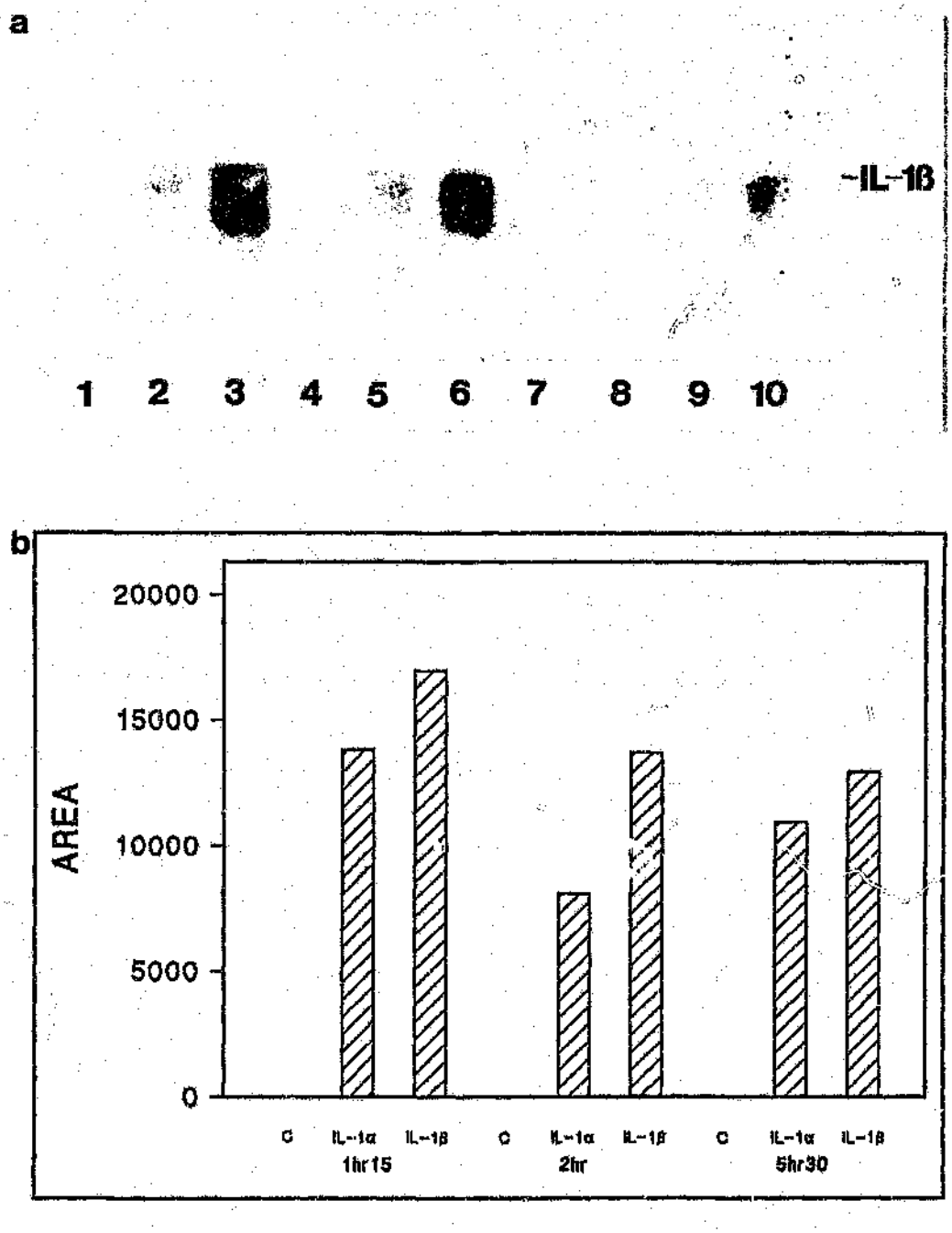


Figure 3.4. Northern blot analysis of IL-1ß mRNA expression in PBMNCs.

a. Autoradiograph of an IL-1ß Northern blot. Lanes 1-3, 4-6, 7, and 8-10 represent IL-1ß mRNA after 1 hour 15 minutes, 2 hours, 3 hours, and 5 hours 30 minutes respectively (n=1). Stimuli: lanes 1, 4, 8: control; lanes 2, 5, 7, 9: IL-1α (10 ng/ml); lanes 3, 6, 10: IL-1ß (10 ng/ml)
b.Densitometric scanning data of the bands appearing in figure a. The relative absorbance of each band has been converted to an area (in arbitrary units).

In the following experiment, adherence enriched peripheral blood monocyte fractions expressed IL-1ß in control cells (unstimulated cells) after 4 hours incubation at 37°C. These cells may have been mechanically stimulated or stimulated during the isolation process by LPS (possibly due to contamination of the anticoagulant heparin with LPS). Adherence to the plastic petri dish induces the expression of IL-1ß mRNA, but not the translation of this message (Auron and Webb 1994). A 4 hour exposure to PMA did not alter the expression of IL-1ß. Dexamethasone alone and IL-4 alone appeared to decrease the amount of IL-1ß mRNA, while IL-1ß appeared to stimulate its own expression. (see figure 3.5.). Large inter-personal differences in the expression of IL-1ß mRNA were observed. Donor 3 (lanes 11 -15 in figure 3.5.) appeared to have a generally lower IL-1ß mRNA expression than the other two donors (0,5 μg mRNA was loaded onto the gel per donor).

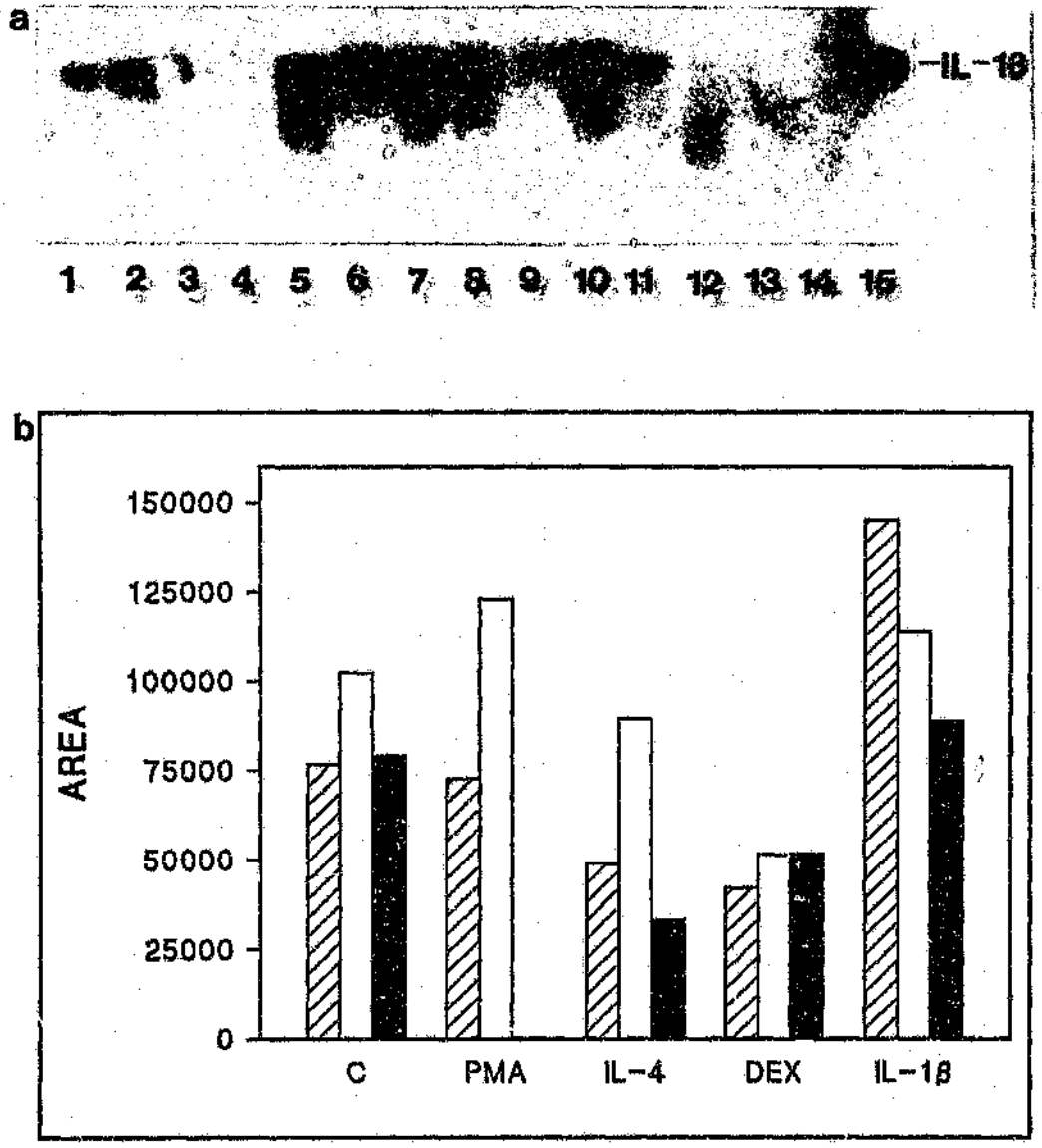


Figure 3.5. Northern blot analysis of IL-1ß mRNA expression in adhered monocytes.
a. Autoradiograph of IL-1ß northern blot (n=3). Adhered monocytes were stimulated for 4 hours with the following stimuli: lanes 1, 6, 11: unstimulated; lanes 2, 7, 12: PMA (10 nM); lanes 3, 8, 13: IL-4 (10 ng/ml); lanes 4, 9, 14: dexamethasone (100 nM); lanes 5, 10, 15: IL-1ß (100 ng/ml). Lanes 1-5, 6-10, and 11-15 represent the IL-1ß mRNA expressed in the adhered monocytes from donors 1 to 3 respectively.
b. Densitometric scanning analysis of the autoradiograph in a. The relative absorbance of each band was converted to an area (in arbitrary units). Donor 1: hatched bars; donor 2: clear bars; and donor 3; solid bars.

IL-4 inhibits the expression of IL-1ß in monocytes when that expression is stimulated by LPS (Estrov *et al.* 1993b and Banchereau et al. 1994). In adhered monocytes (see figure 3.6.), it was found that unstimulated cells produced low levels of IL-1ß mRNA (confirmed in figure 3.4.). IL-1ß (10 ng/ml) and LPS (1 μg/ml) induced IL-1ß mRNA expression. IL-4 (10 ng/ml) inhibited the stimulation of IL-1ß by IL-1ß and by LPS, although this inhibition did not reduce the IL-1ß mRNA to the amounts found in unstimulated cells (n=2) (see figure 3.5.).

In figure 3.6.a., the IL-1ß bands detected on the Northern blot appear to have different sizes, indicated by different mobilities in the gel. It is possible that the amount of IL-1ß mRNA in each lane differed to such a great extent (due to LPS stimulation for example), that the mobility of the IL-1ß mRNA occurring at high concentrations was retarded in the gel. It would have been useful to check the equal loading of the total cellular RNA onto the gels, by probing for the GAP-DH housekeeping gene on the same blot. Unfortunately this experiment was not successful in this regard (and for figure 3.5.).

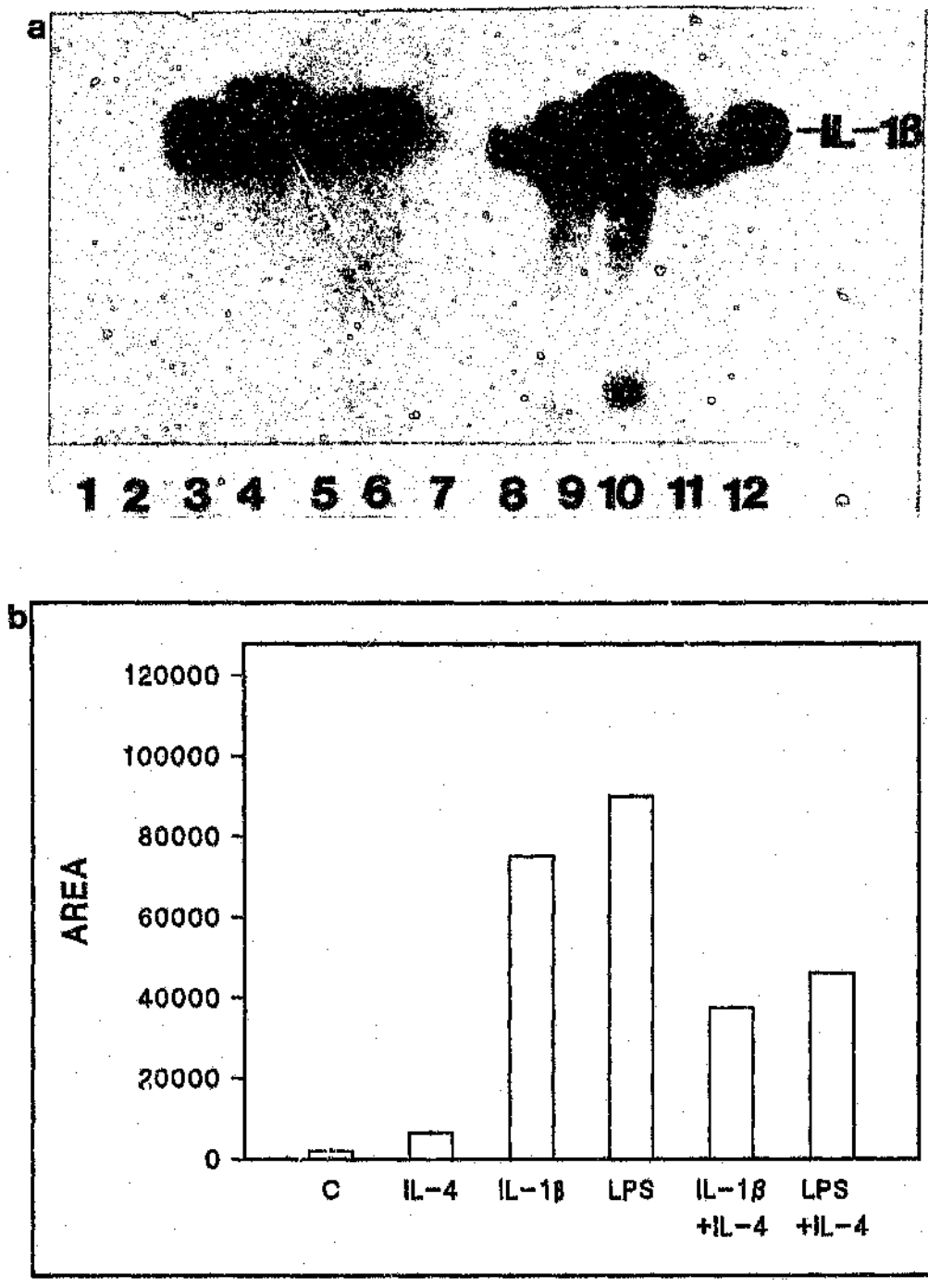


Figure 3.6. Northern blot analysis of the effects of IL-4 on the expression of IL-1ß mRNA in adhered monocytes.

a. Autoradiograph of IL-1ß northern blot. Adhered monocytes were stimulated for 2 hours. Stimuli: lanes 1, 7: unstimulated; lanes 2, 8: IL-4 (10 ng/ml); lanes 3, 9: IL-1ß (10 ng/ml); lanes 4, 10: LPS (1 µg/ml); lanes 5, 11: IL-1ß and IL-4; lanes 6, 12: LPS and IL-4. Lanes 1-6 and 7-1 represent the results obtained from 2 donors.

b. Densitometric scanning analysis of the autoradiograph in a. The relative absorbance of each band was converted into an area (in arbitrary units). The mean of the two sets of data is represented.

3.6. NORTHERN BLOT ANALYSIS OF IL-1RtII mRNA EXPRESSION

Dexamethasone increases the expression of IL-1RtII in neutrophils (Shieh *et al.* 1993 and Colotta and Mantovani 1994b). IL-1RtI and IL-1RtII expression could not be detected by the method of northern blotting in Percoll enriched monocyte fractions of cells (see 2.1.4.) (data not shown). Possible IL-1RtII mRNA (approximately 1200 bp) was detected in THP-1 cells but not in HL-60 cells (see figure 3.7.). It was not clear if this was a specific band for IL-1RtII as the radiolabelled oligonucleotide probe (IL-1RtII PCR product) bound nonspecifically to the blot, especially to the 28S rRNA and 18S rRNA bands, as was determined by the staining of the blot with methylene blue (data not shown). It would have been useful to increase the stringency of the washes in order to decrease the non-specific binding of the probe to the 28S and 18S rRNA bands.

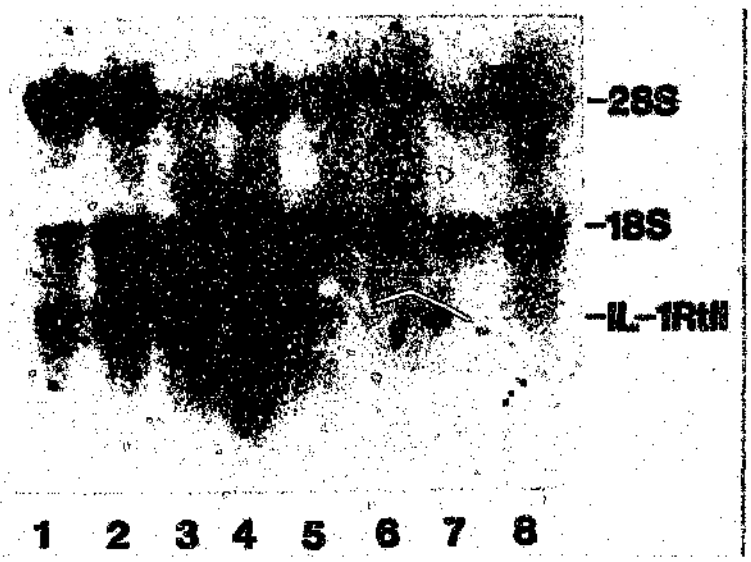


Figure 3.7. The Northern blot analysis of IL-1RtII mRNA expression in THP-1 cells and HL60 cells.
An autoradiograph of IL-1RtII mRNA expression (one of two experiments). Lanes 1-4 represent THP-1 cells and lanes 5-8 represent HL60 cells. Lanes 1 and 5 represent the expression of IL-1RtII mRNA from untreated cells and lanes 2 and 6 from dexamethasone (100 nM) treated cells incubated at 37°C for 4 hours. Lanes 3 and 7 represent mRNA from untreated cells and lanes 4 and 8 from dexamethasone treated cells incubated for 24 hours at 37°C.

