# THE ROLE OF ARACHIDONIC ACID MOBILISATION IN MYELOID CELLS

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**1999**

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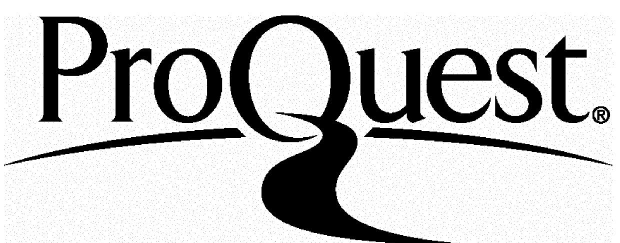

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*To*

*My parents*
*&*
*Dear brother, MEHDI,*
*&*
*In memory of*
*Ferreydon Soudavar*

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## ABSTRACT

Haemopoietic growth factors (GF) are important for regulating the proliferation and differentiation of immature cells, but granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 3 (IL-3) also have a role in regulating the function of mature phagocytic cells. The study presented in this thesis investigated the GF regulation of phospholipase $A_2$ ($PLA_2$) in immature and mature haemopoietic cells.

In mature neutrophils, respiratory burst (NADPH oxidase) and $PLA_2$ activity stimulated by the agonist FMLP can be enhanced (primed) by GF. A comparison was made between the mechanism of priming of both $PLA_2$ and NADPH oxidase by GM-CSF and TNFα. GM-CSF and TNF both stimulate the phosphorylation and activation of $p42^{ERK2}$ and p38 MAP kinase (MAPK) in neutrophils. To investigate the role of these MAPKs in priming $PLA_2$ and NADPH oxidase, inhibitors of $p42^{ERK2}$ (PD098059) and p38 MAPK (SB203580) were used. Inhibition of $p42^{ERK2}$ blocked neither superoxide generation nor cytokine-mediated priming, but partially inhibited cytokine-mediated priming of $PLA_2$. In contrast inhibition of p38 MAPK blocked primed and unprimed NADPH oxidase activity, but only partially inhibited primed $PLA_2$ activity. The dissociation of the priming of these two enzymes systems indicates that they may activated by different mechanisms.

Inflammation of the vascular endothelium is part of the pathogenic process in the crisis phase of sickle cell disease (SCD). This study investigated whether the priming of neutrophil $PLA_2$ and NADPH oxidase activity in response to *in vitro* GM-CSF and TNFα was altered in both the steady state and crisis of SCD. The data showed raised resting levels of neutrophil $PLA_2$ even in the steady state, indicating an ongoing activation of these cells. But there were reduced responses of $PLA_2$ and NADPH oxidase to GF priming in both steady state and crisis, and this may contribute to the susceptibility of these patients to infection.

Immature cells were also studied. A range of myeloid and lymphocytic cell lines were screened for the presence of $PLA_2$ activity measured by arachidonate release stimulated by calcium ionophore. Immature myeloid cells (HL-60 and K562) had extremely low $PLA_2$ activity which was not enhanced by GF. Immature lymphocytic cells, Daudi and IL-2-dependent CTLL cells also released little arachidonic acid, and $PLA_2$ activity was not further enhanced by stimulation with IL-2. However, the GF dependent myeloid cell lines (TF-1, THP-1) and purified human $CD34^+$ stem cells showed higher levels of $PLA_2$ activity which was increased by GM-CSF and IL-3. This suggests that $PLA_2$ activity may be important for mediating the effects of myeloid growth factors.

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## ABBREVIATIONS

| | |
|---|---|
| AA | Arachidonic acid |
| AA-CoA | Arachidonoyl-coenzyme A |
| ARF | ADP-ribosylation factor |
| ASK | Apoptosis signal regulating kinase |
| ATF2 | Activating transcription factor 2 |
| Bis | N'N'-bis-methylene-acrylamide |
| BSA | Bovine serum albumin |
| $CaCl_2$ | Calcium chloride |
| CAMs | Cell surface adhesion molecules |
| CGD | Chronic granulomatous disease |
| $cPLA_2$ | Cytosolic $PLA_2$ |
| CSBP | CSAID binding protein |
| CSF | Colony-stimulating factor |
| DAG | Diacylglycerol |
| DIFP | Diisopropyl fluorophosphate |
| DTT | Dithiotreitol |
| EBV | Epstein-Barr virus |
| EDTA | Ethylenediamine-tetraacetic acid disodium salt |
| EET | Epoxyeicosatrienoic acids |
| Epo | Erythropoietin |
| ERK | Extracellular-signal-regulated kinase |
| FAD | Flavin adenine dinucleotide |
| FCS | Foetal calf serum |
| FLT-3 | fms-like tyrosine kinase-3 |
| FMLP | n-Formyl-Met-Leu-Phe |
| G-CSF | Granulocyte colony-stimulating factor |
| G-protein | Guanosine triphosphate binding protein |
| GDI | GDP-dissociation inhibitor factor |
| GDP | Guanosine 5'-diphosphate |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| Grb2 | Growth factor receptor-bound protein 2 |
| GTP | Guanosine triphosphate |
| $H_2O_2$ | Hydrogen peroxide |
| HMP | Hexose monophosphate shunt |
| HPETE | Hydroperoxyeicosatetraenoic acid |
| hsp25/27 | Heat shock protein 25/27 |

| | |
|---|---|
| ICAM | Intercellular adhesion molecule |
| IL-2 | Interleukin-2 |
| IL-3 | Interleukin-3 |
| IL-8 | Interleukin-8 |
| $IP_3$ | Inositol trisphosphate |
| $iPLA_2$ | Calcium-independent $PLA_2$ |
| ISGF | Interferon stimulated gene factor |
| JAK | Janus kinase |
| JNK/SAPK | *c-jun* $NH_2$-terminal kinase/Stress-activated protein kinases |
| LPS | Lipopolysaccharide |
| LTB4 | Leukotriene B4 |
| M-CSF | Macrophage colony-stimulating factor |
| MAPK | Mitogen-activated protein kinase |
| MAPKAPK | MAPK-activated protein kinase |
| MCF | Mean cell fluorescence |
| MPO | Myeloperoxidase |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NaF | Sodium fluoride |
| NEM | n-ethyl maleimide |
| NF-κB | Nuclear factor-κB |
| NP-40 | Nonidet-40 |
| $O_2^-$ | Superoxide |
| PAF | Platelet activating factor |
| PAGE | Polyacrylamide gel electrophoresis |
| PAK | p21-activated kinase |
| PBS | Dulbecco's phosphate buffered saline |
| PC | Phosphatidylcholine |
| PDGF | Platelet-derived growth factor |
| PE | Phosphatidylethanolamine |
| PI | Phosphatidylinositol |
| PI3K | Phosphoinositide-3 kinase |
| $PIP_2$ | Phosphatidylinositol 4,5-bisphosphate |
| PKC | Protein kinase C |
| $PLA_2$ | Phospholipase $A_2$ |
| PMN | Polymorphonuclear |
| PMSF | Phenylmethylsulfonylfluoride |
| PS | Phosphatidylserine |
| ROI | Reactive oxygen intermediates |