3.7. OPTIMISING THE CONDITIONS FOR RT-PCR

Conditions for the different RT-PCR reactions were optimised by altering variables such as the $MgCl_2$ concentration, the concentration of the primers, and the annealing temperature. For each primer set, these variables need to be altered to give optimal detection and amplification of the cDNA sequences.

Two $MgCl_2$ concentrations (2,0 mM and 3,5 mM) were used in the PCR reaction mixture. Figure 3.8 shows the effect of a $MgCl_2$ concentration of 3,5 mM (lane 1) and 2,0 mM (lane 2) on the amplification of IL-1ß (811 bp) in adhered monocytes. The amplification was more efficient when the lower

concentration of $MgCl_2$ (2 mM) was used in the reaction mixture, and hence this was the concentration used for IL-1ß PCR. No GAP-DH (internal control) was amplified in this reaction. The cDNA for the two PCR reactions came from the same RT reaction, thus eliminating variations in amplification due to different amounts of cDNA being present in the PCR reaction mixture. This does not, however, eliminate tube to tube variations occurring during the PCR. This effect of the $MgCl_2$ concentration was noted on a regular basis during the many RT-PCR reactions performed in the laboratory. $MgCl_2$ concentrations higher than 3,5 mM (eg. 10 mM) completely inhibited the PCR, as no bands could be detected on a ethidium bromide stained agarose gel (data not shown).

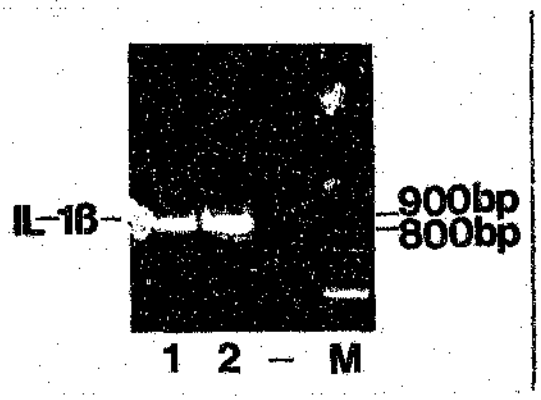


Figure 3.8. The effect of different $MgCl_2$ concentrations on the efficiency of amplification of IL-1ß cDNA.
IL-1ß cDNA was amplifi CR in 30 cycles, and the PCR product (811 bp) was run on a 2 % non-denaturing aga . The annealing temperature was 58°C and the IL-1ß primer concentration was 0,25 µM (see 2.8.2.2.). The $MgCl_2$ concentration in lane 1 was 3,5 mM and in lane 2 it was 2,0 mM. The blank was clear (not shown). The molecular mass marker appears in lane M.

The concentrations of the primer sets were altered to optimise the efficiency of amplification during PCR (Dukas *et al.* 1993).

Often two sets of primers were used in the same PCR reaction mixture to coamplify two cDNA sequences, one sequence being the internal standard GAP-DH. The target sequence (eg. IL-1RtII) and the internal standard sequence (eg. GAP-DH) must be amplified at the same rate so that the internal standard can be used as a reference to ensure that equal amounts of total cellular RNA were added to the RT reaction. Thus separate PCR reactions can be compared to one another, minimising the effects of tube to tube variation (Dukas *et al.* 1993).

IL-1RtII was expressed in very low concentrations in THP-1 cells, as up to 40 PCR cycles were needed to amplify enough cDNA to detect the PCR product on an agarose gel. When the target sequence such as IL-1RtII was present in low concentrations, the GAP-DH cDNA concentrations were therefore relatively higher. Thus the GAP-DH primer concentrations were lowered (from 0,1 μM down to 0,01 μM). At the same time the target sequence primers concentrations were high, at 0,25 μM to 0,5 μM. This resulted in similar rates of amplification, as both IL-1RtII and GAP-DH products could be detected in the same PCR reaction. If the two PCR products accumulate at similar rates, neither one will use up one of the limited components of the PCR mixture, thus both PCR product would be amplified to a detectable level. In a similar reaction, if a GAP-DH primer concentration of 0,1 μM was used, the increased formation of the GAP-DH PCR product appeared to inhibit the amplification of the less prevalent target sequence (data not shown). This inhibition was possibly caused by exhausting a limiting component of the PCR reaction mixture, before the target sequence has been amplified to a detectable concentration on an agarose gel.

IL-1RtII mRNA was expressed at higher concentrations in adhered monocytes, as only 30 cycles were needed to detect the PCR product on an agarose gel. In this case a higher concentration of GAP-DH primers (0,1 μM) was required to achieve a similar rate of amplification. In figure 3.14. (where IL-1RtII and GAP-DH were detected in adhered monocytes) a GAP-DH primer concentration of 0,01 μM was used in the PCR reaction mixture, while the IL-

1RtII primers were at 0,25 μM. As can be seen, the IL-1RtII PCR product appeared to have suppressed the formation of the GAP-DH PCR product, possibly by depleting the PCR reaction mixture of dNTPs. Thus a higher GAP-DH primer concentration of 0,1 μM was used when the target sequence was expressed at higher levels in the cell.

Figure 3.9. shows that changing the primer concentrations of the target sequence effects the efficiency of amplification. A high primer concentration (0,5 μM in lane 1, figure 3.9.) resulted in the formation of primer dimer which was amplified during the PCR cycles. The formation of high amounts of primer dimer could have depleted one of the limited resources in the reaction mixture, thus preventing the amplification of the IL-1RtII PCR product after a certain number of cycles. When 0,25 μM primers was used (lane 2), primer dimer formation was reduced and the PCR product band was more intense. When a still lower primer concentration was used (0,1 μM), the primer dimer was almost absent. The low primer concentration reduced the amplification of the target sequence, thus resulting in a less intense PCR product band. The aim of optimising the PCR reaction is not, however, to produce the most intense bands on an agarose gel. Less intense bands are easier to quantitate using densitometric scanning, than very intense bands. Subtle differences in band intensities are more obvious or detectable in moderately intense bands (eg. lane 3, figure 3.9.).

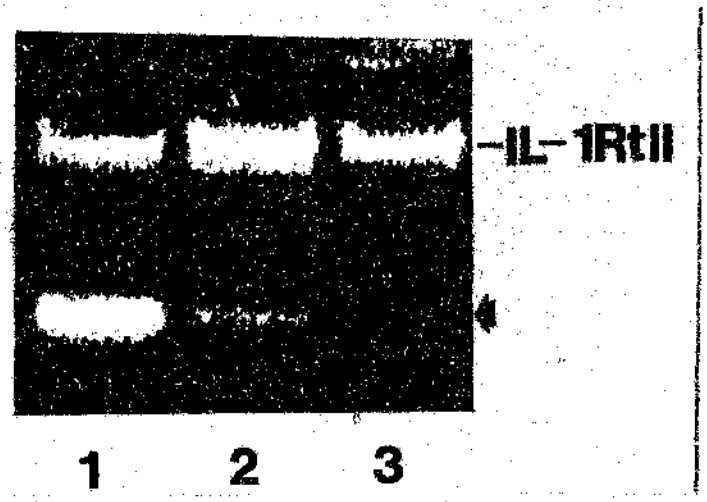


Figure 3.9. The effect of changing primer concentrations on the efficiency of amplification during PCR.
Alternate IL-1RtII primers were used (see 2.8.2.1.) at 0,5 µM (lane 1), 0,25 µM (lane 2), and at 0,1 µM (lane 3). This IL-1RtII expression was found in unstimulated lymphocytes (nonadherent fraction of cells) (see 2.1.3) after 30 cycles. The annealing temperature was 55°C. The arrow indicates primer dimer. The PCR products were run on a 2 % agarose gel.

There is an optimal temperature at which the primers anneal to the template cDNA where the binding of the primers is sequence specific. At too high annealing temperatures the primers either do not bind or they bind nonspecifically to the cDNA. The annealing temperature for a primer set was taken as ± 10°C below the average melting point of the two primers. For the IL-1RtI primers the average melting point was 63°C. Using the IL-1RtI primers an annealing temperature of 57°C resulted in the formation of multiple PCR product bands. An annealing temperature of 51°C resulted in the formation of one specific band of the correct size (data not shown).

3.8. RT-PCR ANALYSIS OF IL-1ß mRNA LEVELS

PBMNCs were adhered for 2 hours and the nonadherent cell (mainly lymphocytes) and adherent cells (monocyte rich) were analysed for IL-1ß mRNA expression. The purpose of dividing the PBMNCs into monocyte rich and lymphocyte rich fractions, was to determine if there was any difference in the expression and regulation of IL-1ß between the two populations.

3.8.1. IL-1ß mRNA EXPRESSION IN ADHERED MONOCYTES

Adhered monocytes from 3 donors were stimulated with PMA (10 nM), IL-4 (10 ng/ml), dexamethasone (100 nM), or IL-1ß (100 ng/ml) for 4 hours at 37°C. Total cellular RNA was isolated and analysed for changes in IL-1ß mRNA expression by RT-PCR (figure 3.10.). The PCR was performed at an annealing temperature of 58°C for 30 cycles, using IL-1ß (0,25 μM) and GAP-DH (0,1 μM) primers. Unstimulated cells exhibited high IL-1ß expression. There was little difference between the IL-1ß mRNA levels in the cells after 4 hours of stimulation. Dexamethasone tended to decrease IL-1ß expression.

The similarities in the band intensities may be due to the RT-PCR technique, and have little relation to the actual IL-1ß mRNA levels in the cell. The difficulties with the RT-PCR technique are discussed in the section 4.5.

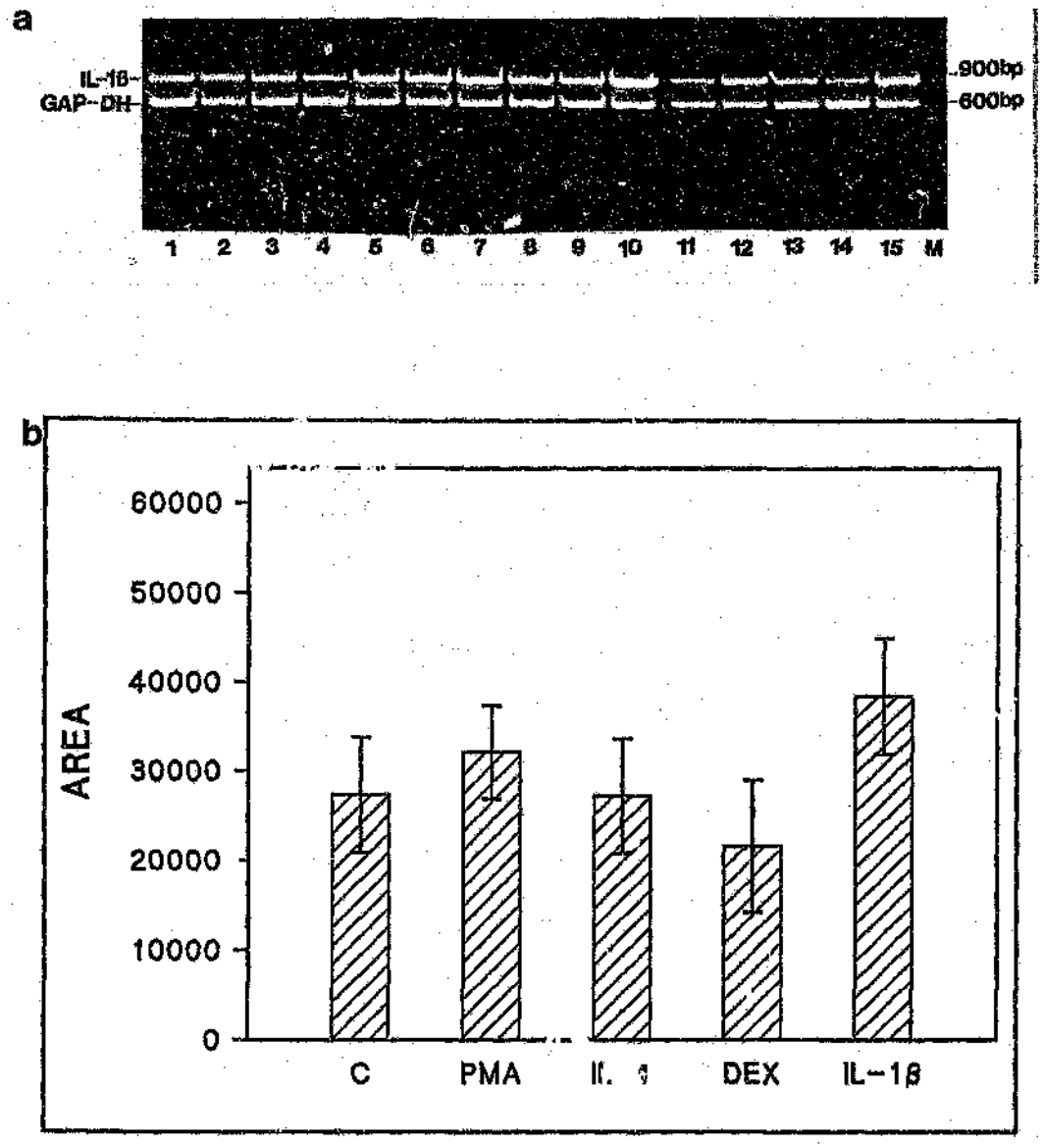


Figure 3.10. RT-PCR analysis of IL-1ß mRNA expression in adhered monocytes.
a. A 2 % agarose gel of IL-1ß (811 bp) and GAP-DH (587 bp) PCR products, where GAP-DH is the internal standard. The adhered monocytes from 3 donors were stimulated for 4 hours at 37°C. Stimuli: lanes 1, 6, 11: unstimulated; lanes 2, 7, 12: PMA (10 nM); lanes 3, 8, 13: IL-4 (10 ng/ml); lanes 4, 9, 14 dexamethasone (100 nM); and lanes 5, 10, 15: IL-1ß (100 ng/ml). The blank showed no contamination (not shown). Lane M: molecular mass marker.
b. Densitometric scanning analysis of the ethidium bromide stained gel in a. The relative absorbance of each band was converted into an area (in arbitrary units) (n=3; mean ± SEM).

3.8.2. IL-1ß mRNA EXPRESSION IN NONADHERENT LYMPHOCYTES

Nonadherent lymphocytes were isolated from 3 donors (see 2.1.3.) and stimulated for 4 hours at 37°C with PMA (10 nM), IL-4 (10 ng/ml) dexamethasone (100 nM), or IL-1ß (10 ng/ml). The mRNA levels were analysed by RT-PCR (see 3.7.1 for PCR conditions). GAP-DH was not used as an internal standard in this experiment, as I was not able to optimise this reaction with respect to GAP-DH and IL-1ß primer concentration. The RNA was quantified (as with the other reactions) spectrophotometrically and 0,5 µg of RNA was added to each RT reaction. A fixed amount (10 µl) of each RT reaction was amplified by PCR under the same conditions. The results of one donor are discussed. The unstimulated cells show high IL-1ß expression, possibly due to mechanical stimulation, LPS contamination of the anticoagulant heparin during the isolation procedure. PMA (10 nM) appeared to increase IL-1ß mRNA levels, while IL-4 and dexamethasone appeared to inhibit IL-1ß mRNA expression (see figure 3.11.).

Unfortunately, no statements on the effect of these stimuli can be made with any certainty due to the lack of a GAP-DH standard. For example, in this particular donor (lymphocytes), dexamethasone appears to decrease IL-1ß mRNA. Dexamethasone might be cytotoxic to lymphocytes, therefore, the decrease in IL-1ß mRNA may simply be due to a decrease in total cellular RNA caused by cell death. Although the RNA concentration in each sample was measured, and a constant amount of RNA was added to each reaction, the spectrophotometric technique is possibly not as sensitive as the RT-PCR

internal standard in detecting small changes in RNA concentration. A small difference in the amount of RNA added to the RT-PCR is amplified by the PCR reaction, resulting in large differences in the PCR product bands. The difficulties with the RT-PCR technique are discussed in section 4.5. No IL-1ß expression could be detected in HL-o0 cells (see section 4.5. for a discussion of this finding).

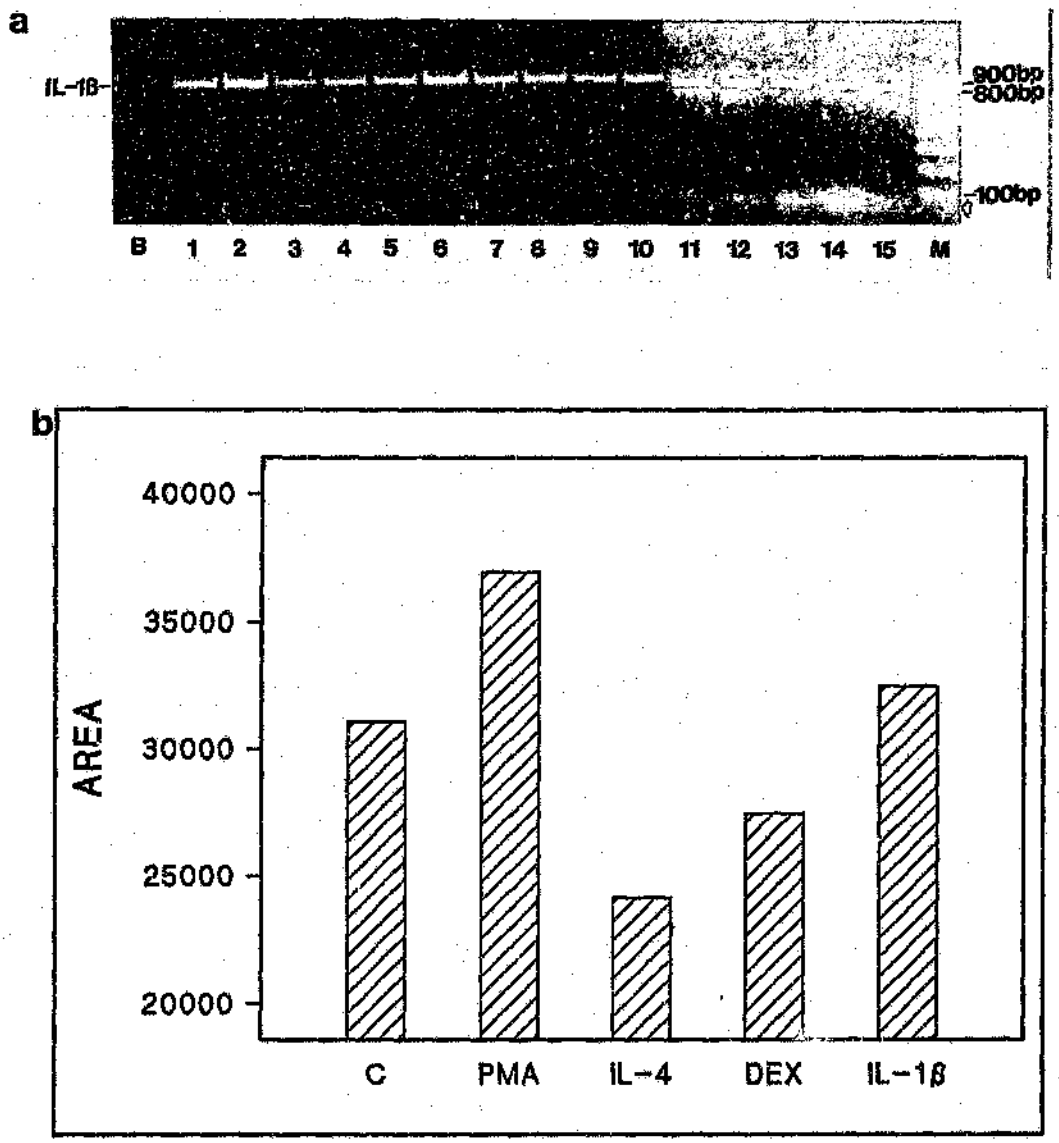


Figure 3.11. RT-PCR analysis of IL-1ß mRNA expression in nonadherent lymphocytes.

a. A 2 % agarose gel of IL-1ß PCR product. The 3 donors' nonadherent lymphocytes were treated as in figure 3.10. The lanes are labelled as in figure 3.10. except that there are no GAP-DH bands here. The blank showed no contamination (lane B). The molecular mass marker appears in lane M.

b. Densitometric scanning of the bands on the agarose gel in a. (lanes 1-5 only ie. donor 1). The relative absorbance of each band was converted to an area (in arbitrary units).

3.9. RT-PCR ANALYSIS OF IL-1RtI mRNA LEVELS

IL-1RtI mRNA could not be detected by Northern blotting using a PCR generated oligonucleotide probe. Possibly, the PCR product probe failed to hybridise with the IL-1RtI mRNA. In order to generate data on the expression of IL-1RtI in monocytes and the leukaemic HL60 and THP-1 cells, IL-1RtI mRNA was detected by specific primers using the RT-PCR technique.

3.9.1. IL-1RtI mRNA EXPRESSION IN ADHERED MONOCYTES

Adhered monocytes (see 2.1.3.) from 3 donors were stimulated for 4 hours at 37°C with either PMA (10 nM), IL-1ß (10 ng/ml), dexamethasone (100 nM), or IL-1ß (100 ng/ml). The RNA was analysed by RT-PCR (2.7.) for 30 cycles using IL-1RtI primers (0,25 μM). The annealing temperature was 51°C and the $MgCl_2$ concentration was 2,0 mM. GAP-DH was used as internal standard (0,1 μM).

Unstimulated cells expressed similar levels of IL-1RtI mRNA as stimulated (eg. with PMA, IL-4, dexamethasone, or IL-1ß) cells indicating constitutive expression of IL-1RtI in adhered monocytes. After 4 hours of stimulation with the various reagents, there was no difference in the IL-1RtI mRNA levels expressed in these cells (see figure 3.12.a.). PMA after 4 hours induced a greater degree of adherence (a morphological change) to the plastic petri dish. Adhered monocytes exposed to PMA for 4 hours appeared more flattened and they had more cytoplasmic projections, appearing more macrophage - like than freshly isolated monocytes. This increase in differentiation was not

accompanied by a change in the IL-1RtI mRNA levels in the cells.

3.9.2. IL-1RtI mRNA EXPRESSION IN HL-60 CELLS

Cultured HL-60 cells from (n=3) were stimulated with either PMA (10 nM), IL-4 (10 ng/ml), dexamethasone (100 nM), or IL-1ß (100 ng/ml) for 4 hours at 37°C. The RNA was analysed by RT-PCR using the same conditions as in 3.9.1. except that 0,01 μM GAP-DH primers were used as an internal standard. No differences in the expression of IL-1RtI mRNA after 4 hours of stimulation with the various reagents with respect to the control could be detected (see figure 3.12.b.). PMA did not induce adherence of the HL-60 cells after 4 hours, indicating that the cells had not differentiated towards macrophages. This indicates that PMA exposure for 4 hours was insufficient to induce differentiation in the HL60 cells. Perhaps a longer PMA stimulation for HL-60 cells was required or a different differentiating agent such as 12-O-tetradecanoylphorbol-13-acetate (TPA). The unstimulated cells expressed similar levels of IL-1RtI compared to stimulated cells indicating constitutive expression of IL-1RtI in HL-60 cells. See section 4.6. for a discussion of these results.

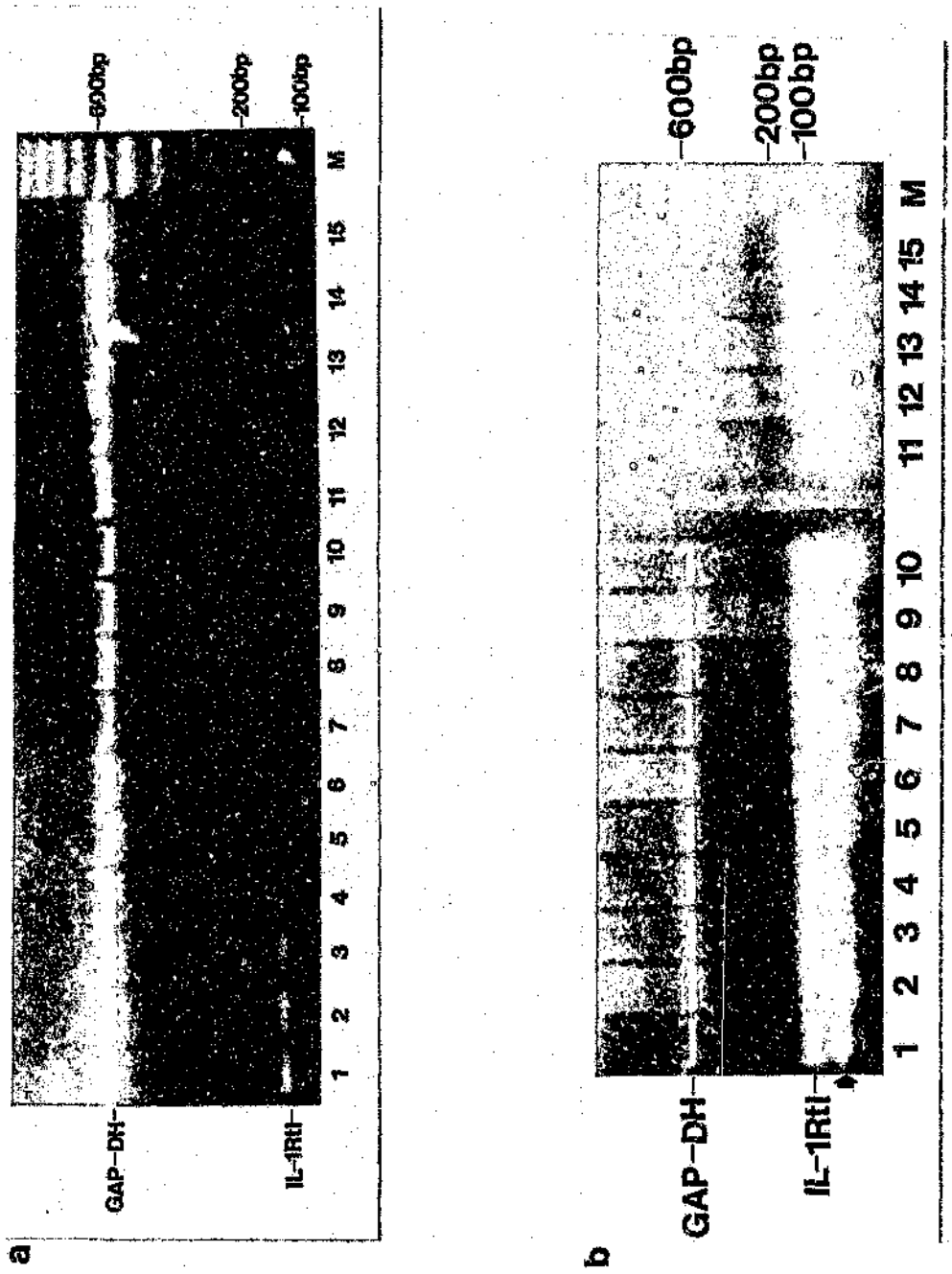


Figure 3.12. RT-PCR analysis of IL-1RtI mRNA levels in adhered monocytes and HL-60 cells. Nondenaturing 2 % agarose gels of IL-1RtI (379 bp) and GAP-DH (579 bp) PCR products in (a.) adhered monocytes and in (b.) HL-60 cells. Stimuli for both gels: lanes 1, 6, 11: unstimulated; lanes 2, 7, 12: PMA (10 nM); lanes 3, 8, 13: IL-4 (10 ng/ml); lanes 4, 9, 14: dexamethasone (100 nM); lanes 5, 10, 15: IL-1ß (100 ng/ml) (n=3). The PCR conditions were as in 3.8.1. The arrow in b. indicates primer dimer. The blank showed no contamination (not shown). Lane M: molecular mass marker.

3.9.3. IL-1RtI mRNA EXPRESSION IN THP-1 CELLS

THP-1 cells (n=2) were stimulated with PMA (10 nM) for 4 and 24 hours. The PCR conditions were the same as in 3.8.1. except that the GAP-DH primers were used at a concentration of 0,01 μM. Unstimulated cell expressed the IL-1RtI mRNA, indicating constitutive IL-1RtI expression (see figure 3.13. lanes 1 and 5). PMA induced adherence and thus differentiation of the THP-1 cells after 4 and 24 hours. From the data in figure 3.13. it was difficult to ascertain whether PMA stimulation altered the expression of IL-1RtI mRNA. Densitometric scanning of the bands was not possible, as the bands were too faint.

Figure 3.13. demonstrates the importance of determining the RNA concentration in each sample spectrophotometrically before proceeding with the RT-PCR. It is not sufficient to rely on the use of an internal standard such as GAP-DH to semi-quantitate the bands appearing on the agarose gel. It makes it difficult to visually estimate the effect of various stimuli, when densitometric scanning of the results is not possible.

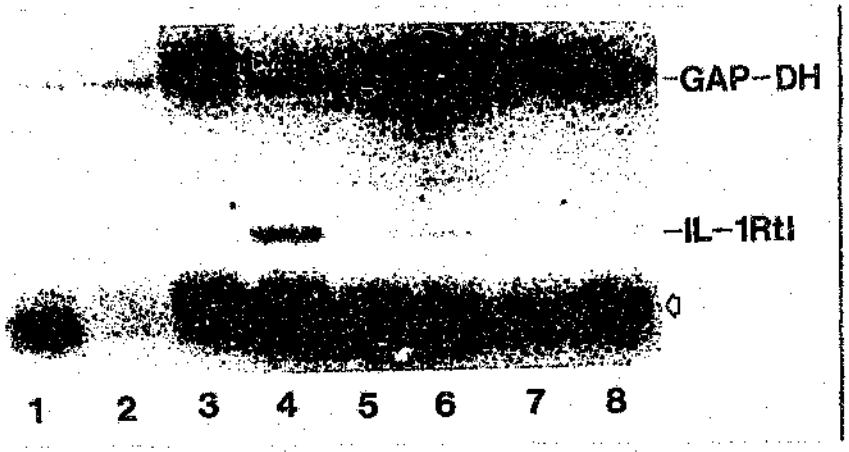


Figure 3.13. RT-PCR analysis of IL-1RtI mRNA levels in THP-1 cells.
A 2 % agarose gel of IL-1RtI and GAP-DH PCR products. Stimuli: lanes 1, 5: unstimulated (incubated for 4 hours); lanes 2, 6: PMA (10 nM, 4 hours); lanes 3, 7: unstimulated (incubated for 24 hours), and lanes 4 and 8 from PMA (10 nM, 24 hours). The arrow indicates primer dimer. The blank showed no contamination (not shown).

3.10. RT-PCR ANALYSIS OF IL-1RtII mRNA LEVELS

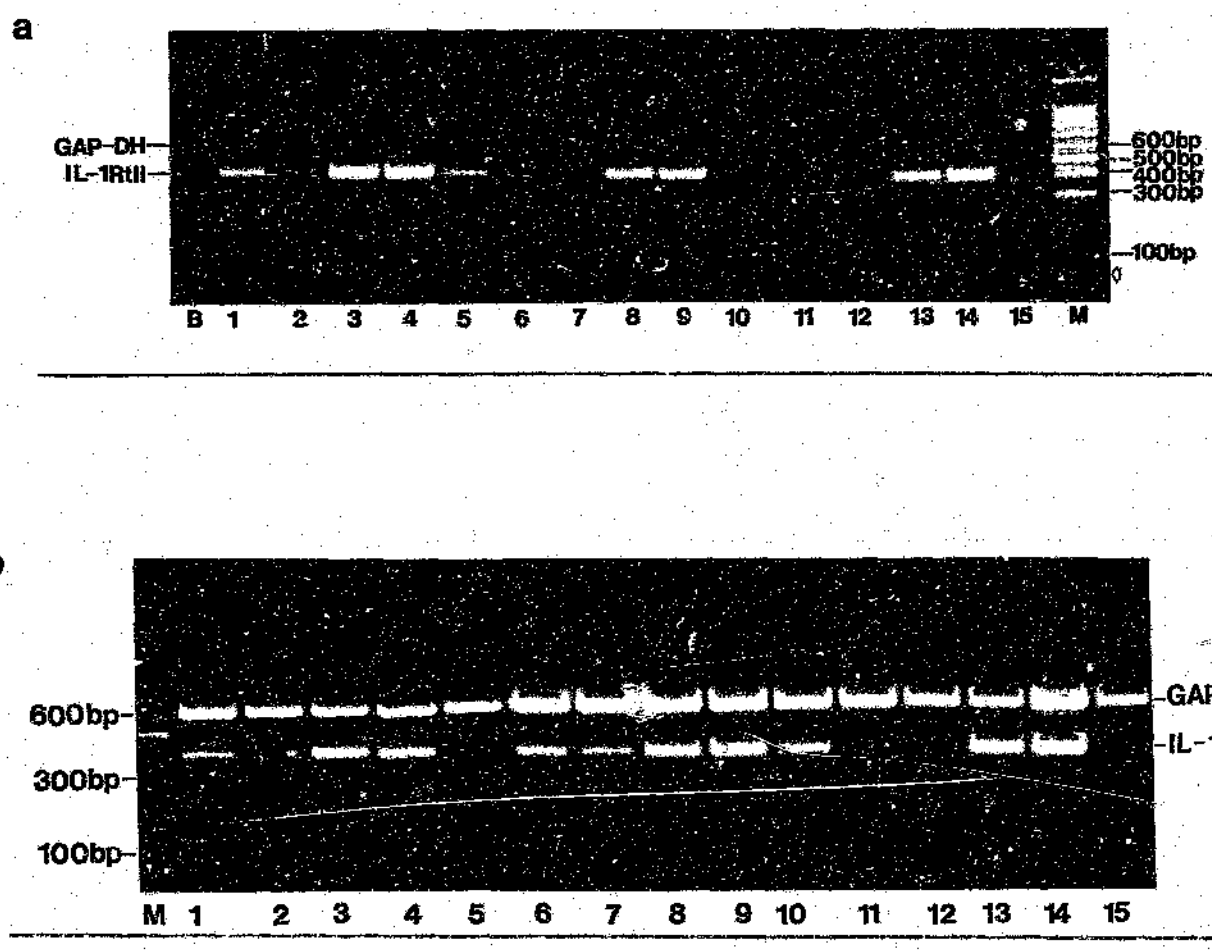
3.10.1. IL-1RtII mRNA EXPRESSION IN ADHERED MONOCYTES

Adhered monocytes from 3 different donors were stimulated for 4 hours at 37°C with either PMA (10 nM), IL-4 (10 ng/ml), dexamethasone (100 nM), or IL-1ß (100 ng/ml). The isolated RNA was analysed by RT-PCR (see 2.7.) for 30 cycles. The annealing temperature was 51°C, the $MgCl_2$ concentration was 2,0 mM, and 0,25 μM IL-1RtII primers and 0,01 μM or ., μM GAP-DH primers were used in the PCR reaction mixture.

Figure 3.14.a. shows the PCR products obtained when 0,01 μM GAP-DH primers were used. The GAP-DH PCR product was barely visible, suggesting that a higher GAP-DH primer concentration was required. Despite the lack of internal standard, the RNA added to each RT reaction was kept constant (0,5 μg). In addition the RNA isolated from the samples was of a high purity, as the ratio of the absorbance of the sample at $A_{260\ nm}$: $A_{280\ nm}$ was close or equal to 2 : 1. This ratio indicates negligible amounts of contaminating protein, ie. with a ratio of 2 : 1, the absorbance at 260 nm gives an accurate estimate of the amount of RNA present. (Glasel 1995). Thus, trends in the results can be noted. PMA did not affect the expression of the IL-1RtII mRNA levels after 4 hours compared to unstimulated cells, suggesting that differentiation for 4 hours with PMA (indicated by an increase in adherence) towards macrophages does not result in altered IL-1RtII expression in the cells. Both IL-4 and dexamethasone dramatically increased the amount of IL-1RtII mRNA. This increase could be due to an increase in IL-1RtII gene expression or an increase in the stability of the mRNA. IL-1ß did not alter the expression of IL-1RtII mRNA in the cells compared to unstimulated cells.