| | |
|---|---|
| SCD | Sickle cell disease |
| SCF | Stem cell factor |
| SDS | Sodium dodecyl sulphate |
| SH2 | Src homology 2 domain |
| SH3 | Src homology 3 domain |
| SOD | Superoxide dismutase |
| $sPLA_2$ | Secretory $PLA_2$ |
| SRE | Serum response element |
| STAT | Signal transducers and activators of transcription |
| TBS | Tris buffered saline |
| TBST | Tris buffered saline tween-20 |
| TEMED | Tetra-methylethylenediamine |
| $TNF\alpha$ | Tumor necrosis factor $\alpha$ |
| TRE | TPA response element |
| Tris | Tris (hydroxymethyl) methylamine |
| $TXA_2$ | Thromboxane $A_2$ |

# CHAPTER 1

# INTRODUCTION

## 1.1 HAEMOPOIESIS & DEVELOPMENT OF PHAGOCYTES

In adult life all the cells that populate circulating blood are derived from a small pool of multipotential haemopoietic stem cells that reside in the bone marrow. It also contains haemopoietic tissue which is composed of stromal cells (macrophages, fibroblasts, endothelial cells, fat cells) and an extracellular matrix containing adhesive proteins, collagen and proteoglycans. The stem cells are anchored in the extracellular matrix by the expression of cell surface adhesion molecules (CAMs). Stem cells are capable of continuous self-renewal. However, they can become committed to differentiate into various blood cell lineages which give rise to the eight main types of mature effector cells present in the circulation (Figure 1.1). These differentiated cells have a finite life span ranging from days for erythrocytes to years for some lymphocytes. The intermediate-stage cells that are committed to specific lineages but still capable of considerable proliferation are known as progenitor cells. To maintain the steady-state number of mature blood cells there is a constant production of these from progenitor cells, a process known as haemopoiesis which is regulated by complex interlinked mechanisms involving both cellular and humoral (colony stimulating factors) events. The commitment of stem cells to specific lineages appears to be a stochastic process with haemopoietic growth factors and cytokines enhancing the probability of commitment to a particular lineage (Metcalf and Nicola, 1995). Differentiation of pluripotent stem cells can give rise to either myeloid or lymphoid progenitors (Abramson *et al.*, 1977). The myeloid progenitors are the precursors of erythrocytes, megakaryocytes, monocytes and granulocytes. The latter two cell types are collectively known as phagocytes.

Phagocytes are a group of specialised haemopoietic cells which have a role as primary defenders of the host against attack by micro-organisms. In addition, phagocytes are capable of destruction of tumor cells and apoptotic cells and hence aid tissue repair. Polymorphonuclear (PMN) granulocytes are divided into three groups according to their staining characteristics and granule contents; neutrophils, eosinophils and basophils, whereas, mononuclear phagocytes are of two types; monocytes and macrophages. Once a myeloid progenitor cell is committed to a precursor of a myeloid cell type, it undergoes extensive proliferation passing through different stages of maturation and development. The earliest identifiable cells of the neutrophil lineage are the myeloblasts. Thereafter, different stages of maturation form promyelocytes, myelocytes, metamyelocytes and finally the fully mature polymorphonuclear neutrophils (Bainton, 1980).

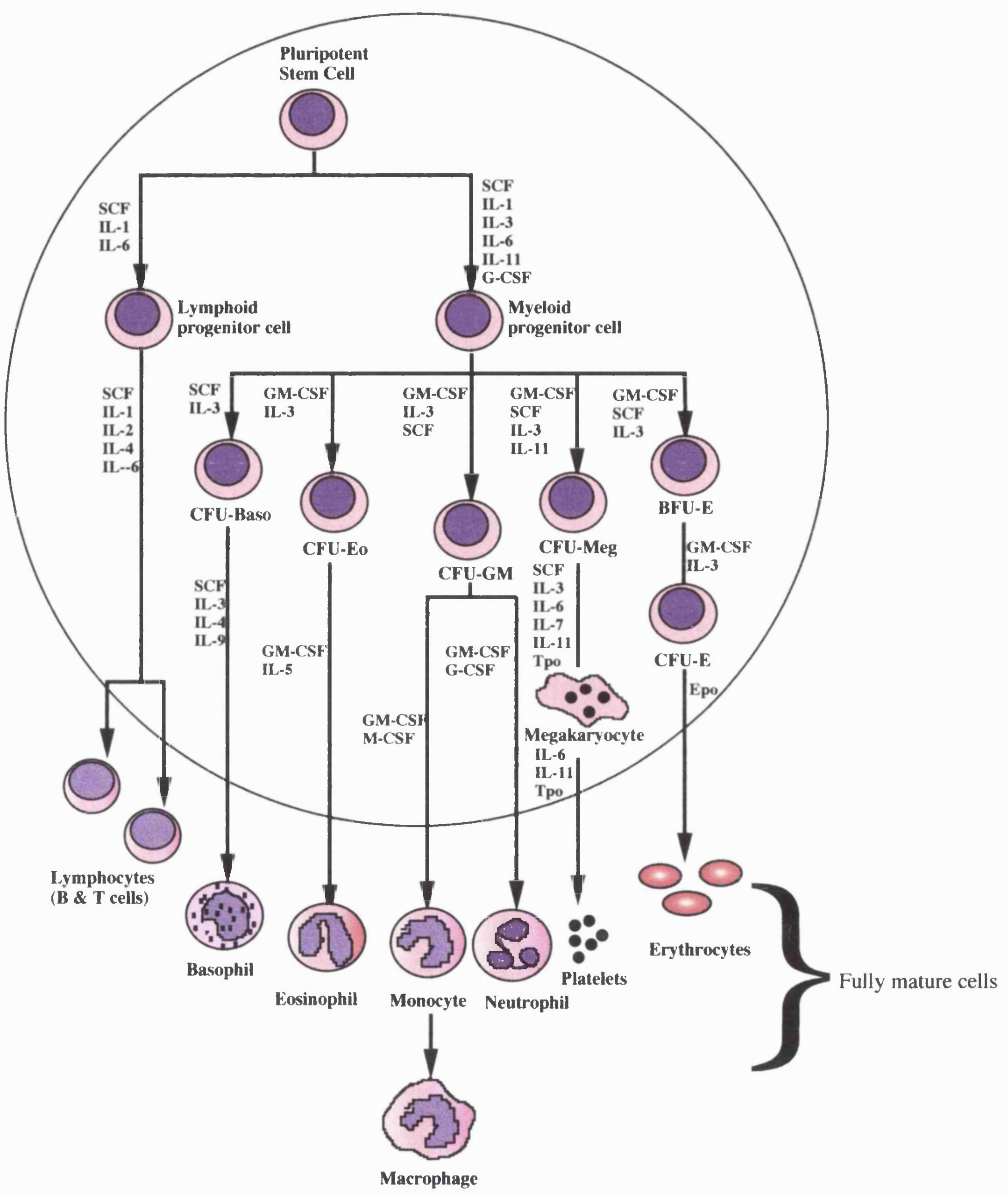



**Figure 1.1: Schematic presentation of haemopoiesis**

BFU-E: Erythroid Burst Forming Unit
CFU-E: Erythroid Colony Forming Unit
CFU-Meg: Megakaryocyte Colony Forming Unit
CFU-Eo: Eosinophil Colony Forming Unit
Baso: Basophil
GM: Granulocyte-Macrophage
(Adapted from Metcalf and Nicola, 1995)