Since the GAP-DH PCR product bands were too faint for densitometric scanning, additional *taq* polymerase (0,5 U) and GAP-DH primers (0,1 μM) were added to the reaction mixture after an aliquot of the completed PCR for IL-1RtII was run on an agarose gel to give the results in figure 3.14.a. The reaction then was allowed to continue for an additional 30 cycles. The resulting gel revealed distinct and intense GAP-DH bands, as well as the

original IL-1RtII bands, whose relative intensities had not altered to a great extent during the additional 30 cycles. With the GAP-DH PCR product then visible, effects of unequal RT and loading of the gel could be eliminated within one donor but not between donors. The reason for this is that GAP-DH mRNA levels (in figure 3.14.b.) vary between donors. A fairly constant amount of mRNA (0,5 μg) was added to each RT-PCR reaction, yet it is clear that donor 1 (lanes 1 - 5) has less GAP-DH than donor 2 (lanes 6 - 10). The upregulation of the IL-1RtII mRNA levels by IL-4 and dexamethasone was confirmed. The scanning data for both gels appears in figure 3.14.c. and d.


a
GAP-DH
IL-1RtII
600bp
500bp
400bp
300bp
100bp
B 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 M
b
600bp
300bp
100bp
GAP-DH
IL-1RtII
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

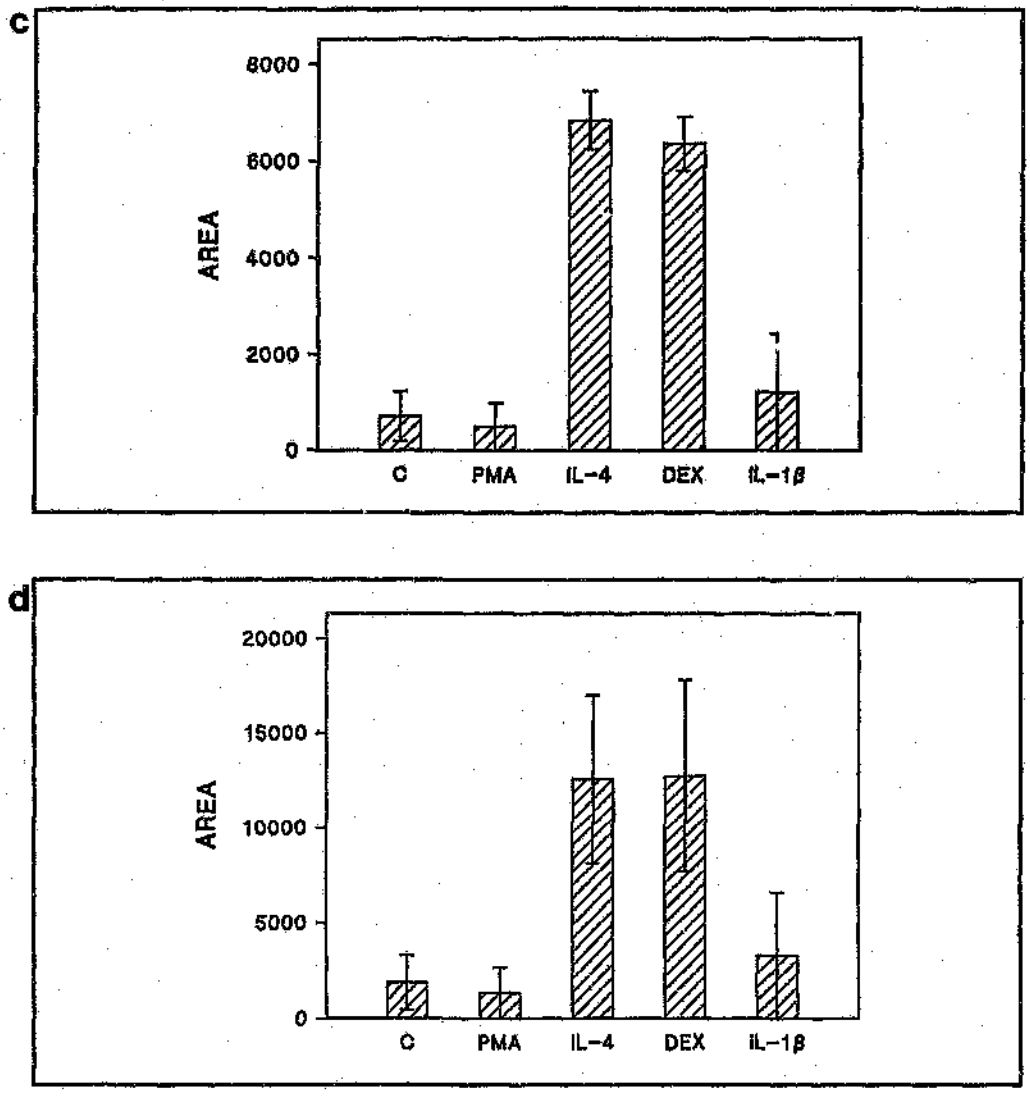


Figure 3.14. RT-PCR analysis of IL-1RtII mRNA in adhered monocytes.
Nondenaturing agarose gels (2 %) of IL-1RtII (379 bp) and GAP-DH (579 bp) PCR products. The adhered monocytes from 3 donors were stimulated for 4 hours at 37°C in the following way: lanes 1, 6, 11: unstimulated; lanes 2, 7, 12: PMA (10 nM), lanes 3, 8, 13: IL-4 (10 ng/ml); lanes 4, 9, 14: dexamethesone (100 nM); lanes 5, 10, 15: IL-1ß (100 ng/ml). The PCR reaction mixtures for **a.** contained 0,01 µM GAP-DH as internal standard. The PCR mixes for **b.** were the PCR reaction mixes from a. after 30 cycles, with the addition of 0,1 µM GAP-DH and 0,5 U DNA polymerase. The reaction was then allowed to proceed for another 30 cycles. The blanks showed no contamination (lane B). Lane M: molecular mass marker.
c. and **d.** Densitometric scanning of the bands in a. and b. respectively. The relative absorbance of each band was converted to an area (in arbitrary units).

3.10.2. IL-1RtII mRNA EXPRESSION IN THP-1 CELLS

THP-1 cells were stimulated with IL-4 (10 ng/ml) for 4 hours. In one of two experiments, IL-4 stimulated an increase in IL-1RtII mRNA found in the cells, as determined by RT-PCR (see 2.7.). Unstimulated cells expressed low levels of IL-1RtII mRNA.

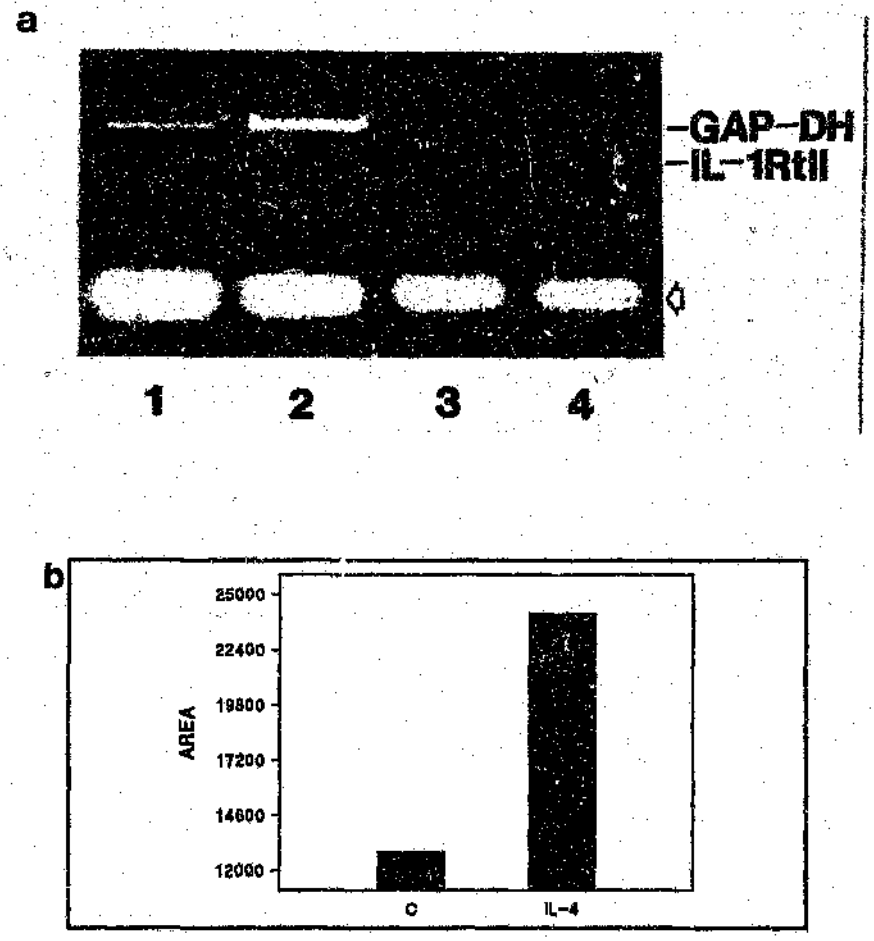


Figure 3.15. RT-PCR analysis of IL-1RtII mRNA expression in THP-1 cells treated with IL-4.
a. A nondenaturing agarose gel (2 %) of IL-1RtII and GAP-DH PCR products. Stimuli: lanes 1, 3: unstimulated (incubated at 37°C for 4 hours); lanes 2, 4: IL-4 (10 ng/ml) for 4 hours at 37°C. The blank showed no contamination.
b. Densitometric scanning of the bands in a. (lanes 3 and 4). The relative absorbance of each band was converted into an area (in arbitrary units).

3.11. ENDONUCLEASE RESTRICTION OF PCR PRODUCTS

In order to determine if the primer pairs are annealing specifically to the template cDNA, the PCR product is cleaved at specific sites in its sequence. If cleavage with a specific endonuclease generated fragments of the predicted sizes, it is assumed that the correct PCR product has been amplified. All of the primer pairs generated PCR products of the predicted size, indicating that the primers were recognising their specific cDNA sequences.

Two primer pairs were available to analyse IL-1RtII mRNA expression. The alternate IL-1RtII PCR product (1221 bp) (see 2.8.2.1.) was cleaved by Eco RI, generating fragments of 475 and 736 bp (see figure 3.16.). In figure 3.16. the two digestion fragments appear slightly larger than the predicted sizes, but this was possibly due to retardation of the mobility of the DNA fragments in the gel. Both IL-1ß and GAP-DH primers had been previously established as producing the correct PCR products in the laboratory and were not tested. IL-1RtI and IL-1RtII (387 bp) products were not digested, as I was unable to isolate the PCR products from the agarose gel. The PCR products were run on an agarose gel in order to separate them from primer dimer which would obscure the results of the digestions. The IL-1RtI (Ferrari *et al.* 1993) and IL-1ß (Larrick *et al.* 1989) primer sequences were obtained from published papers. It was therefore assumed that they generated the correct PCR products.

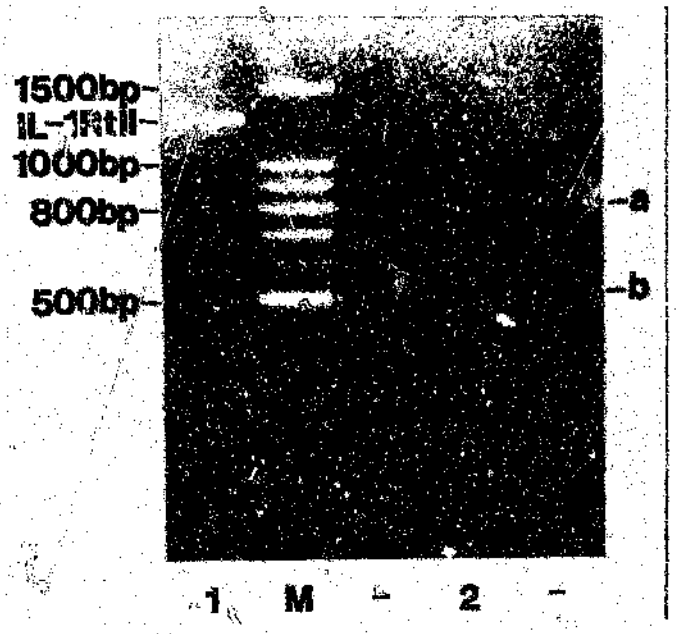


Figure 3.16. Endonuclease restriction of the alternate IL-1RtII PCR product.
A 2 % nondenaturing agarose gel of the alternate IL-1RtII PCR product (1211 bp) (lane 1) cleaved with Eco RI to generate two fragments of 736 bp (a) and 475 bp (b) (lane 2). The molecular mass marker is in lane M.

3.12. RIA ANALYSIS OF IL-1ß PROTEIN EXPRESSION IN PBMNCS

PBMNCs were stimulated with either IL-4 (30 ng/ml), IL-1α (10 ng/ml), LPS (1 μg/ml), a combination of IL-1α and IL-4, or a combination of LPS and IL-4 for 24 hours. The cell lysates and supernatants were analysed for the presence of IL-1ß protein using a RIA (see 2.8.). Cell lysate IL-1ß represented intracellular IL-1ß and cell supernatant IL-1ß represented secreted IL-1ß.

IL-4 (30 ng/ml) down regulated the expression of cell associated IL-1ß (which was possibly induced by mechanical or LPS stimulation) to an undetectable level compared to unstimulated cells after 24 hours (see figure 3.17.a.). IL-1α (10 ng/ml) and LPS (1 μg/ml) upregulated the expression of cell associated IL-1ß, but the addition of IL-4 (30 ng/ml) inhibited this production.

IL-4 (30 ng/ml) also downregulated the amount of IL-1ß that was secreted compared to unstimulated cells after 24 hours (figure 3.17.b.). As was the case with cell associated IL-1ß, LPS (1 μg/ml) upregulated the amount of secreted IL-1ß, while IL-4 (30 ng/ml) inhibited this upregulation. IL-1α increased the amount of secreted IL-1ß compared to cell associated IL-1ß. IL-4 inhibited the slight increase in IL-1ß secreted due to IL-1α. LPS stimulated secreted IL-1ß (lane D in figure 3.17.b.) to a greater extent than cell associated IL-1ß (lane D in figure 3.17.a.). Thus, LPS is efficient at inducing the synthesis and release of IL-1ß.

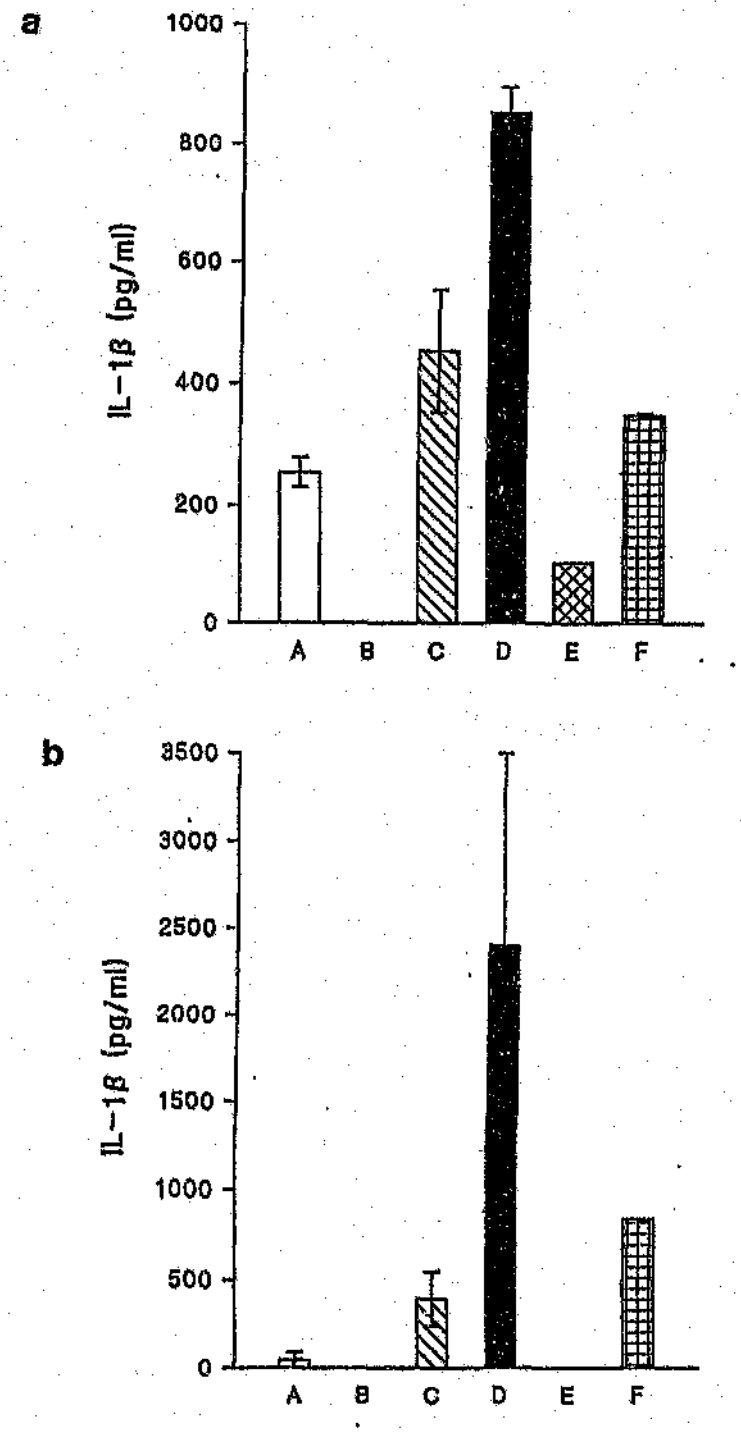


Figure 3.17. RIA analysis of cell associated and secreted IL-1ß protein in PBMNCs.
a. Detection of IL-1ß in cell lysates of PBMNCs stimulated for 24 hours (n=3).
b. Detection of IL-1ß in cell supernatants of PBMNCs stimulated for 24 hours (n=3).
A: control (unstimulated cells); B: IL-4 (30 ng/ml); C: IL-1α (10 ng/ml); D: LPS (1 µg/ml); E: IL-1α and IL-4; F: LPS and IL-4 (mean ± SEM).

3.13. ANALYSIS OF IL-1RtI PROTEIN BY WESTERN BLOTTING AND IMMUNODETECTION

It was difficult to detect differences in IL-1RtI mRNA levels by RT-PCR, possibly because the stimuli used did not affect the expression of IL-1RtI at the mRNA level. IL-1RtI protein expression was therefore analysed by conventional Western blotting and immunodetection.

Total cellular protein was isolated from various cells and quantified by the Bradford reaction. Protein (75 μg) was run on a 10 % SDS PAGE gel and Western blotted. IL-1RtI protein was detected by a specific IL-1RtI polyclonal antibody and the streptavidin - peroxidase / 4 chloro-1-naphthol immunodetection system (see 2.10.).

3.13.1. IL-1RtI PROTEIN EXPRESSION IN PBMNCS

PBMNCs were isolated from the blood of 3 healthy donors (see 2.1.2.). The PBMNCs were stimulated with either PMA (10 nM), IL-4 (10 ng/ml), dexamethasone (100 nM), or IL-1ß (100 ng/ml) for 24 hours at 37°C. IL-1RtI protein was detected in unstimulated PBMNCs, and PBMNCs stimulated with either PMA, IL-4, dexamethasone, or IL-1ß (data not shown).

3.13.2. IL-1RtI PROTEIN EXPRESSION IN THE NONADHERENT LYMPHOCYTE FRACTION

The effect of PMA, IL-4, dexamethasone, and IL-1ß on the expression of IL-1RtI protein was analysed in nonadherent lymphocytes.

PBMNCs were therefore adhered to plastic petri dishes, and the nonadherent fraction of cell (mainly lymphocytes) were isolated (see 3.11.). The nonadherent cells were stimulated with either PMA (10 nM), IL-4 (10 ng/ml), dexamethasone (100 nM), or IL-1ß (100 ng/ml) for 24 hours at 37°C. Figure 3.18. presents the results of the Western blot and immunodetection of IL-1RtI protein in these cells. IL-1RtI was detected in the lymphocytes as approximately a 80 - 88 kDa protein, as determined using the relative mobilities of the marker proteins on the blot. The background was dark on this particular blot which made it difficult to use densitometric scanning of the bands to determine their relative intensities. Based on a visual assessment of the bands in figure 3.18. dexamethasone (lane 2 and 12) appeared to upregulate the expression of IL-1RtI protein compared to the expression in unstimulated cells (lane 5 and 15). This upregulation of IL-1RtI by dexamethasone occurred in two of three donors.

The IL-1RtI antibody was a polyclonal antibody raised in rabbit. This antibody routinely bound to more than one protein band on the blot in all the IL-1RtI Western blots tested. Only one of the bands detected represented a protein of approximately the correct size. The antibody did detect proteins of other molecular masses (see figure 3.18), eg. a smaller protein (< 66 kDa) and a relatively large protein (< 220 kDa). I cannot say whether these extra bands are specific (ie. the antibody has detected IL-1RtI protein which has been digested or formed dimers) or nonspecific (the antibody has bound to proteins other than IL-1RtI). In an effort to clarify the situation, the blots were probed

with non-immune rabbit serum. None of the major bands appearing on the blots probed with the immune rabbit serum (containing anti-IL-1RtI) were detected using the non-immune serum. This suggested that the extra bands appearing on the blot in figure 3.18. were specific. The additional bands may be due to different degrees of glycosylation of the IL-1RtI, the formation of receptor dimers, or partial degradation of the protein.

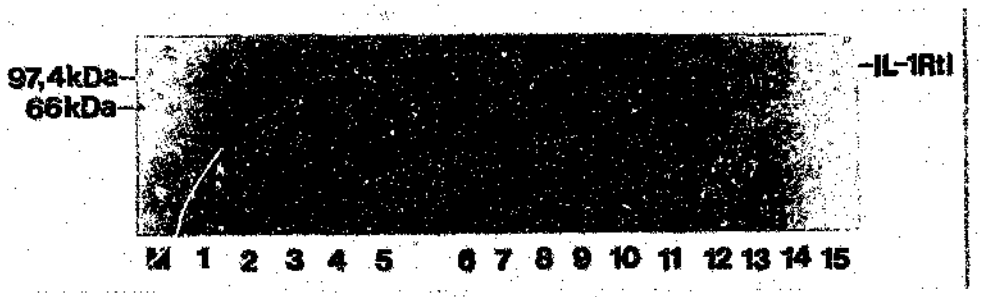


Figure 3.18. Western blotting and immunodetection of IL-1RtI protein in nonadherent lymphocytes.
Proteins (75 µg) were separated by denaturing (SDS) PAGE (4% stacking gel and a 10 % resolving gel), Western blotted, and IL-1RtI protein levels analysed (see 3.11). Stimuli (24 hours): lanes 5, 10, 15 unstimulated; lanes 4, 9, 14: PMA (10 nM); lanes 3, 8, 13: IL-4 (10 ng/ml); lanes 2, 7, 12: dexamethasone (100 nM); and lanes 1, 6, 11: IL-1ß (100 ng/ml). Lane M: rainbow molecular mass marker.

3.13.3. IL-1RtI PROTEIN EXPRESSION IN HL-60 CELLS

It was not certain at what time point changes in IL-1RtI protein expression would be detected in HL-60 cells. IL-1RtI protein was probed for after 4 and 24 hours exposure to dexamethasone (see figure 3.19.). Dexamethasone was chosen as the stimulus at it appeared to be the stimulus that upregulated the expression of IL-1RtI in lymphocytes (figure 3.18.).

Unstimulated cells (control at 0 hours) showed the highest expression of IL-1RtI protein, while unstimulated cells incubated for 4 hours and for 24 hours both showed a decrease in the IL-1RtI protein. Dexamethasone downregulated the IL-1RtI protein expression at 4 hours (53,8 % of control at 4 hours) and at 24 hours (55,2 % of control at 24 hours).

It is interesting to note the decreasing amounts of IL-1RtI protein in unstimulated from the 0 hour time point to the 24 hour time point. A possible explanation could be that there were too many cells in the petri dish (3×10^6 cells/ml), and that the cells began to die. This seems unlikely as the cells appeared healthy on morphological examination after the 24 hour incubation, although a trypan blue cell viability study should have been performed to confirm this.

It has been shown by Re *et al.* (1994) that dexamethasone upregulates IL-1RtI in neutrophils, and the data in figure 3.18. indicates that dexamethasone also upregulates IL-1RtI in a mixed population of lymphocytes. Dexamethasone downregulated IL-1RtI in HL-60 cells. Thus, dexamethasone appears to act in a different manner in HL-60 cells compared to lymphocytes It must be taken into account that this is a single result. There is the possibility that dexamethasone (100 nM) was cytotoxic to the HL-60 cells, although 100 nM of dexamethasone had no reported adverse effects on the neutrophils (Re *et al.* 1994). There is the possibility that dexamethasone truly down regulated the expression of IL-1RtI in HL-60 cells. See section 3.12.3. for a discussion of the multiple bands appearing on the blot in figure 3.19.

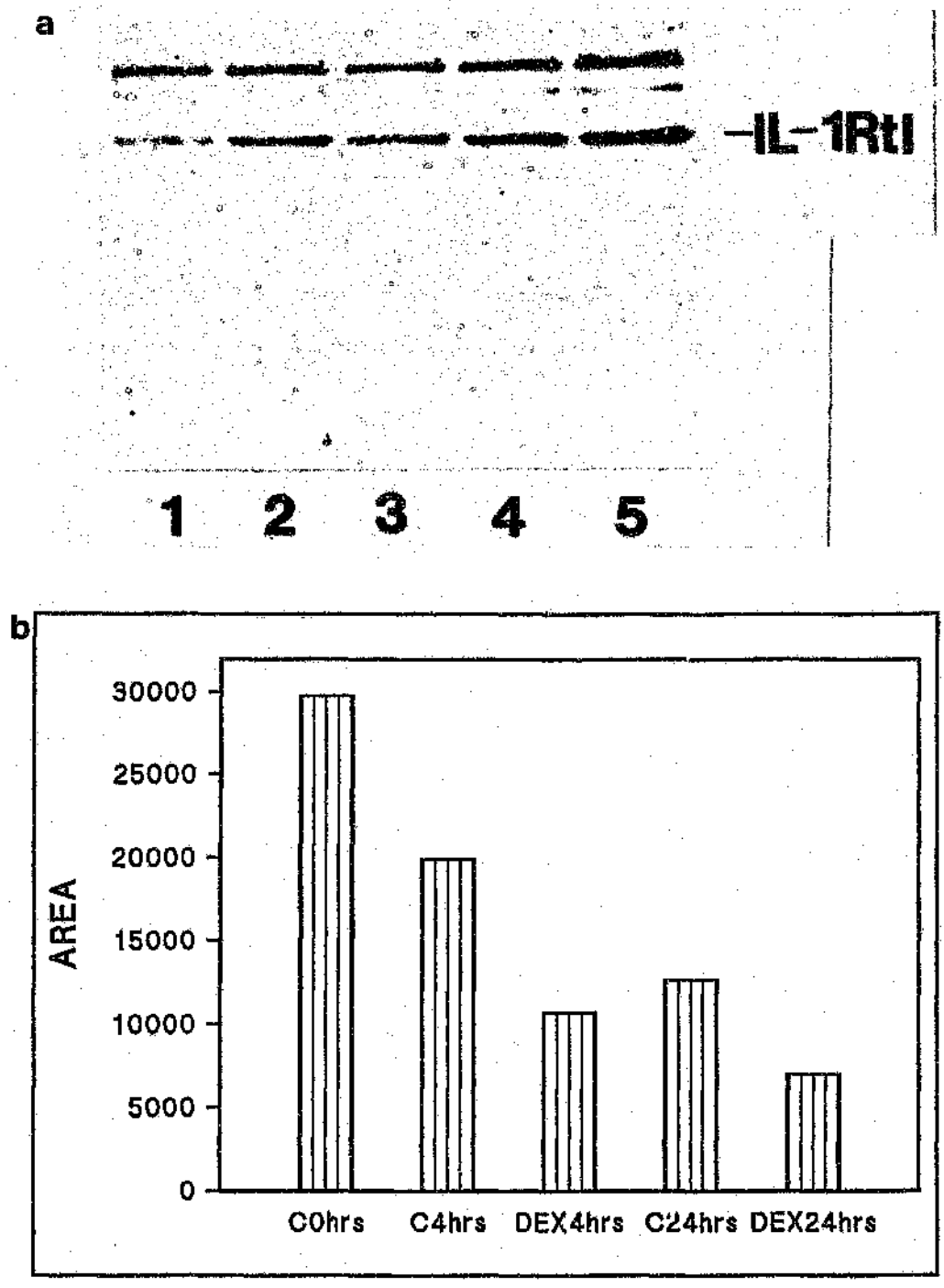


Figure 3.19. The effect of dexamethasone on the expression of IL-1RtI protein in HL-60 cells.
a. Western blot and immunodetection of IL-1RtI protein. Protein (100 ng) from HL-60 cells were separated by denaturing (SDS) PAGE (4 % stacking gel and a 10 % resolving gel), and Western blotted (see 3.11). Stimuli: lane 5: unstimulated (0 hours); lane 4: unstimulated (4 hours); lane 3: dexamethasone (100 nM, 4 hours); lane 2: unstimulated (24 hours); lane 1: dexamethasone (100 nM, 24 hours). Rainbow molecular mass marker not shown.
b. Densitometric scanning of the blot in a. The relative absorbance of each band was converted into an area (in arbitrary units).

HL-60 cells exposed to PMA (10 nM) for 24 hours did show some degree of adherence to the plastic petri dishes. This adherence was greatly increased if the cells were exposed to PMA for 48 hours, indicating a differentiation towards a more macrophage - like state.

Figure 3.20.a. shows that PMA (10 nM) upregulated the expression of IL-1RtI protein after 24 hours (lanes 4, 9, and 14) compared to unstimulated cells incubated for 24 hours without PMA (lanes 5, 10, and 15). The other stimuli, namely, IL-4 (10 ng/ml), dexamethasone (100 nM), and IL-1ß (100 ng/ml) did not affect the expression of IL-1RtI.

The results represented in figure 3.20.b and 3.20.c. confirm the results obtained in figure 3.20.a. PMA (10 nM) upregulated the expression of IL-1RtI protein in HL-60 cells (lane 8). Co-stimulation with PMA and IL-4 (10 ng/ml) did not markedly alter the level of IL-1RtI protein expressed (lane 7) compared to cells stimulated with PMA alone (lane 8). In figure 3.20.c. stimulation with PMA for 24 hours increased IL-1RtI expression (lane 7). PMA stimulation for 48 hours did not markedly change the expression of IL-1RtI compared to 24 hours, and neither did costimulation with PMA and a combination of IL-1α, IL-1ß, or IL-4.

Thus, PMA (10 nM, 24 hours) clearly upregulates the expression of IL-1RtI (n=5) compared to unstimulated cells. In these experiments, IL-1RtI is not detectable in unstimulated cells, unlike in figure 3.19. This is possibly because

100 μg of total cellular protein (from 3 x 10^6 cells/ml) was loaded on to the gel in figure 3.19., while 75 μg of total cellular protein (from 2 x 10^6 cells/ml) was used for these three experiments. PMA seemed to have little effect on the adherence of the HL-60 cells, but PMA had a marked effect on the expression of IL-1RtI protein. Thus an increase in differentiation towards macrophages (but not complete differentiation) results in an increase in IL-1RtI expression in HL-60 cells. It is possible that the differentiation of HL-60 cells by PMA, and the increase in IL-1RtI expression due to PMA are separate events.

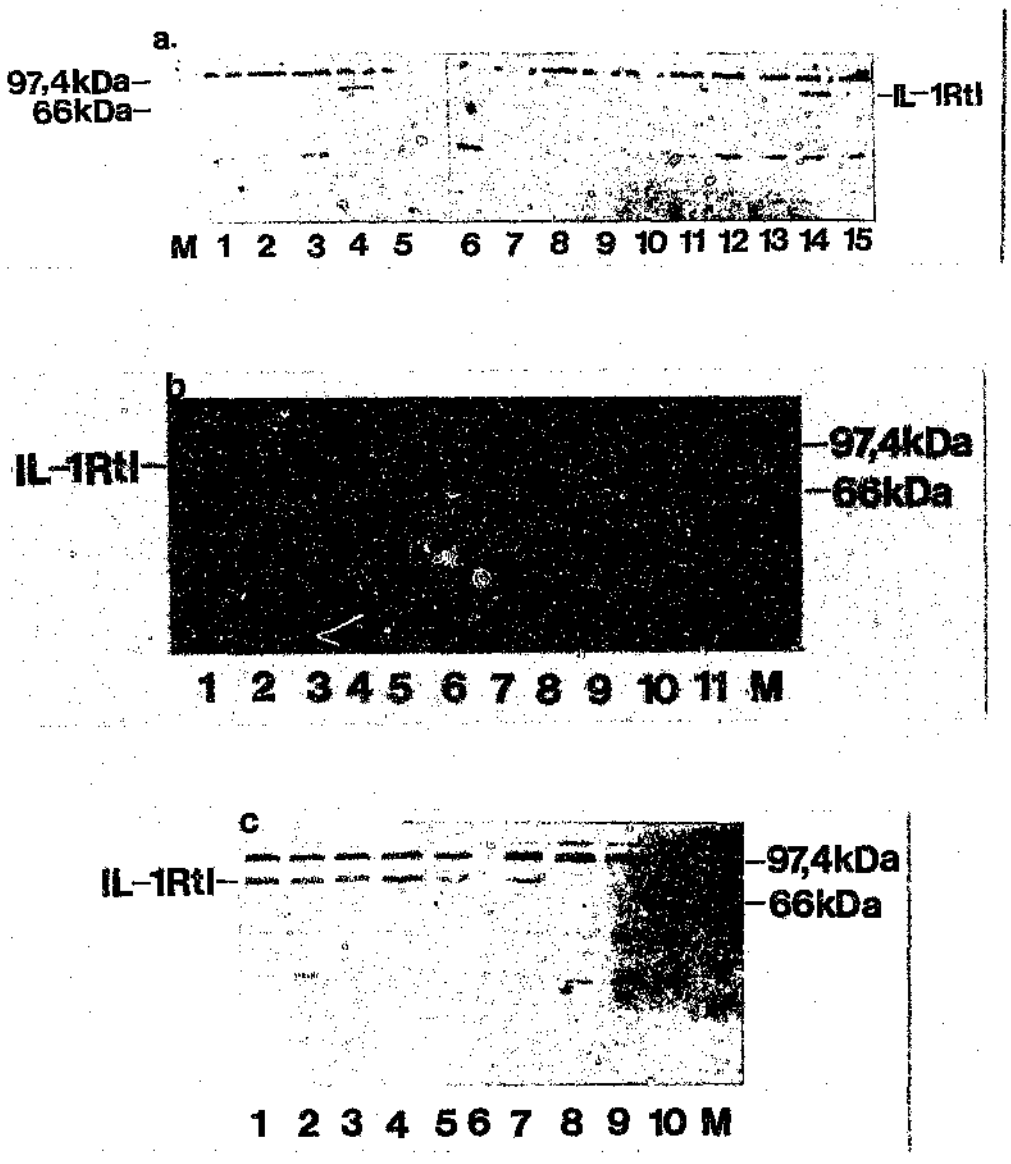


Figure 3.20. The effect of PMA on the expression of IL-1RtI protein in HL-60 cells.
Western blot and immunodetection of IL-1RtI protein after 24 hours stimulations. Proteins (75 μg) were separated by denaturing (SDS) PAGE (4 % stacking gel and a 10 % resolving gel). Lane M: molecular mass marker.
a. Lanes 5, 10, 15: unstimulated; lanes 4, 9, 14: PMA (10 nM); lanes 3, 8, 13: IL-4 (10 ng/ml); lanes 2, 7, 12: dexamethasone (100 nM); lanes 1, 6, 11: IL-1ß (100 ng/ml) (n=3).
b. Stimuli: lane 11: unstimulated (0 hours time); lane 10: unstimulated (24 hours); lane 9: IL-4 (10 ng/ml); lane 8: PMA (10 nM); lane 7: PMA + IL-4; lane 6: IL-1α (10 ng/ml); lane 5: IL-1α + IL-4; lane 4: IL-1ß (10 ng/ml); lane 3: IL-1ß + IL-4; lane 2: dexamethasone (100 nM); lane 1: dexamethasone + IL-4.
c. Stimuli: lanes 10, 9: unstimulated (0 and 24 hours respectively); lane 8: IL-4 (10 ng/ml, 24 hours); lane 7: PMA (10 nM, 24 hours). Lanes 6-1 were exposed to PMA for 48 hours. After 24 hours of exposure to PMA the cells had the following stimuli added to them: lane 6: IL-4; lane 5: PMA; lane 4: IL-1α (10 ng/ml); lane 3: IL-1α + IL-4; lane 2: IL-1ß (10 ng/ml); lane 1: IL-1ß + IL-4.