SCF: Stem Cell Factor
Tpo: Thrombopoietin
Epo: Erythropoietin
IL-3: Interleukin-3

Myeloblasts are undifferentiated cells which are capable of proliferation. They are characterised by a large nucleus with two to five nucleoli. Neutrophil granules which contain microbicidal and degradative enzymes are formed during the promyelocyte and myelocyte stages. At the metamyelocyte stage, the nucleus become kidney shaped and there is an increase in the specific granules within the cytoplasm. From this stage onward there is no further cell division but progressive changes toward a mature cell. The final stage is the mature neutrophil which is recognised by an elongated and segmented nucleus.

## 1.2 HAEMOPOIETIC GROWTH FACTORS

Studies on cultured haemopoietic cells have shown the requirement for a group of regulatory molecules, collectively known as haemopoietic growth factors (HGF). Multiple HGFs are capable of regulating cells in any one lineage, due to simultaneous expression of specific receptors for the different HGFs on individual cells (Metcalf, 1992). Together extracellular matrix and HGFs (cytokines) play a crucial role in proliferation and maturation of haemopoietic cells (Metcalf, 1991).

Pluznik and Sachs, in 1965 and Bradley and Metcalf in 1966 developed the semi-solid culture techniques to support and grow colonies of haemopoietic cells in the presence of conditioned media. Using these invaluable assays, they were able to show the requirement for the colony stimulating factors (CSFs) that support the growth of haemopoietic cells. Because of the low concentration of these CSFs it was difficult to purify these molecules by traditional biochemical techniques for *in vivo* experiments. The development of recombinant techniques in mid 1980s made it possible to isolate complementary DNA (cDNA) encoding different colony stimulating factors namely, erythropoietin (Epo) (Jacobs *et al.*, 1985), granulocyte-macrophage colony-stimulating factor (GM-CSF) (Gough *et al.*, 1984), macrophage colony-stimulating factor (M-CSF) (Kawasaki *et al.*, 1985), granulocyte colony-stimulating factor (G-CSF) (Souza *et al.*, 1986) and interleukin (IL)-3 (Yokota *et al.*, 1986). Large-scale production and availability of the recombinant CSFs made it possible to study their physiological roles in the haemopoietic system.

Growth factors (cytokines) are a group of at least 20 acidic glycoproteins with a molecular mass of 14-39 kDa, that regulate production and function of haemopoietic cells. There is little amino acid sequence homology between the group but analysis of the three-dimensional molecular structure indicate that many exhibit similar structural conformations consisting of four $\alpha$-helices, in which two helices combine to produce the active binding domain (Bazan, 1990). It was originally thought that each cytokine exerts a specific effect on its particular target cell. However, this is not the case. A

characteristic feature of cytokines is their functional pleiotropy (one cytokine can control cells in more than one lineage) and redundancy (different cytokines can act on the same cell type to mediate similar effect) (Metcalf, 1988). For example, neutrophil production can be stimulated, at least *in vitro*, by G-CSF, and GM-CSF. Similarly, eosinophil production can be stimulated by IL-3, GM-CSF and IL-5. Figure 1.1 shows a simplified schematic presentation of haemopoiesis, indicating the involvement of different cytokines at various stages of lineage development. Some factors affect the early primitive progenitor cells, while others affect cells at an intermediate stage of differentiation, with late acting factors being more lineage specific such as; Epo, IL-5, G-CSF and M-CSF which control the maturation of cells committed to one lineage. The direction in which stem and progenitor cells differentiate depends largely on the growth factors to which they are exposed, but whether this is due to directed differentiation or selected survival remains controversial. Growth factors not only regulate the proliferation and differentiation of immature cells but also modulate the function of mature cells.

## 1.3 CYTOKINE RECEPTOR FAMILY

The biological effects of cytokines are mediated by binding to specific surface receptors on the target cells thereby initiating intracellular signal transduction. Many cytokine receptors have been cloned and sequenced and their common structural homology has been recognised. These receptors are divided into different classes;

### 1.3.1 Receptor tyrosine kinases (RTK)

All the members of the haemopoietic RTK family share a common structural and functional feature which is a single polypeptide chain divided into an extracellular segment, responsible for binding to its corresponding cytokine, a short transmembrane portion and an intracellular domain consisting of a tyrosine kinase domain. The intrinsic tyrosine kinase portion of the receptor becomes phosphorylated upon ligand binding with subsequent activation of signal transduction proteins. Several of the haemopoietic growth factors that bind to this class of receptor family are dimeric and therefore capable of bringing two receptor chains together, thus forming stable receptor dimers. Examples of haemopoietic growth factors which bind to this class of receptor are stem cell factor (SCF), M-CSF and FLT-3 ligand.

### 1.3.2 Class I cytokine receptors

This family includes the receptors for GM-CSF, G-CSF, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7 which lack the tyrosine kinase domain but associate with cytoplasmic protein tyrosine kinases instead. They share a conserved sequence of 200 amino acid residues in their extracellular domain which contains multiple cysteine residues and a conserved amino acid motif (Trp-Ser-X-Trp-Ser, where X is a non conserved amino acid) with two fibronectin type III-like motifs (Patthy, 1990). An interesting feature of this class of receptor is that subgroups of cytokines in this class use a common signal-converting chain. For example, GM-CSF, IL-3 and IL-5 receptors have a common transmembrane $\beta$-subunit which with the specific $\alpha$-chain forms a high affinity signalling complex in the presence of ligand. It has recently been shown that the unique $\alpha$-chain of IL-3, GM-CSF and IL-5 receptors are also signal transduction molecules and this may give an element of signalling specificity (Polotskaya *et al.*, 1993)

### 1.3.3 Class II cytokine receptors

This class of receptor family consists of receptors for IFN-$\alpha$, IFN-$\gamma$ (Novick *et al.*, 1994)), IL-10 (Ho *et al.*, 1993) and coagulation factor III (Bazan, 1991). These receptors are multimeric with similar structural features to the class I family, but are more divergent, containing an additional cysteine pair, and several conserved prolines and tyrosines. The multicomponent nature of class I and II receptor families is another similarity between the two classes, enabling further characterisation by the number of receptor subunits or use of common signalling subunits.