3.13.4. IL-1RtI PROTEIN EXPRESSION IN THP-1 CELLS

IL-1RtI protein was expressed in unstimulated THP-1 cells (n=1) which were incubated for 24 hours at 37°C (data not shown). Figure 3.21. shows that unstimulated cells at the 0 hour time point did not express detectable amounts of IL-1RtI protein (lane 7), but at 24 hours (without an additional stimuli) they appear to express higher levels of IL-1RtI (lane 6). IL-4 (10 ng/ml) appeared to upregulate the expression of IL-1RtI protein after 24 hours (lane 5) compared to unstimulated cells (lane 7). A statement on this result cannot be made with certainty, because the protein loading appears unequal.

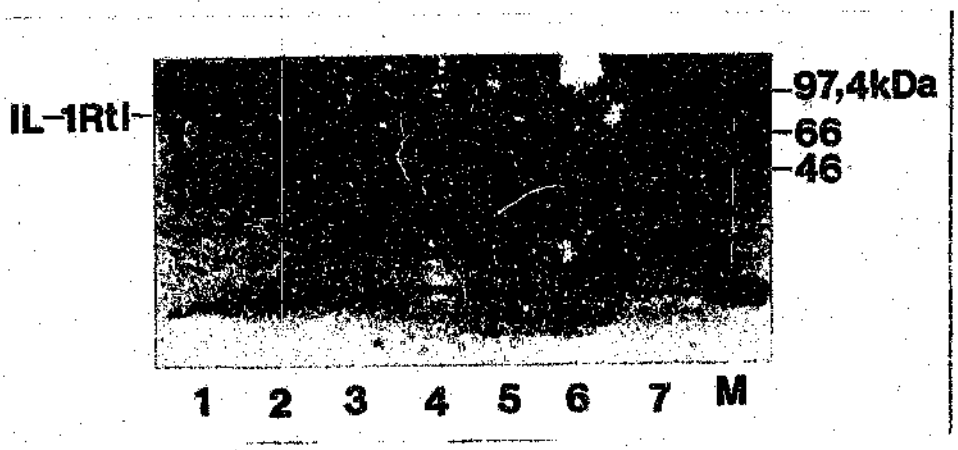


Figure 3.21. The expression of IL-1RtI protein in THP-1 cells
Western blot and immunodetection of IL-1RtI protein after 24 hour stimulations (n=1). Proteins (75 µg) were separated by denaturing (SDS) PAGE (4 % stacking gel and a 10 % resolving gel). Stimuli: lanes 7, 6: unstimulated (0 and 24 hours respectively); lane 5: IL-4 (10 ng/ml); lane 4: IL-1α (10 ng/ml); lane 3: IL-1α + IL-4; lane 2: IL-1β (100 ng/ml); lane 1: IL-1β + IL-4. Lane M: rainbow molecular weight marker.

3.14. IMMUNOPRECIPITATION OF IL-1RtI AND IL-1RtII PROTEIN

Total cellular protein was isolated from adhered monocytes and HL-60 cells and quantified (see 2.9.). Either IL-1RtI or II was detected by specific primary antibodies. IL-1RtI or II was then precipitated out of solution with a secondary antibody linked to protein G (see 2.11.). After the removal of protein G the precipitated protein (either IL-1RtI or II) was separated by denaturing (SDS) PAGE and either stained with Coomasie blue or a silver stain, or Western blotted and immunodetected as in 2.10.

3.14.1. IMMUNOPRECIPITATION OF IL-1RtI PROTEIN FROM ADHERED MONOCYTES

It was proposed that protein bands of greater intensity would result if the IL-1RtI protein was immunoprecipitated. IL-1RtI protein was immunoprecipitated (see 3.11.1.) from total cellular protein (50 μg) isolated from adhered monocytes. The adhered monocytes had been previously stimulated with 10 ng/ml IL-4 from 3 donors and the protein run on a denaturing (SDS) PAGE gel (10 %). The immunoprecipitated protein was Western blotted and immunodetected by a specific IL-1RtI antibody (see 2.10.).

Figure 3.22. shows that in 2 of the 3 donors (donors 2 and 3), IL-4 (10 ng/ml) upregulated the expression of IL-1RtI protein (80 - 88 kDa protein in lanes 4 and 2), while IL-4 downregulated the expression in donor 1 (lane 6) compared to unstimulated cells (lanes 7, 5, and 3).

It is interesting to note that the large protein (< 220 kDa), which appeared on the conventional Western blots, still appears here after the immunoprecipitation and Western blotting. It is possible that the anti-IL-1RtI recognises an epitope in this unrelated protein, or this large protein represents a complex either of two IL-1RtI molecules or other co-regulatory molecules. It seems unlikely that complexes would form under these strongly denaturing conditions. The heavy and light chains of the primary antibody can be seen on the gel (indicated by the arrows).

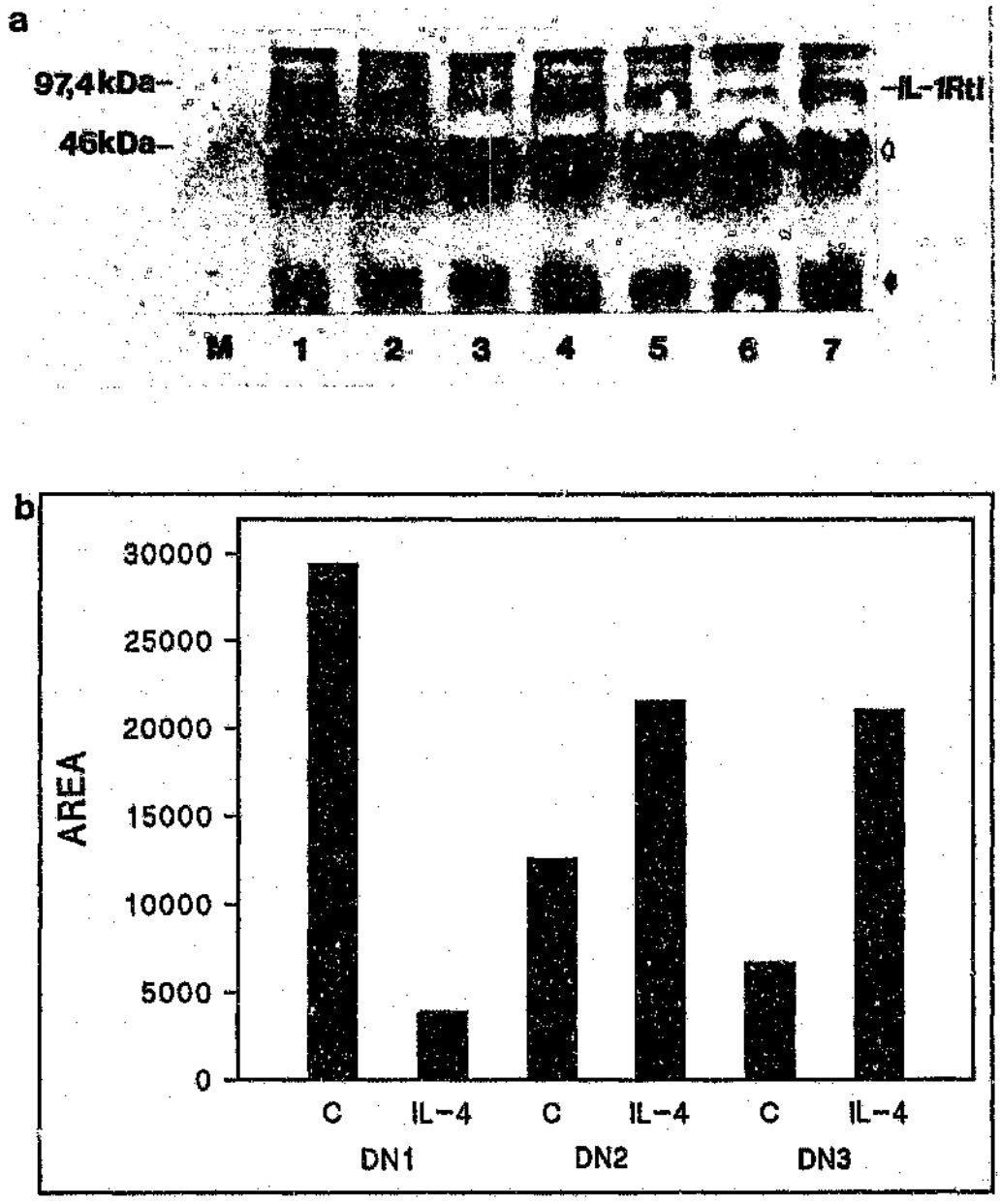


Figure 3.22. The immunoprecipitation and immunodetection of IL-1RtI protein in adhered monocytes.

a. Western blot of immunoprecipitated IL-1RtI protein after 24 hour stimulations (see 3.12.1.). Lanes 7-6: donor 1; lanes 5-4: donor 2; lanes 3-2: donor 3. Stimuli: lane 1: dexamethasone (100 nM) (nonadherent lymphocytes); lanes 7, 5, 3: unstimulated; lanes 6, 4, 2: IL-4 (10 ng/ml). Lane M: rainbow molecular mass marker. The arrows (◊ ♦) indicate the heavy and light chains, respectively, of the primary antibody.

b. Densitometric scanning of the bands in a. The relative absorbance of each band was converted into an area (in arbitrary units).

3.14.2. IMMUNOPRECIPITATION OF IL-1RtII PROTEIN FROM ADHERED MONOCYTES AND HL-60 CELLS

The primary antibody directed against the IL-1RtII protein was not suited to Western blotting, and unfortunately it was the only one commercially available. The IL-1RtII Western blots showed no specific bands (data not shown). Thus the anti-IL-1RtII was used instead to immunoprecipitate the IL-1RtII protein.

During the procedure of immunoprecipitation (see 2.11.) it was important to incubate the isolated protein with the protein G - secondary antibody complex before proceeding with the primary antibody step. It is important to perform this preliminary step to remove non-specific proteins, and therefore improve the results obtained.

Figure 3.23. represents a SDS PAGE gel stained with Coomasie blue. Lanes 2 and 3 show the multiple bands due to the nonspecific binding of the protein G - secondary antibody complex to other proteins in the sample. Lanes 4 and 1 show the positions of the primary and secondary antibody on the PAGE gel.

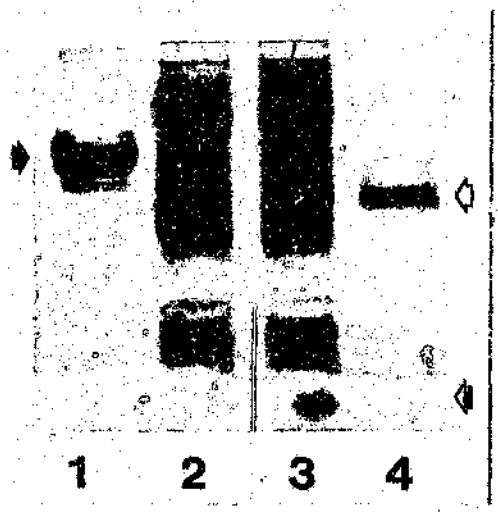


Figure 3.23. The effect of nonspecific binding of proteins by the protein G - secondary antibody complex on the immunoprecipitation of IL-1RtII protein.
Immunoprecipitated protein separated by denaturing (SDS) PAGE (4 % stacking gel and 10 % resolving gel). The gel was stained with Coomasie blue in order to visualise the bands. Lane 2 and 3 represent immunoprecipitated protein from adhered monocytes. Lane 1 represents secondary antibody (5 µg) (◗) and lane 4 represents primary antibody (1 µg) (◊◊heavy and light chains).

Total cellular protein was isolated from adhered monocytes and HL-60 cells. IL-1RtII protein was immunoprecipitated from the protein isolate and run on a denaturing (SDS) PAGE gel (10 %).

Figure 3.24.A. shows the Coomasie blue stained gel, and figure 3.24.B. shows the same gel restained with a silver stain (see 2.11.3.). In both figures IgG antibody bands (approximately 55 kDa) were seen (open and solid arrows). In the silver stained gel (figure 3.24.B.) additional bands could be visualised

with the more sensitive stain (thin arrow). One of these bands (± 63 kDa) which appears just below the band indicated by the thin arrow was possibly the IL-1RtII protein, as the reported size of the type II receptor is 60 - 68 kDa (Benjamin and Dower *et al.* 1990).

Duplicate PAGE gels of the gels in figure 3.24.A. and B. were run in order that the gels could be Western blotted and the immunoprecipitated IL-1RtII protein could be immunodetected. As the IL-1RtII primary antibody was not suited to immunoblotting, no specific IL-1RtII band could be detected on the Western blot (figure 3.24.C.). As a result it could not be confirmed that the ± 63 kDa band detected on the silver stained gel was IL-1RtII protein.

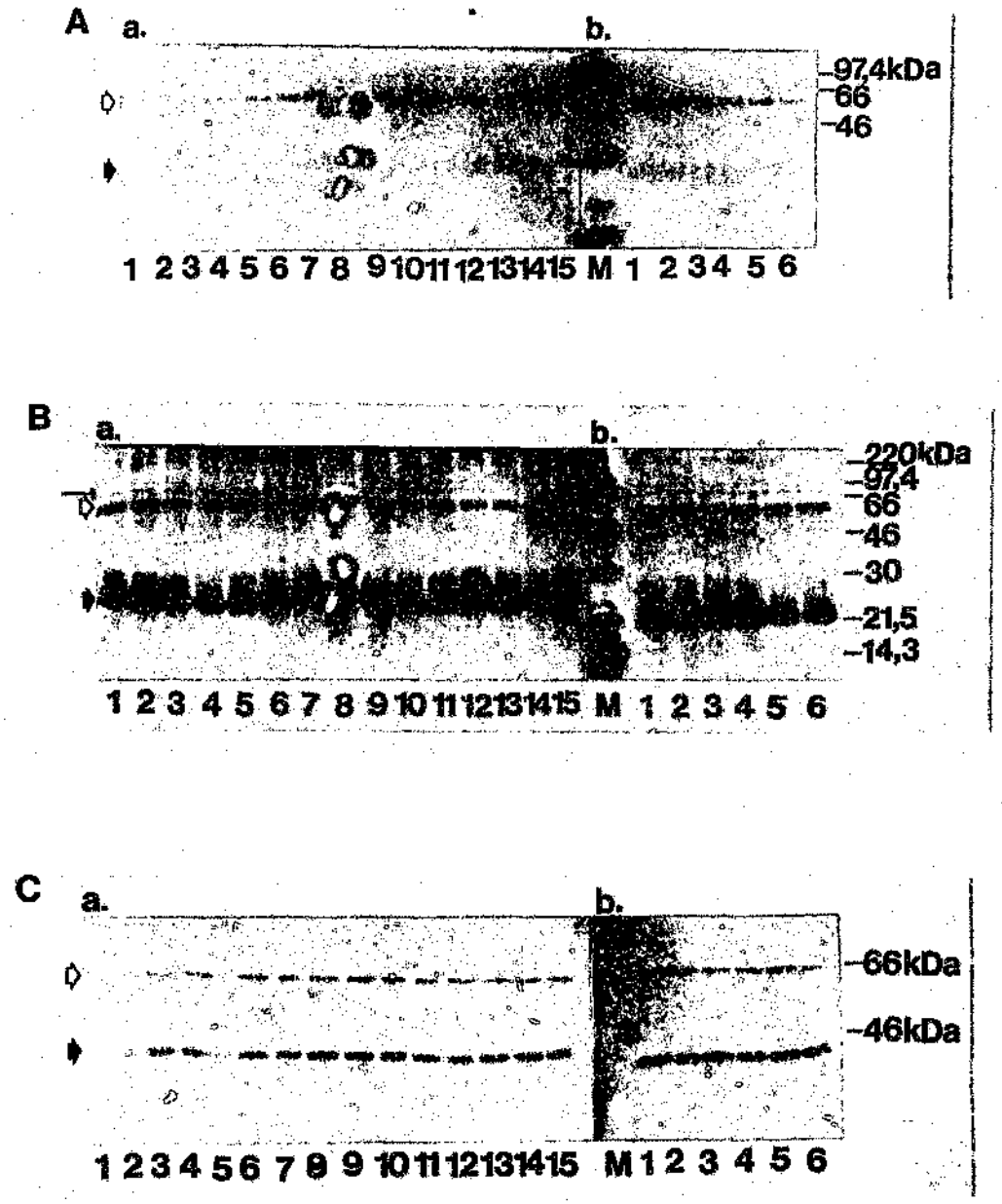


Figure 3.24. The immunoprecipitation and immunoblotting of IL-1RtII protein in adhered monocytes and HL-60 cells.
Denaturing (SDS) PAGE gels (4 % stacking and 10 % resolving) of immunoprecipitated IL-1RtII protein from adhered monocytes (b, lanes 1-15) and HL-60 cells (a, lanes 1-6). The gels were stained with Coomasie blue (A.) and then with a silver stain (B.). ◊ indicates the heavy chain of the secondary antibody, while ◆ indicates the position of the light chain. A ± 63 kDA band was detected (just below the→) after silver staining. The rainbow molecular mass marker appears in lane M. A duplicate gel of the gel in A. and B. was Western blotted and an attempt was made to immunodetect the IL-1RtII protein (C.). Again the secondary antibody was detected due to nonspecific binding of the secondary antibody during immunodetection.

SECTION 4

DISCUSSION

This report discusses the effects of PMA, IL-4, dexamethasone, and IL-1ß on the expression of IL-1ß, IL-1RtI, and IL-1RtII in peripheral blood monocytes and the leukaemic cell lines, HL-60 and THP-1. IL-1RtI is the functional, signalling IL-1R, whereas IL-1RtII is reported to be a nonsignalling "decoy" IL-1R which as a molecular trap for IL-1ß, hence inhibiting its action on a cell (Sims *et al.* 1994).

The main objective of this project was to confirm findings in the literature (Colotta *et al.* 1993a) about the antiinflamatory effects of IL-4 and dexamethasone on monocytic cells with respect to IL-1ß, IL-1RtI and IL-1RtII expression. The effects of these agents have been studied in neutrophils (Colotta *et al.* 1993a and Re *et al.* 1994). If it is found that IL-1RtI and II are regulated by IL-4 and dexamethasone in a similar manner in HL-60 and THP-1 cells compared to normal monocytes, then these two cell lines could be used in future as a model for studying anti-inflammatory agents on monocytes. On the other hand, if a difference is found in the regulation of IL-1RtI and II in the HL-60 and THP-1 cells, then these differences could be investigated further. An altered regulation of expression of IL-1Rs in leukaemic cells indicates the possible altered regulation of the action of IL-1 on these cells, since IL-1RtII acts as an inhibitor of IL-1 action. It has been shown that IL-1

is constitutively produced by many monocytic leukaemias and that IL-1 may act in an autocrine, paracrine, and endocrine manner to induce proliferation in these cells (Dinarello and Wolff 1993). An alteration in the regulation of IL-1's action on leukaemic cells (possibly caused by an altered IL-1RtI and II expression) would affect the proliferative activity of the cells. The degree of proliferation correlates with the clinical course of the disease (Sullivan 1993).

The effects of differentiation on the expression of the IL-1Rs was also analysed, ie. is the expression of IL-1Rs affected by differentiation?

4.1 LYMPHOCYTE ACTIVATING FACTOR ASSAY

Before beginning with experiments, the biological activity of the IL-1ß to be used was determined using the lymphocyte activating factor (LAF) assay (Gery *et al.* 1972). Proliferation due to IL-1ß (0,156 - 10 ng/ml) stimulation was measured by [^{3}H]-thymidine uptake into the thymocyte DNA. IL-1ß caused thymocyte proliferation in a dose dependent manner, the higher the IL-1ß concentration, the more the proliferation (see figure 3.1.). These results showed that the IL-1ß was biologically active.

4.2. CD14 AND CD11b SURFACE ANTIGEN EXPRESSION

The cells chosen for this were cells of the myelocytic cell lineage. Normal peripheral blood monocytes were studied, as well as the promyelomonocytic cell line HL-60 and the acute monocytic cell line THP-1. HL-60 (Collins 1987) and THP-1 (Tsuchiya *et al.* 1980) cells have been used as models for studying

monocytic cells under conditions of differentiation. The cell lines were used in this project to study the antiinflammatory effects of IL-4 and dexamethasone and to determine if the leukaemic cells differed in any way to normal monocytes, which might help to explain their leukaemic state.

The purpose of determining the CD14 surface antigen expression in the cells was twofold. Firstly, to determine the degree of purity of monocyte enriched fractions of cells, and secondly, to characterise the cells in the case of the HL-60 and THP-1 cell lines, as CD14 is a monocytic cell marker (Horejsi 1991).

Of the two methods used to produce a monocyte rich fraction of cells, the Ficoll - Percoll density gradient centrifugation method (see 2.1.4.) produced the highest purity of CD14 positive (monocytic) cells (75,5 %, n=1), but the number of cells recovered was too low to utilise for experiments. An enriched monocyte fraction consisting of adherent cells (see 2.1.3.) was made by adhering the monocytes to plastic. Unfortunately this fraction was contaminated with a CD14 negative group of cells which were possibly B-cells. Thus the adherent fraction could only be considered as a monocyte enriched fraction.

As it was difficult to obtain a relatively pure fraction of monocytes from blood, the monocytic cell lines, HL-60 and THP-1 were used to study the effects of PMA, IL-4, dexamethasone, and IL-1ß on the expression of IL-1ß, IL-1RtI, and IL-1RtII. The CD14 and CD11b surface antigen expression was

determined in these cells, in order to characterise the cells. It was found that the THP-1 cells cultured here expressed low CD14 levels, therefore the CD11b surface antigen expression was analysed. The original THP-1 cell line expressed CD11b (C3b receptor) (Tsuchiya *et al.* 1980). The THP-1 cell line we used expressed very low CD11b antigen levels (8,8 - 11,6 %) as determined by flow cytometry. Differentiation (as indicated by an increase in adherence of the cells) with PMA only slightly increased this expression, indicating that the THP-1 cells had possibly altered their characteristics to a certain extent while in culture. It was possible that the PMA was not functional, although this seems unlikely as PMA did induce adherence in THP-1 cells after 24 hours.

The HL-60 cell line too showed very low CD11b expression. Differentiation of HL-60 cells to more mature monocytic cells leads to an increase in CD11b surface antigen expression (Collins 1987). Again PMA did not upregulate the CD11b expression in these cells after 24 hours. Either the 24 hours exposure to PMA was not long enough to induce differentiation in these cells (only a small number of the HL-60 cells became adherent after 24 hours, while the cells were almost completely adherent after 48 hours), or the HL-60 cells had also possibly altered their characteristics. Despite this, the THP-1 and HL-60 cells were used to study the expression of IL-1R types in monocytic cells. Possibly an alternative differentiating agent for HL-60 cells should have been used, which would have induced adherence and therefore an increase in the monocytic marker CD11b after 24 hours. TPA was used originally by Collins

(1987) to differentiate HL-60 cells.

4.3. NORTHERN BLOT ANALYSIS OF IL-1ß mRNA EXPRESSION

IL-1ß mRNA levels were first analysed in monocytic cells in this project to confirm findings in the literature, namely that IL-4 (Donnelly *et al.* 1991) and dexamethasone (Lee *et al.* 1988) both act as antiinflammatory agents by decreasing the expression of IL-1ß mRNA.

The initial aim here was to determine if IL-1α or IL-1ß was a more efficient inducer of IL-1ß expression. This result would then determine which of these two cytokines would be used in future experiments. The IL-1RtII is a nonsignalling "decoy" receptor which has a higher affinity for IL-1ß than IL-1α (Sims *et al.* 1994). Thus it would be expected that IL-1α would be a more potent inducer of IL-1ß mRNA expression than IL-1ß, since the signalling receptor has a higher affinity for IL-1α. At 3 different time points (1 hour 15 minutes, 2 hours and 5 hours 30 minutes), IL-1ß appeared to induce IL-1ß mRNA expression to a greater extent than IL-1α (see figure 3.3.). It is difficult to interpret this result, as the experiment was only done once, and the large diffuse bands on the autoradiograph made the densitometric scanning difficult. Despite these drawbacks a clear trend can be seen.

IL-4 (Donnelly *et al.* 1991) and dexamethasone (Lee *et al.* 1988) act as anti-

inflammatory agents by inhibiting the expression of proinflammatory IL-1ß in monocytic cells. To confirm this data, adhered monocytes were treated with IL-4 or dexamethasone for 4 hours. A trend could be seen where IL-4 and dexamethasone inhibited the production of IL-1ß mRNA compared to unstimulated cells (figure 3.5.), which expressed IL-1ß mRNA. The high level of IL-1ß mRNA in unstimulated cells indicated that the cells had been either mechanically stimulated during the isolation procedure, or that they had been stimulated due to contamination with LPS of the anticoagulant, heparin. The GAP-DH bands on the autoradiograph were difficult to scan, possibly because the GAP-DH expression in the cells was low, or because the GAP-DH probe did not hybridise efficiently during Northern blotting. There was also a large degree of variation between donors with respect to GAP-DH levels. The above results confirm the findings in the literature that IL-4 and dexamethasone act as antiinflammatory agents by decreasing the expression of IL-1ß mRNA. IL-1ß has many proinflammatory properties (Dinarello 1994), thus a decrease in IL-1ß production would help to inhibit the inflammatory process.

LPS stimulated the production of IL-1ß compared to unstimulated cells (figure 3.6.), and this increase in IL-1ß mRNA expression was inhibited by IL-4. IL-4 also inhibited the increased expression of IL-1ß mRNA when the stimulus was IL-1ß. This confirms data generated by Estrov *et al.* (1993b) and Banchereau *et al.* (1994). The antiinflammatory effects of IL-4 can partly be explained by its ability to decrease IL-1ß mRNA levels. This data gives no indication

whether this result is due to an decrease in IL-1ß gene expression or if it is due to a destabilising effect on the IL-1ß mRNA itself. Donnelly *et al.* (1991) have shown that IL-4 affects IL-1ß mRNA levels at the transcriptional and posttranscriptional level (ie. affecting IL-1ß mRNA stability). Although this result was only obtained from two donors, the trend that IL-4 inhibits the expression of IL-1ß is clear.

IL-4 and dexamethasone not only act by decreasing IL-1ß mRNA levels, but also by upregulating the expression of the IL-1RtII. It has now been established (using antibodies directed against IL-1RtI and II) that IL-1RtII acts as a decoy target for IL-1ß, and is a nonfunctional IL-1 receptor. Thus the upregulation of the IL-1RtII inhibits the action of IL-1. This has been extensively studied in neutrophils (Colotta *et al. 1993)*, but not to any great extent in monocytic cells. Unfortunately, attempts to detect IL-1RtI and II by Northern blotting were unsuccessful. The Northern blot radiolabelled probe for IL-1RtII appeared to be unsuitable for hybridisation, as a high degree of nonspecific binding occurred (see 3.7.). Thus in order to study the expression and regulation of IL-1RtI and II mRNA levels, the method of RT-PCR was employed. Although RT-PCR is a sensitive method for the detection of specific mRNA species, it was difficult to quantitate the results using this technique.

4.4. OPTIMISING CONDITIONS FOR RT-PCR

Many variables exist in a PCR reaction mixture. These include the $MgCl_2$, primer, and dNTP concentrations, as well as the relative abundance of the target sequences being amplified. Since a PCR results in the exponential amplification of a target cDNA sequence, changing the PCR conditions even slightly can greatly alter the end result.

The PCR conditions for each set of primers has to be optimised in order to prevent the occurrence of false negative results, ie. where the mRNA species exists in the cell but the PCR conditions are such that this message is not converted to cDNA or is not amplified sufficiently to be detected.

The $MgCl_2$ in the PCR mixture is required for the functioning of the DNA polymerase. It was found, in this study, that $MgCl_2$ concentrations of 3,5 mM in the PCR mixture inhibited the amplification of the target cDNA sequence, compared to the lower concentration of 2,0 mM (see figure 3.8.). This latter $MgCl_2$ concentration was found to suit all of the primer sets.

An internal standard (the ubiquitous house-keeping gene, GAP-DH) was used in the PCR to ensure that equal RNA was added to the RT reaction, and to monitor the first-strand synthesis and amplification of the sequences during RT-PCR. GAP-DH and, for example, IL-1RtII primers were used to co-amplify sequences in the same reaction tube so as to remove the effect of tube to tube variations (Dukas *et al.* 1993 and Larrick *et al.* 1988). This method is

the PCR mixture. Here a similar rate of amplification of the cDNA sequences will be achieved. Unfortunately in HL-60 and THP-1 cells the GAP-DH mRNA levels appeared to be relatively more numerous compared to IL-1RtI and II mRNA levels. Thus the GAP-DH primer levels had to be reduced in order to co-amplify both target sequence and internal standard. The GAP-DH primer concentration was lowered to 0,01 μM, while the target sequence (eg. IL-1RtII) primer concentration was maintained at 0,25 μM. Under these conditions the rate of amplification of GAP-DH PCR product was decreased due to a low primer concentration, and both PCR products could be visualised on the agarose gel. If the GAP-DH primer concentration was increased to 0,1 μM, the GAP-DH PCR product accumulated rapidly, and possibly this high concentration of PCR product inhibited the amplification of the target sequence, as only a GAP-DH PCR product band could be detected on the agarose gel.

In addition, a high primer concentration resulted in the formation of primer dimer. Here the primers bind to each other and are amplifed to form a small PCR product. This primer dimer PCR product can accumulate rapidly and possibly inhibit the amplification of the target sequence (see figure 3.9.). Lowering the primer concentration decreases the primer dimer formation, which in turn results in a higher yield of target PCR product, thus primer dimer formation influences the quantification of RT-PCR.

specifically to their complementary sequences and no other. The annealing temperature for the primers was taken as 8 - 10°C below the average of the melting points of the two primers. If the annealing temperature was nearer the average melting points then the primers were more likely to bind nonspecifically to other areas in the cDNA sequences. Multiple PCR product bands resulted after the IL-1RtI primer annealing temperature was set at 2°C below the average melting point of the primers. When the annealing temperature was lowered by a further 7°C one specific PCR product of the correct size was amplified.

As mentioned above GAP-DH was used as an internal control for the equal loading of RNA to Northern blots and as an internal standard or reporter gene in RT-PCR. The important characteristic of a reporter gene, is that its expression remains at a constant level under conditions where the expression of other genes change. Dukas *et el.* (1993) stated that GAP-DH expression did not vary to a great extent during cell growth, and it was based on this that GAP-DH was chosen as the internal standard for RT-PCR. It has been noted that the GAP-DH gene expression is controlled by the cell cycle. GAP-DH levels were shown to increase up to 19 fold under conditions inducing DNA synthesis. Thus Mansur *et al.* (1993) state that GAP-DH can be used as a reporter gene when the "proliferative state" of the cells is kept constant. Thus GAP-DH must be used in differentiation experiments with caution. Bhatia *et al.* (1994) found that GAP-DH mRNA levels differed markedly in different

compare results between cell lines using GAP-DH. In this study GAP-DH levels were found to differ in the monocytes from different donors, making the quantitation of results difficult. GAP-DH levels do not always remain the same in cells. Endothelial cells exposed to hypoxia show elevated expression of GAP-DH (Graven *et al.* 1994), while the differentiating agent TPA also altered GAP-DH levels in the cell (Spanakis 1993). Considering the above, an alternative to GAP-DH as reporter gene should possibly have been used, for example, the 28S rRNA gene (Khan *et al.* 1992).

4.5. RT-PCR ANALYSIS OF IL-1β mRNA LEVELS

RT-PCR is a sensitive technique able to detect mRNA species occurring at very low concentrations in the cell. Care must be taken when this technique is used to detect an mRNA produced in abundance, as amplification of the target sequence is exponential and therefore these PCR products accumulate rapidly. The intensity of a PCR product band as visualised on a agarose gel should increase with an increase in the number of PCR cycles in a linear fashion (Dukas *et al.* 1993). This linear relationship is lost when high concentrations of the PCR product inhibit the DNA polymerase, or when one or more of the PCR mixture components (eg. the dNTPs or primers) is exhausted. Thus the accumulation of PCR product reaches a plateau, ie. as the cycles proceed the band intensity no longer increases at the same rate. Unfortunately this plateau effect can occur in different tubes at different times, often obscuring the results.

The results of the IL-1ß Northern blot in figure 3.5. and the RT-PCR IL-1ß results in figure 3.10. and 3.11. were compared. The RT-PCR data showed no difference between stimulated and unstimulated cells in the RT-PCR experiments, while clear differences were obtained using the Northern blot technique. The IL-1ß PCR product in figure 3.10. and 3.11. was obtained after a 30 cycle PCR. The linear relationship between the PCR product band intensity on an agarose gel and the number of PCR cycles has most likely been lost. The rate of amplification of samples with a higher IL-1ß mRNA concentration would begin to decrease or plateau (due to product inhibition or exhaustion of a component in the PCR mixture) while the rate of amplification of samples with less numerous IL-1ß mRNA would only plateau at a later stage. Thus the intensities of the PCR product bands would begin to approach each other as the number of PCR cycles increased. This could explain why similar band intensities resulted. The other explanation could be that the IL-1ß mRNA levels were similar in all the samples, but in light of the Northern blot data in figure 3.5. this seems unlikely. Decreasing the number of PCR cycles and determining the linear range of cycles would possibly improve the data from this experiment (Dukas *et al.* 1993).

4.6. RT-PCR ANALYSIS OF IL-1RtI mRNA LEVELS

IL-1RtI is the signalling, functional IL-1R (Colotta *et al.* 1993a). IL-1 responsive cells express IL-1RtI on their cell surfaces. It has been shown by Simon *et al.* (1994), that IL-1ß upregulates the expression of the IL-1RtI in

endometrial stromal cells. The aim here was to determine if IL-4 and dexamethasone modulate the expression of IL-1RtI.

Since no Northern blot data on the effects of IL-4 and dexamethasone on IL-1RtI mRNA levels could be generated, IL-1RtI mRNA levels were detected using RT-PCR. IL-1RtI was detected in adhered monocytes, HL-60 cells, and THP-1 cells. Up to 40 cycles were needed to detect the IL-1RtII mRNA species in THP-1 cells, indicating a low expression. There was no difference in the expression of IL-1RtI mRNA after 4 hours of stimulation with PMA, IL-4, dexamethasone, or IL-1β in adhered monocytes, HL-60, and THP-1 cells. Does this result reflect the situation within the cell, ie. is there IL-1RtI mRNA is unstimulated cells (as the data would suggest), or is this result an artifact of the RT-PCR technique used? The number of cycles used to detect the IL-1RtI mRNA may be too high and that the amplification of the band verses the number of PCR cycles is no longer linear. This would result in PCR product bands of similar intensities on a agarose gel. This seems unlikely as in THP-1 cells, the bands are faint and up to 40 cycles were needed to detect IL-1RtI mRNA. Thus it is probable that THP-1 cells express low levels of IL-1RtI. The data does suggest that IL-1RtI mRNA is constitutively produced in all three cell types, as IL-1RtI mRNA was detected in unstimulated cells. The expression in adhered monocytes could have been due to mechanical stimulation or contamination of heparin (the anticoagulant used in the isolation of monocytes) with LPS, but this could not explain the expression in HL-60 and THP-1 cells.