### 1.3.4 Tumor necrosis factor (TNF) receptor family

TNF has been shown to stimulate a variety of responses such as activation of a lipid signalling cascade involving ceramide (Obeid *et al.*, 1993; Hannun, 1994) and activation of phospholipase $A_2$ (Seeds *et al.*, 1998). This multifunctional cytokine exerts its effects by binding to two distinct receptors, the 55-kDa TNF receptor (TNFR-1/CD120a or the 75-kDa TNFR-2/CD120b) (Tartaglia *et al.*, 1992; Vandenabeele *et al.*, 1995). Other receptors that belong to this family are Fas and CD40 (Rabizadeh and Bredesen, 1994). TNF binding results in receptor trimerisation and the consequent initiation of signal transduction (Vandenabeele *et al.*, 1995). Following receptor triggering, the ligand-TNFR55 complex is internalised by clathrin-coated pits and is degraded in lysosomes (Mosselmans *et al.*, 1988). Unlike TNF-

R55, TNF-R75 contains no tyrosine residues in its intracellular domain, therefore lacks a consensus sequence for rapid cellular internalisation through coated pits (Collawn *et al.*, 1990). The cytoplasmic domain of these two receptors has no homology, indicating that they utilise different signalling molecules. The intracellular extension of TNFR-1 contains a death domain which is critical for several signalling programs including apoptosis. The TNF receptor family is characterised by the presence of three to six cysteine-rich motifs approximately 40 amino acids in the extracellular domain, which provide the motif for binding to shared structures in the ligands (Mallett and Barclay, 1991).

## 1.4 NEUTROPHIL FUNCTION

Neutrophils are the primary line of host defence against infection, due to their ability to kill micro-organisms. Following maturation and storage in the bone marrow they are released and circulate quiescently in the blood stream. Growth factors not only regulate the proliferation and differentiation of immature phagocytes but also regulate the functions of the mature phagocytic cells such as adherence and migration, phagocytosis, and microbicidal activity.

### 1.4.1 Migration

Recruitment of neutrophils to an inflammatory site is dependent on physical and functional properties of both leukocytes and endothelial cells. Different stages involved in emigration of neutrophils from the circulation to the tissue are; initial contact between neutrophil and endothelium, neutrophil rolling, adhesion and finally migration by chemotaxis through extravascular tissues. Under normal conditions of blood flow, neutrophils "roll" along the endothelial surface. The recruitment of neutrophils from the blood stream into infected tissues depends on their ability to respond to chemoattractants generated at the site of infection, but also requires interaction with the endothelial cells. They marginate to the endothelium and migrate through the tissues by adherence and rolling over cell surfaces, which depends on cell adhesion molecules (CAMs). There are three major families of CAMs; the selectins, the integrins and the immunoglobulin-like superfamily (IGSF) of adhesion molecules (Table 1.1). The selectins are most important in the early stages of leukocyte adhesion. The initial step is the induction of a rolling interaction with the endothelial lining of blood vessels (Carlos and Harlan, 1994; Bevilacqua and Nelson, 1993). The weak adhesion forces of the selectins are suited for the rolling phenomenon. The surface expression of selectins is inducible by inflammatory mediators. Rolling is followed by

**Table 1.1: General features of major adhesion molecules**

| *Name* | *Types (other name)* | *Occurrence* |
|---|---|---|
| Selectins | | |
| | L-selectin (LECAM-1) | Neutrophils, lymphocytes, |
| | E-selectin (ELAM-1) | Endothelium |
| | P-selectin (CD62) | Endothelium, platelets |
| Integrins | | |
| | CD11a/CD18 (LFA-1) | All human leukocytes |
| | CD11b/CD18 (CR3) | Neutrophils, monocytes, NK cells |
| | CD11c/CD18 (p150, 95) | Monocytes, macrophages, neutrophils |
| | CD49d/CD29 (VLA-4) | Monocytes, eosinophils, lymphocytes |
| IGSF | | |
| | ICAM-1 (adhesion mol-1) | Endothelial cells |
| | ICAM-2 | Endothelial cells |
| | VCAM-1 | Endothelial cells |

VLA-4=very late antigen-4, LFA-1=lymphocyte function-associated molecule, IGSF=immunoglobulin-like superfamily, ICAM=intercellular adhesion molecules, VCAM-1=vascular cell adhesion molecule,

firm adhesion to the endothelial cell surface which is mediated by the cytokine-regulated expression of CAMs on the surface of both neutrophils and endothelial cells. Transition of neutrophils to the rolling state is stimulated by chemokines such as IL-8, cytokines and chemotactic factors. The firm adhesion of neutrophils to endothelial cells is via the binding of integrins to their respective ligand on activated endothelial cells. The final step involves transmigration between endothelial cells and penetration of the basement membrane (Figure 1.2).

### 1.4.2 Phagocytosis

Phagocytosis is the ingestion of particles by phagocytes and this protects the host against infection. This process is comprised of; recognition and binding of the particle to the membrane; cell activation; extension of the peripheral cytoplasm to form pseudopodia and formation of a phagocytic vacuole or phagosome (Griffin *et al.*, 1976). Recognition and binding of the particles to the phagocyte membrane is via membrane receptors, either for antigens on the particles or for opsonins such as complement and immunoglobulin, which have bound to the particle. The attachment of antigen to phagocyte receptors does not always lead to phagocytosis and a cytokine-mediated step is required in order to ensure efficient binding and engulfment. Phagocytosis of a particle requires the sequential interaction of plasma membrane receptors with particle-bound ligands not involved in the initial attachment process, the so called "zipper mechanism" (Griffin *et al.*, 1975, Griffin *et al.*, 1976). During phagocytosis the local changes in the plasma membrane that result in the formation of the phagocytic vacuole are due to changes in the cytoskeleton. Rac, a 21-kDa GTP-binding protein, have been shown to regulate actin filament polymerisation during phagocytosis. This is supported by experiments where microinjection of an activated mutant form of Rac into fibroblasts induced the accumulation of macropinocytotic vesicles and membrane ruffling, accompanied by formation of polymerised actin in ruffles at the plasma membrane (Ridley *et al.*, 1992).