4.7. RT-PCR ANALYSIS OF IL-1RtII mRNA LEVELS

Shieh *et al.* (1993) demonstrated that IL-4 increased serum glucocorticoids, and that the synthetic glucocorticoid dexamethasone upregulated IL-1RtII protein (in neutrophils). Colotta *et al.* (1993a) showed that IL-4 increases the expression of IL-1RtII mRNA in neutrophils.

IL-1RtII mRNA levels were detected using RT-PCR in adhered monocytes and THP-1 cells. Unfortunately no data for HL-60 cells was obtained. The data appearing in figure 3.14. was obtained by RT-PCR using adhered monocyte RNA. Clear differences can be seen in the IL-1RtII PCR bands (indicating that the rate of amplification of the PCR product had not yet plateaued). The data from figure 3.14. shows that IL-4 and dexamethasone increase the amount of IL-1RtII mRNA found in adhered monocytes after 4 hours of stimulation. The result is from 3 different donors, with each donors' monocytes reacting in a similar fashion. From these results it cannot be determined if this increase in IL-1RtII due IL-4 or dexamethasone was due to an increase in IL-1RtII gene transcription or due to a stabilisation of the IL-1RtII mRNA. It has been shown that IL-4 (Donnelly *et al.* 1991) and dexamethasone (Lee *et al.* 1988) both act at the transcriptional and post transcriptional level to decrease TNF-1ß mRNA levels. It is possible that these agents act in a similar manner to increase the steady state levels of IL-1RtII mRNA.

Figure 3.15. shows that IL-4 increases the expression of IL-1RtII mRNA in THP-1 cells (n=1). In the above experiments, an increase in IL-1RtII mRNA is seen upon stimulation with the antiinflammatory agents IL-4 and dexamethasone. An increase in the IL-1RtII "decoy" receptor suggests an additional mechanism for the anti-inflammatory properties of IL-4 and dexamethasone. IL-1ß preferentially binds to the IL-1RtII "decoy", which acts as a molecular trap for IL-1ß. With ar increase in the IL-1RtII, there will be an inhibition of the effects of the proinflammatory IL-1ß on the cell. The presence of an mRNA species in a cell does not guarantee the translation of that message. However, an increase in the production of IL-1RtII mRNA might imply that IL-4 and dexamethasone will cause an increase in the amount of IL-1RtII protein on the cell surface.

The IL-1ß was shown not to affect the IL-1RtII mRNA levels in adhered monocytes after 4 hours. Dubois *et al.* (1991) generated data using haematopoietic bone marrow cells. It was suggested that IL-1 indirectly increases IL-1RtII on these cells, possibly by inducing accessory cells to secrete growth factors such as GM-CSF, which would in turn increase IL-1RtII expression. Therefore in a mixed population of cells, IL-1ß may be unable to affect IL-1RtII expression, as accessory cells / factors are required. IL-1ß possibly increases IL-1RtII expression as part of a negative feedback mechanism to regulate the action of IL-1ß.

4.8. RIA ANALYSIS OF IL-1ß PROTEIN

The anti-inflammatory effects of IL-4 were demonstrated at the mRNA level, ie. it was shown that IL-4 decreased the IL-1ß mRNA levels when IL-1ß was stimulated by itself or by LPS (see figure 3.5.). An RIA was used to measure the levels of IL-1ß protein after stimulation with IL-4 in PBMNCs. IL-4 was shown to inhibit the expression of IL-1ß compared to unstimulated cells after 24 hours. In addition when IL-1α or LPS was used to induce the expression of IL-1ß, IL-4 inhibited this increase in IL-1ß production. It can therefore be said that IL-4 acts to decrease the production of IL-1ß at the mRNA and at the protein level. IL-1ß is mainly a secreted protein (Kostura *et al.* 1989). This is confirmed by the RIA data here. IL-1α induces the synthesis of cell associated and secreted IL-1ß, but it induces the synthesis and secretion of secreted IL-1ß to a greater extent. Secreted IL-1ß is then able to play an important role in mediating the local and systemic responses to infection (Molloy *et al.* 1993). This data confirms previous studies that show that IL-4 decreases IL-1ß protein levels (Fenton *et al.* 1992). Thus, the data presented here indicates that, at the mRNA and protein level, IL-4 acts to decrease IL-1ß production, thus modulating the action of this proinflammatory cytokine.

4.9. ANALYSIS OF IL-1RtI PROTEIN BY WESTERN BLOTTING AND IMMUNODETECTION

The data on the regulation of IL-1RtI mRNA by IL-4 and dexamethasone was inconclusive, ie. it could not be determined whether these stimuli affected IL-1RtI at the mRNA level. The production of IL-1RtI protein was analysed by Western blotting and immunodetection. (see 2.9.). The presence of the IL-1RtI protein is difficult to demonstrate. Most of the studies have used inhibitory IL-1RtI antibodies to demonstrate the presence and function of IL-1RtI (Colotta *et al.* 1993a). Re *et al.* (1994) showed that IL-4 increased Il-1RtI in neutrophils, and Monick *et al.* (1994) showed that dexamethasone did not decrease IL-1RtI.

IL-1RtII protein was detected in PBMNCs (n=1) by Western blotting. PBMNCs represent a mixed population of cells, therefore the role of the lymphocytes in the mixed PBMNC population was analysed. Based upon visual assessment of the Western blot, IL-4 also seemed to upregulate the expression of IL-1RtI in lymphocytes. IL-4 has been implicated in the proliferation of T cells (Mori *et al.* 1995). Therefore this increase in IL-1RtI could play a part in that activation. When the nonadherent lymphocyte population was stimulated with dexamethasone, there appeared to be an increase in the production of IL-1RtI protein. These results complement the findings in the literature, although it must to taken into account that in the experiment only 2 of 3 donors responded in this way. IL-4 upregulated (n=1) the expression of IL-1RtI in THP-1 cells (see figure 3.21.).

IL-4 increases the mRNA for the "decoy" IL-1RtII, thus inhibiting the action of IL-1 on the cell. It has also been found (both here and in the literature that IL-4 increases the expression of the signalling IL-1RtI. It is possible that this effect on IL-1RtI protein found in THP-1 cells is part of a system of checks and balances, were the responsiveness of the cells to IL-1ß is modulated.

A time course of IL-1RtI production in HL-60 cells was performed. The HL-60 cells were found to constitutively express IL-1RtI protein, as the protein was detected in unstimulated cells. Dexamethasone inhibited the expression of IL-1RtI in these monocytic cells (n=1). At both 4 hours and 24 hours, dexamethasone inhibited the production of IL-1RtI protein by 50 % (see 3.19.). It is possible that dexamethasone was cytotoxic to the HL-60 cells (although the cells appeared healthy), and that dexamethasone did not truly downregulate IL-1RtI. In addition, Re *et al.* (1994) found that dexamethasone increased the expression of IL-1RtI in neutrophils.

Phorbol esters such as 12-o-tetradecanoyl phorbol-13-acetate (TPA) are known to differentiate HL-60 cells towards a more macrophage - like state (Collins *et al.* 1987). The effect of the phorbol ester and differentiating agent, PMA, on the expression of IL-1RtI protein was investigated. PMA had little effect on the levels of IL-1RtI mRNA in the cells tested. The IL-1RtI protein levels were therefore analysed after 24 hours of exposure to PMA. HL-60 cells began adhering to the plastic petri dishes after 24 hours of PMA treatment, indicating that the cells were beginning to differentiate towards cells with

more macrophage - like characteristics. PMA upregulated the expression of IL-1RtI protein compared to unstimulated cells (n=5), indicating that differentiation results in an increase in the signalling IL-1R in HL-60 cells.

4.10. IMMUNOPRECIPITATION OF IL-1RtI PROTEIN

No data could be obtained by Western blotting about the effects of IL-4 on the expression of IL-1RtI protein. IL-1RtI was therefore immunoprecipitated from a total cellular protein extract from adhered monocytes. The immunoprecipitated IL-1RtI protein was then Western blotted and immunodetected. This increased the amount of IL-1RtI protein that could be loaded on to the PAGE gels, which therefore increased the intensities of the protein bands detected after Western blotting and immunodetection. In two of the three donors, IL-4 upregulated the expression of IL-1RtI protein, while in the other donor, IL-4 markedly decreased the expression of IL-1RtI protein.

IL-4 upregulated IL-1RtI protein expression (in adhered monocytes, THP-1 cells, and HL-60 cells). Some of the experiments were only performed once and therefore their results must be interpreted with caution. Thus, IL-4 not only increases the expression of the inhibitory IL-1RtII, but also the signalling IL-1RtI.

4.11. IMMUNOPRECIPITATION OF IL-1RtII PROTEIN

Unfortunately, the IL-1RtII antibody available to me was not suitable for immunodetection after Western blotting. Thus the antibody was used to immunoprecipitate the IL-1RtII protein from a total cellular protein extract from adhered monocytes and HL-60 cells. The protein G - secondary antibody complex bound nonspecifically to many proteins in the sample. This can be seen when this nonspecific binding was not removed before starting with the specific precipitation. This can be seen in figure 3.24. which shows the multiple proteins bands visualised on the SDS PAGE gel stained with Coomasie blue.

The immunoprecipitated IL-1RtII protein from adhered monocytes and HL-60 cells were run on a SDS PAGE gel and stained with Coomasie blue. This stain was not sufficiently sensitive, as only the precipitating antibody was detected. The gel was then stained with a silver stain which proved to be far more sensitive. A protein band was detected which, using Rf values, was found to be approximately 63 kDa. This protein could possibly have been the IL-1RtII protein.

It was thought that a higher concentration of protein loaded on to the gel, would successfully result in the immunodetection of IL-1RtII protein after blotting. No protein band of the correct size was detected. This was either because no IL-1RtII was expressed in the cells, or because the IL-1RtII primary antibody was unsuitable for Western blotting and immunodetection.

In order to optimise the conditions for Western blotting, the following steps were taken. Between incubations with the primary antibody, the secondary antibody, and the streptavidin peroxidase, the blot was washed with great care so as not to dislodge the weakly binding IL-1RtII antibody. In addition, the detergent, Tween-20, was not added to the washes, but the antibody was still washed off and no specific IL-1RtII protein band was detected. The primary antibody (anti-IL-1RtII) did bind nonspecifically to the secondary antibody bands (heavy and light chains). As a result, no data on the expression of the IL-1RtII protein expression in these cells could be obtained. It is possible that there was no IL-1RtII protein to be detected in the cells, but this seems unlikely based on the IL-1RtII mRNA data and based on the findings of other groups (Colotta *et al.* 1993).

In order to solve this problem, the first step would be to repeat the experiment, possibly using a higher number of cells. As the IL-1RtII primary antibody is not suitable for immunoblotting, an alternative technique could be used in the future. The method of metabolic labelling with ^{35}S-methionine followed by the immunoprecipitation of the radiolabelled IL-1RtII protein would greatly enhance the level of detection. It is important to confirm that IL-4 and dexamethasone increase IL-1RtII protein expression. The mRNA data for IL-1RtII expression indicates that this is the case, but it cannot be assumed that a change in the expression of a gene at the mRNA level automatically means that the expression of that protein at the cell surface is altered in the same way.

SECTION 5

CONCLUSIONS AND FUTURE PROSPECTS

IL-1 plays an important role in the immune and inflammatory responses. IL-1 has many systemic and local effects, including fever, inducing the acute phase response, and affecting the expression of various genes which are also involved in the immune and inflammatory responses. IL-1 has been described as a proinflammatory cytokine, which can have deleterious effects when it is over produced (eg. IL-1 and TNF-α have been implicated in septic shock). Thus the regulation of the synthesis and action of this potent cytokine needs to be tightly controlled. There is more than one regulatory system for IL-1, which include the production of a unique IL-1ra, cytokines such as IL-4 and IL-10 down regulate IL-1 expression, and the regulation of IL-1 at the receptor level. IL-1 has two receptors, namely, IL-1RtI and IL-1RtII. IL-1RtI has been shown to be a functional receptor, expressed at low concentrations on the cell surface. Binding of IL-1 to this receptor elicits a response in the cell. IL-1RtII, on the other hand, is a nonsignalling receptor or "decoy" receptor which acts as a molecular trap for IL-1ß, thus preventing IL-1 from binding to the IL-1RtI. This represents an additional method of inhibiting the action of IL-1.

The main object of this project was to study the effects of IL-4, dexamethasone and IL-1ß, on the expression of IL-1RtI, IL-1RtII, and IL-1ß in monocytic cells. The cytokine, IL-4, and the synthetic glucocorticoid,

dexamethasone, have antiinflammatory properties, and have been reported to inhibit the expression of IL-1ß. In addition they have been implicated in the regulation of the IL-1R's in neutrophils, T helper-2 cells, and monocytes.

To confirm data in the literature, the effect of IL-4, dexamethasone, IL-1ß, and LPS on IL-1ß expression was investigated. IL-4 and dexamethasone both down regulated the expression of IL-1ß after 4 hours in adhered monocytes at the mRNA level as determined by Northern blotting (section 3.5.). Although no certain statement can be made, the results in figure 3.6. indicate that IL-4 decreased the expression of IL-1ß at the mRNA level, when this expression was induced by LPS or IL-1ß. At the protein level in adhered monocytes, LPS and IL-1α increased the expression of IL-1ß after 24 hours, and this increase was inhibited by IL-4 (section 3.12.). The results in figure 3.4 indicated that IL-1ß induced more IL-1ß than IL-1α was able to induce. Thus IL-4 and dexamethasone have been shown to have anti-inflammatory activities by inhibiting the expression of IL-1ß in monocytes. Thus the overproduction of IL-1ß can be inhibited by IL-4 or dexamethasone.

Using the method of RT-PCR it was found that IL-1RtI mRNA was expressed in unstimulated adhered monocytes and in the leukaemic HL-60 cells. Clark (1992) showed that IL-4 increased the expression of IL-1RtI in T helper-2 cells, and Re *et al.* (1994) showed that dexamethasone also increased the expression of IL-RtI. This study confirmed that IL-4 increased the expression of IL-1RtI protein (section 3.14.1.) in the adhered monocytes of two out of

three donors. The results also suggested that dexamethasone increased the expression of IL-1RtI protein in nonadherent lymphocytes (section 3.13.2.). Dexamethasone possibly decreased IL-1RtI protein in HL-60 cells, although this was a single experiment, and it was possible that dexamethasone was toxic to the cells (section 3.13.3.). Therefore the downregulation of IL-1RtII by dexamethasone was possibly not a true result. Thus IL-4 and dexamethasone not only increase the expression of IL-1RtII, but they paradoxically also increase the expression of IL-1RtI. An increase in IL-1RtII would inhibit IL-1's action, but an increase in IL-1RtI should have the opposite effect. Possibly the increase in IL-1RtI is part of a regulatory mechanism, controlling the action of these two anti-inflammatory agents, ensuring that they do not inhibit the responses of the cells to IL-1 completely.

IL-4 and dexamethasone increase the expression of the nonsignalling IL-1RtII in neutrophils (Colotta *et al.* 1993a). An increase in the expression of IL-1RtII inhibits the action of IL-1 on the cell. This study found that (using RT-PCR) IL-4 and dexamethasone after 4 hours increased the expression of IL-1RtII at the mRNA level in adhered monocytes. The results also suggested that (using RT-PCR) IL-4 increased the expression of IL-1RtII mRNA in THP-1 cells, although this was a single experiment. Thus IL-4 and dexamethasone act as antiinflammatory agents by increasing the expression of "decoy" IL-1RtII in adhered monocytes and maybe THP-1 cells.

The effects of differentiation on the leukaemic cell lines HL-60 and THP-1 on the expression of IL-1RtI and IL-1RtII was investigated. No data could be obtained for IL-1RtII at the protein level. Although differentiation of HL-60 cells with PMA for 24 hours did not induce a marked change in the adherence of HL-60 cells to the petri dishes, PMA did increase the expression of IL-1RtI to detectable levels using the Western blot technique. Thus it seems that differentiation of the leukaemic cell line HL-60 towards a more macrophage-like state results in an increases expression of IL-1RtI. One cannot say whether this would increase the responsiveness of the HL-60 cells to IL-1, as it could not be shown whether PMA caused a similar increase in IL-1RtII.

Thus from the above study it can be seen that IL-4 and dexamethasone do have antiinflammatory properties with respect to inhibiting IL-1 by increasing the expression of thee nonsignalling "decoy" IL-1RtII in adhered monocytes. It was asked at the beginning of this study if the leukaemic cell lines differed in their regulation of IL-1Rs compared to adhered monocytes. Although it cannot be said for certain, the results showed that IL-4 and dexamethasone had similar effects on adhered monocytes, HL-60 and THP-1 cells, with respect to the expression of IL-1RtI and II. Thus HL-60 and THP-1 cells could possibly be used as a model for studying the anti-inflammatory effects of IL-4 and dexamethasone on monocytes. A model for the study of monocytes is required, as pure fractions of peripheral blood monocytes are difficult to obtain, therefore it would be timesaving and convenient to use HL-60 and THP-1 cells instead.

In the future the results in the study need to be confirmed to determine if the results were significant or not. It was only possible to perform some of the experiments once or twice, and in some cases, due to technical difficulties, no data could be obtained. For example, little data could be obtained using the THP-1 cells due to difficulties encountered in culturing them. As THP-1 cells are more mature and therefore more monocytic in nature than HL-60 cells, they would appear more suitable as model for studying monocytic cells.

The regulation of IL-1 at the level of signal transduction, although extensively studied, is still in question. The function of NF-kB and other nuclear factors on IL-1 signal transduction have also been studied. One area of interest in the future would be the immediate events following IL-1 binding to its IL-1RtI.

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