### 1.4.3 Degranulation & killing mechanism

Degranulation is a process by which granules translocate from the cytosol and fuse to the plasma membrane and their contents are released into the phagocytic vacuole during the process of phagocytosis. Neutrophil granules are divided into 1) peroxidase-containing granules known as "azurophil" (primary) granules which contain myeloperoxidase (MPO) (an antimicrobial enzyme), as well as lysozyme and variety of cationic proteins, 2) peroxidase-negative granules, known as "specific"

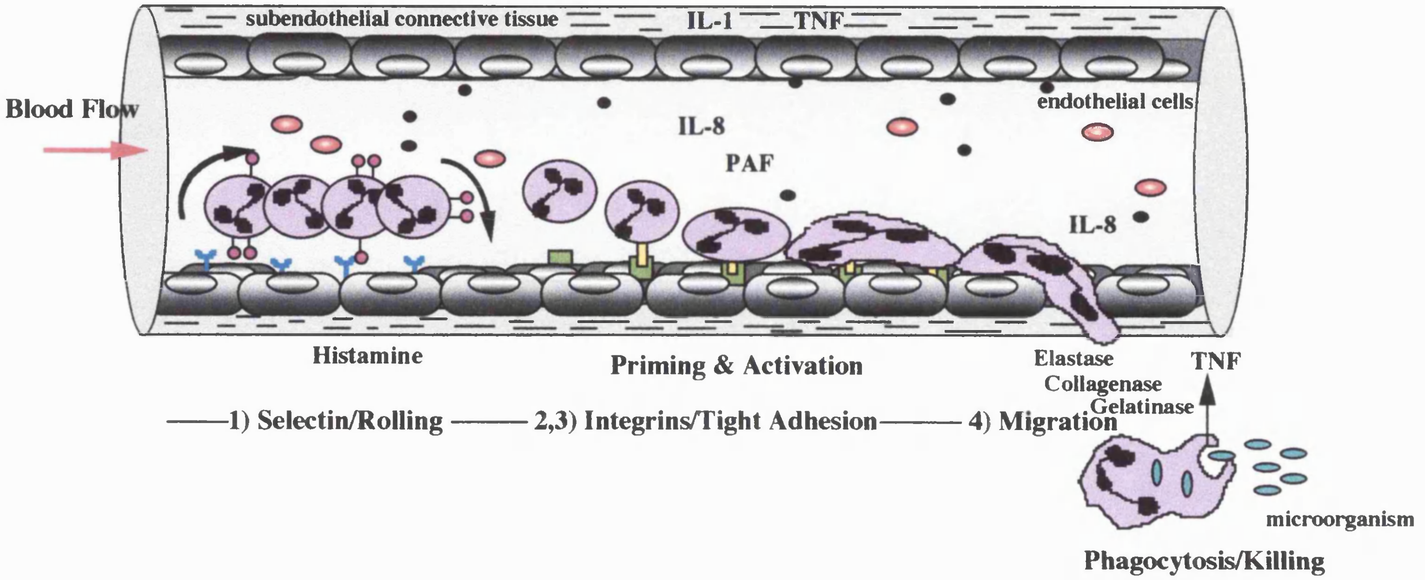



**Figure 1.2: Schematic presentation of neutrophil interaction with vascular endothelium.** 1) circulating neutrophils with representative surface molecules. 2) Rolling neutrophils with loose adherence to endothelium mediated by selectin binding. 3) Adherent neutrophils, with strong adhesion mediated by binding of activated integrin to intercellular cell adhesion molecules (ICAM). Adhesion of neutrophils to endothelial cells is mediated by cytokines, chemokines and chemoattractants. 4) Penetration of the basement membrane mediated by secreted hydrolytic enzymes.

(secondary) granules which contain lactoferrin, vitamin $B_{12}$-binding protein and lysozyme and 3) "Gelatinase containing" (tertiary) granules which contain gelatinase, diacylglycerol lipase, and β-glucuronidase.

Following phagocytosis different mechanisms co-operate to kill micro-organisms; a) one is mediated by the phagocyte NADPH oxidase (see Section 1.5 for details), b) killing mediated by nitric oxide, a highly reactive free radical product of the oxidation of L-arginine and c) killing mediated by antimicrobial proteins such as lysozyme, lactoferrin and cationic proteins. In addition, extracellular phospholipase $A_2$ and phospholipases from granule stores participate in the process of phagocytosis and degradation of microbial phospholipids. The oxidase-independent killing mechanisms, are important in host defence against microbial infection as shown by the ability of neutrophils from patients with chronic granulomatous disease (CGD) that lack the phagocyte oxidase to kill several potentially lethal organisms.

## 1.5 THE RESPIRATORY BURST OF PHAGOCYTES

Oxygen is essential for aerobic forms of life, and its presence is also a vital condition for the effective killing of pathogens by phagocytes, but it is not necessary for phagocytosis itself. Stimulation of phagocytes during phagocytosis is associated with a rapid increase in oxygen consumption during a process known as the "respiratory burst". This respiration is non-mitochondrial. The ability to generate a significant respiratory burst was thought to be a unique feature of phagocytes but it has been shown that non-phagocytic cells such as fibroblasts (Fikrig *et al.*, 1980), lymphocytes (especially natural killer cells) (Babior *et al.*, 1976) and porcine articular chondrocytes (Hiran *et al.*, 1997) are also have the capacity to generate superoxide ($O_2^-$), although at a lower rate than that measured in neutrophils.

Studies on increased oxygen ($O_2$) metabolism by the professional phagocytic cells, neutrophils, monocytes, macrophages and eosinophils, began in 1933 by Baldridge and Gerard. They observed a small but significant increase in the oxygen consumption by canine neutrophils during phagocytosis of bacteria, but the unusual nature of the process was only revealed in 1959, when it was discovered that it was not inhibited by mitochondrial inhibitors like cyanide and azide (Sbarra and Karnovsky, 1959). This observation distinguished the "respiratory burst" from the "mitochondrial respiration" and suggested that the former was involved in some process other than the production of energy required for engulfment of the microorganism, most of which is provided by glycolysis.

An advance in the understanding of the function and molecular mechanisms involved in the respiratory burst occurred when it was discovered that superoxide

production was defective in a condition, known as Chronic Granulomatous Disease (CGD), a rare inherited disorder in which the patients are predisposed to frequent infections by bacteria and fungi. It has been shown that 80% of the infectious deaths were caused by Gram-negative bacilli but other common pathogens are, *Serratia marscescens, Klebsiella* spp, *Salmonella* spp, *Candida* spp, *Aspergillus fumigatus* and *Staphylococci aureus* which accounted for 9% of the fatal infections (Lazarus and Neu, 1975). In patients with CGD, organisms that produce hydrogen peroxide ($H_2O_2$) but are catalase negative (such as, streptococci, pneumococci or lactobacilli) have been shown not to be major pathogens whereas organisms that are catalase positive are pathogenic (Quie, 1973). The manifestations of infection are pneumonia, lymphadenitis, multiple granuloma in a variety of tissues (such as; skin, lung and liver) and persistent fever (Tauber *et al.*, 1983). Insight was provided when neutrophils from these patients were shown to be incapable of killing bacteria efficiently because their phagocytes lacked the respiratory burst activity responsible for the generation of toxic oxygen metabolites (Holmes *et al.*, 1967; Curnutte *et al.*, 1974).

The inheritance of CGD can be either X-linked or autosomal, with defects due to the lack of one or other, of the main components of NADPH oxidase (Table 1.2). The most common form of CGD (approximately two third of the cases) is inherited as an X-linked recessive form and is due to the lack of the large subunit of flavocytochrome *b*, $gp91^{phox}$ (phox: phagocytic oxidase) (Royer-Pokora *et al.*, 1986). The gene for $gp91^{phox}$ has been mapped to chromosome Xp21.1 (Royer-Pokora *et al.*, 1986). Heterozygous carriers develop skin lesions and they may be at increased risk of minor infection. Studies on these patients indicated that the majority of mutations of the flavocytochrome $gp91^{phox}$ led to the loss of both $gp91^{phox}$ and $p21^{phox}$ because the $gp91^{phox}$ was required for stable incorporation of the $p21^{phox}$ subunit of the cytochrome into the plasma membrane (Segal, 1987; Parkos *et al.*, 1989). The autosomal recessive pattern of inheritance in CGD accounts for the lack of the other components of the NADPH oxidase namely, $p47^{phox}$, $p67^{phox}$ and $p21^{phox}$ (Table 1.2).

## 1.5.1 The function of NADPH Oxidase

NADPH oxidase contributes to microbicidal activity of phagocytes at least by two mechanisms. First, the respiratory burst of phagocytes is catalysed by a membrane-bound reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase that is responsible for the reaction:

$$NADPH + 2O_2 \longrightarrow 2O_2^- + H^+ + NADP^+$$

**Table 1.2: Classification of Chronic Granulomatous Disease**

| *Inheritance* | *Component & Chromosome* | *Cases (%)* | *References* |
|---|---|---|---|
| X-linked recessive | $gp91^{phox}$ (Xp21.1) | 56 | Royer-Pokora *et al.*, 1986 |
| Autosomal | $p47^{phox}$ (7q11.23) | 33 | Clark *et al.*, 1989 |
| Autosomal | $p67^{phox}$ (1q25) | 5 | Kenney *et al.*, 1993 |
| Autosomal | $p21^{phox}$ (16q24) | 6 | Clark *et al.*, 1989 |

phox =phagocytic oxidase

The cytochrome component of the oxidase is located on the plasma membrane (although other regulatory components are located in the cytosol and on the membranes of specific granules and secretory vesicles). The NADPH oxidase is dormant in resting cells but becomes activated during the process of phagocytosis. This enzyme acts as an electron transport chain passing electrons from NADPH to molecular oxygen and thus, reducing $O_2$ to superoxide ($O_2^-$) which is toxic and its dismutation leads to the formation of $H_2O_2$, which participates in several chemical reactions resulting in the formation of other reactive oxidant species (Table 1.3). Superoxide and its derivatives can impose damage on tissues therefore it is essential that the NADPH oxidase is tightly regulated. During the process of the respiratory burst, glucose oxidation, through the hexose monophosphate (HMP) shunt, accelerates because of an increase in the rate of production of $NADP^+$. Each glucose molecule that is metabolised via the HMP shunt reduces two molecules of NADP to NADPH, therefore replenishing the supply of reducing agent necessary for the continued operation of the respiratory burst (Klebanoff, 1980; Rossi, 1986). The availability of $NADP^+$ limits the capacity of the HMP shunt.

Following phagocytosis, the fusion of the phagosome membrane with the membrane of cell granules causes discharge of the granule enzymes into the phagosome. One of these enzymes, MPO, which is abundant in the azurophil granules of neutrophils, interacts with $H_2O_2$ in the presence of halide ions (such as, $Cl^-$, $I^-$, $Br^-$ ) to produce more reactive and toxic compounds, such as hypohalite ions. The HOCl formed by the MPO-$H_2O_2$-chloride system of neutrophils can react with taurine or other nitrogen containing compounds to form long-lived oxidants such as chloramines which have microbicidal function (Zgliczynski *et al.*, 1971; Learn *et al.*, 1990).

The primary product of the respiratory burst is the one electron reduced species of oxygen, the superoxide anion ($O_2^-$), which is relatively inactive and long lived, and is able to diffuse some distance from its source. Another extremely reactive product of the reduction of molecular oxygen is the hydroxyl radical ($OH\cdot$), which may be formed in an iron-catalysed Haber-Weiss reaction, in which $O_2^-$ and $H_2O_2$ are reactants (see Table 1.3).

The second function of the NADPH oxidase is to modulate the pH of the phagocytic vacuole for the optimum which is required for activity of antibacterial proteins. NADPH donates electrons to the electron transport chain which results in the outpouring of millimolar quantities of electrons into the phagocytic vacuoles. These rapidly consume available protons, raising the vacuolar pH to about 7.8-8.0 during which enzymes of the tertiary and specific granules are activated. However, the metabolism of NADPH also generates protons and these are rapidly pumped out of the cell into the vacuole causing a fall in pH (around pH 6.0) (acidification phase).

**Table 1.3: Reactions leading to the formation of oxygen-derived metabolites**

| *Oxygen Derived Metabolites* |
|---|
| **1) Reduction of molecular oxygen** |
| $NADPH + 2O_2 \longrightarrow 2O_2^- + H^+ + NADP^+$ |
| **2) Dismutation of superoxide anion** |
| $2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$ |
| $O_2^- + HO_2$ (perhydroxyl radical) $\longrightarrow H_2O_2 + O_2$ |
| **3) Haber-Weiss reaction** |
| $O_2^- + H_2O_2 \longrightarrow O_2 + HO^- + HO\cdot$ (hydroxyl radical) |
| **4) Fenton reaction** |
| $O_2^- + Fe^{3+} \longrightarrow Fe^{2+} + O_2$ |
| $Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^- + HO\cdot$ |
| $O_2^- + H_2O_2 \longrightarrow O_2 + HO^- + HO\cdot$ |
| **5) Myeloperoxidase (MPO)-$H_2O_2$-halide system** |
| $H_2O_2 + Cl^- \longrightarrow H_2O + HOCl$ (hypochlorous acid) |
| $OCl^- + RNH_2 \longrightarrow RNHCl$ (monochloramine) $+ HO^-$ |

Azurophil granule acid hydrolases reach their maximal effectiveness as the pH of the vacuole drops (Segal *et al.*, 1981).

## 1.5.2 Composition of the NADPH oxidase

*i) The electron transport system*

The NADPH oxidase is a multi-protein enzyme complex composed of a number of cytosolic and membrane bound proteins. The NADPH oxidase is dormant in resting cells and its protein components are separated into cytoplasmic and plasma membrane components (Figure 1.3) but after activation all the components assemble as an enzyme complex at the membrane. During the respiratory burst electrons are transferred from NADPH (electron donor) to molecular oxygen (electron acceptor) producing superoxide anions. The redox centre of this enzyme complex is a heterodimeric membrane-associated flavocytochrome *b*, also known as cytochrome $b_{-245}$ (to indicate its low oxidation-reduction mid-point potential) or cytochrome $b_{558}$ (to indicate the absorbance maximum of its $\alpha$ band in the reduced state). Flavocytochrome *b* consists of three subunits (Segal, *et al.*, 1987), two small $\alpha$ subunits (also known as $p21^{phox}$) and a larger $\beta$ subunit of approximately 76-92 kDa (designated $gp91^{phox}$). The gene for these subunits of cytochrome *b* have been cloned and sequenced (Royer-Pokora *et al.*, 1986).

The large subunit of cytochrome *b* ($gp91^{phox}$) is heavily glycosylated and has two major domains (Segal *et al.*, 1992). The N-terminal domain is hydrophobic whereas, the C-terminal domain is much more hydrophilic and lies on the cytosolic side of the membrane, overlying the transmembrane structure. This hydrophilic region of the molecule has a cleft that accomodates the substrate, NADPH, as well as flavin adenine dinucleotide (FAD), to which electrons are passed. From FAD electrons pass onto haems located within the transmembrane domains, attached to one or both of the subunits (Segal *et al.*, 1992; Quinn *et al.*, 1992). It has been shown that CHO cell lines transfected with and expressing the N-terminal fragment containing the transmembrane domains of $gp91^{phox}$, exhibit an arachidonate-activatable $H^+$ flux, indicating a role for $gp91^{phox}$ as the NADPH oxidase $H^+$ channel (Henderson *et al.*, 1997). The structure of $gp91^{phox}$ has strong homology with a number of electron-transporting proteins from different origins such as ferredoxin-$NADP^+$ reductase (FNR) which is an important photosynthetic protein (Segal *et al.*, 1992).

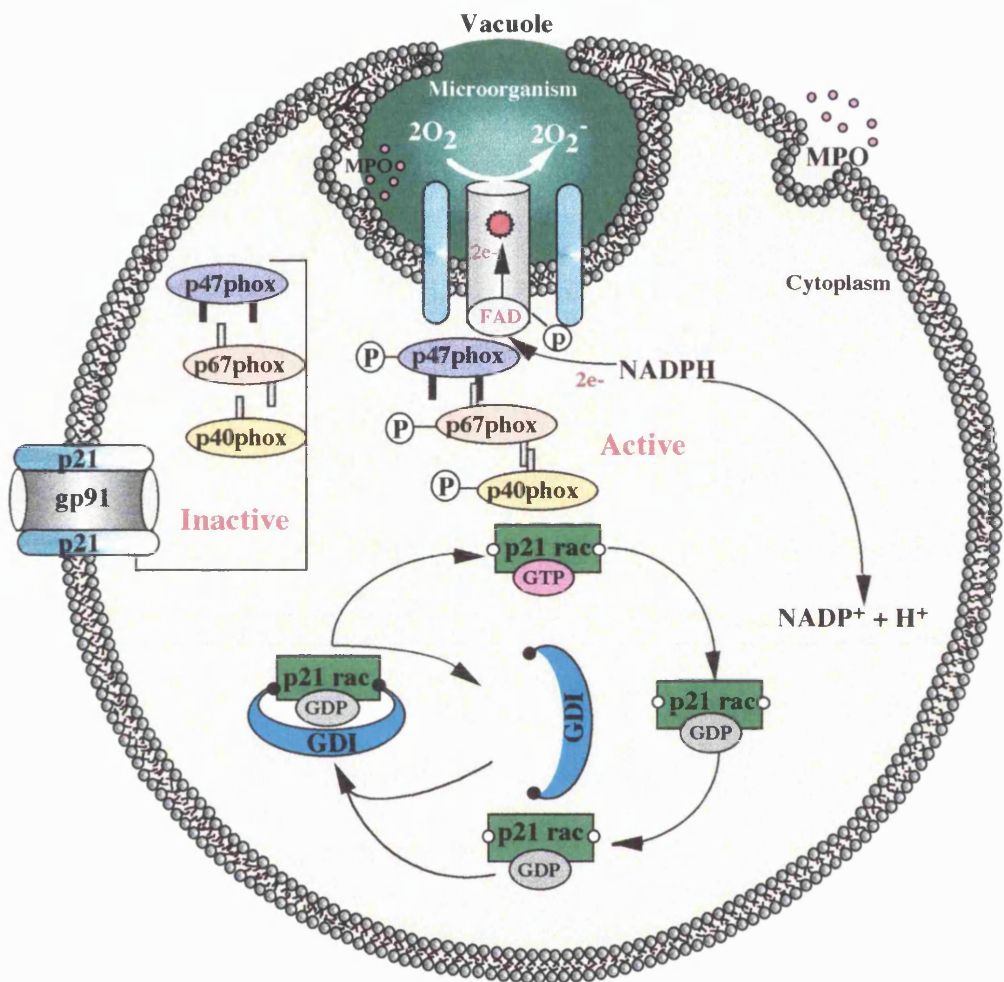



**Figure 1.3: Model of phagocyte NADPH oxidase activation during phagocytosis of microorganisms.** In the inactive state $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ are present in the cytosol as a complex and $p21^{rac}$ and GDI are in another complex. Upon activation $p21^{rac}$ is separated from GDI, converting it to active form. Both $p47^{phox}$ and $p67^{phox}$ are phosphorylated and upon activation all these components attach to flavocytochrome *b* in the vacuole membrane.

phox = phagocyte oxidase, GDI = GDP dissociation inhibitors, ● = Haem

FAD = Flavin adenine dinucleotide, (Adapted from Segal, 1996)

*ii) Cytosolic components*

Activation of the oxidase is regulated by several cytosolic proteins, some of which attach to the flavocytochrome *b*. p47$^{phox}$ (Segal *et al.*, 1985), p67$^{phox}$ (Leto *et al.*, 1990) and p40$^{phox}$ (Wientjes *et al.*, 1993) are specialised components of the NADPH oxidase of "professional" phagocytic cells. They all contain SH3 (Src homology 3) domains, which provide attachment sites to proline rich regions on the other proteins. In resting cells p47$^{phox}$ and p67$^{phox}$ reside in the cytosol in a 240-kDa complex with a third component, p40$^{phox}$ (Someya *et al.*, 1993; Fuchs *et al.*, 1995). Upon activation, the p47$^{phox}$ and p67$^{phox}$ are all phosphorylated and, along with p40$^{phox}$, translocate to the region of plasma membrane forming the phagocytic vacuole, where they associate with hydrophilic regions of flavocytochrome *b* (Heyworth *et al.*, 1991; Tyagi *et al.*, 1992). It has been shown that p40$^{phox}$ is responsible for electron transfer from FAD to the haem centre of flavocytochrome *b* (Cross and Curnutte, 1995). p67$^{phox}$ appears to be associated with the cytoskeleton as it remains in the insoluble pellet after the cell has been permeabilised with detergents (Woodman *et al.*, 1991).

p21rac is a member of the family of small GTP-binding proteins, which act as molecular switches. It is required for activity of the NADPH oxidase in a cell free, *in vitro* system (Abo *et al.*, 1991). The activity of small GTP-binding proteins is regulated by the guanine nucleotide to which they bind. In the cytosol of resting phagocytes, rac is complexed with an inhibitory protein Rho-GDI (Rho GDP-dissociation inhibitor), and when cells are activated rac translocates to the plasma membrane independently of other cytoplasmic components (Heyworth *et al.*, 1994).

Rap1 is a small GTPase which associates with flavocytochrome *b* in neutrophils (Quinn *et al.*, 1989). It is not necessary for the assembly and the functioning of the oxidase system but it has been shown that a dominant negative mutant of Rap1 inhibited the oxidative burst in differentiated HL-60 cells and in an Epstein-Barr virus (EBV)-transformed B-cell line (Gabig *et al.*, 1995; Maly *et al.*, 1994). It has been demonstrated that a variety of stimuli that activate the neutrophil, including FMLP (*N*-formylmethionyl-leucyl-phenylalanine, a chemotactic tripeptide of bacterial origin), GM-CSF and platelet activating factor (PAF), induce a rapid and transient Rap1 activation. In addition, Rap1 is normally activated in neutrophils from patients with CGD that lack cytochrome *b* or p47$^{phox}$ and have a defective NADPH oxidase system, suggesting an independent activation of Rap1 from the respiratory burst (M'Rabet *et al.*, 1998).

*iii) Signal transduction components*

Activation of NADPH oxidase involves translocation of cytosolic proteins from the cytosol to the membrane (reviewed by Robinson and Badwey, 1995). Translocation is initiated by a series of highly regulated signalling events which involves kinases such as phosphoinositol 3-kinase (PI3-k) (Arcaro and Wymann, 1993; Vlahos *et al.*, 1995), mitogen-activated protein kinases (MAPK), protein kinase C and protein kinase A (Dusi *et al.*, 1996; El Benna *et al.*, 1994; El Benna *et al.*, 1996a). The flavocytochrome *b*, $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ components of the oxidase all become phosphorylated during activation of the respiratory burst. PAK (p21-activated kinase, $p65^{pak}$) is a potential downstream target for Rac-1 signalling and it was initially found as a brain serine/threonine kinase (Manser *et al.*, 1994). PAK is activated in phagocytes as a result of its interaction with Rac-GTP and it can phosphorylate the $p47^{phox}$ in a Rac-GTP dependent manner (Knaus *et al.*, 1995). It has been shown that amongst 100 amino acid residues in the C-terminal domain of $p47^{phox}$ there are number of serines flanked with basic amino acids and this is a favourable structure for phosphorylation by protein kinase C (PKC) (Volpp *et al.*, 1989). It has also been shown that stimulation of the neutrophil's respiratory burst is accompanied by the phosphorylation of multiple serine residues in $p47^{phox}$ (El Benna *et al.*, 1994), and this protein is a target for proline directed mitogen activated protein kinases (MAPK) such as extracellular signal-regulated kinase (ERK) and p38 MAPK when neutrophils are exposed to an appropriate stimulus (El Benna *et al.*, 1996b). $p47^{phox}$ binds to flavocytochrome *b*, then $p67^{phox}$ attaches to the $p47^{phox}$-flavocytochrome complex. The sites of attachment of these proteins to flavocytochrome *b* have been partially defined. One of these is the C-terminus of $p21^{phox}$ and the other is a short helix overlying the nucleotide-binding groove of $gp91^{phox}$ (Leusen *et al.*, 1994). Activation might involve moving of this helix and allowing the substrate, NADPH, access to FAD, the first redox cofactor.

### 1.5.3 Activation of the NADPH oxidase

The oxidase is normally inactive but can be rapidly activated by physiological agonists such as, microorganisms opsonised with immunoglobulin, chemotactic factors such as C5a (the active fragment of the fifth component of complement), platelet activating factor (PAF), leukotriene $B_4$ ($LTB_4$), lipopolysaccharide or *in vitro* by synthetic agonists such as phorbol esters or FMLP (Babior, 1988).

The time course of the respiratory burst measured *in vitro* varies according to the stimulant used. For example, the response to PMA is seen after a lag period of about

25 seconds and lasts for several minutes whereas the response to FMLP occurs within 5-10 seconds but is weaker and of shorter duration. It is possible that the difference in lag phase seen with the two different agonists reflects the time taken for phosphorylation and translocation of the oxidase components to the membrane and their association with cytochrome *b* (Sha'afi and Molski, 1988).

In many cases, the extent of reactive oxygen metabolite production is quite low unless the phagocytic cells have been pretreated with agents such as GM-CSF, G-CSF, TNF$\alpha$, interleukins 1,3,5,6,8, interferon $\gamma$, and PAF participate in the process known as "priming", which results in an amplified response to later stimulation.

## 1.5.4 Biological significance of the respiratory burst

Products derived from the reduction of oxygen by the NADPH oxidase are part of a powerful microbicidal system and the ability of macrophages to kill intracellular pathogens is correlated with their oxygen metabolism (Murray and Cohn, 1980). While phagocytes are the primary producers of superoxide, other cells are also capable of generation of reactive oxygen intermediates (ROI). Although the precise role of ROI in these cells is not clear, but there is evidence suggesting that ROI may function as intracellular signalling molecules (Schreck and Baeuerle, 1991). It has been demonstrated that exogenous oxidising agents such as diamide and iodoacetamide were able to regulate the activity of the protein tyrosine kinase *ltk* in lymphocytes (Bauskin *et al.*, 1991). In another study it has been shown that $H_2O_2$ activates transcription factor NF-$\kappa$B by initiating the release of an inhibitory subunit I$\kappa$B from NF-$\kappa$B, causing its activation (Schrech and Baeuerle, 1991). Another transcription factor, AP-1, also responds to oxygen radicals. A study by Devary *et al.* (1991) showed that DNA binding and trans-activation by AP-1 are induced after $H_2O_2$ treatment of HeLa cells. In neutrophils, $H_2O_2$ causes an increase in the level of tyrosine phosphorylation (Fialkow *et al.*, 1993; Brummell *et al.*, 1996), and ROI have also been shown to activate mitogen-activated protein-kinase (MAPK) (Fialkow *et al.*, 1994, Stevenson *et al.*, 1994; Taher *et al.*, 1998). ROI may also trigger apoptosis (Rothstein *et al.*, 1994; Buttke and Sandstrom, 1994).

Many diseases are linked to damage from reactive oxygen species as a result of an imbalance between radical generating and radical scavenging systems a condition known as oxidative stress. Several observations indicate that products of the respiratory burst also have the ability to damage endothelial cells (Jacobs *et al.*, 1980; Dobrina and Patriarca, 1986) or give rise to clinical conditions such as adult respiratory distress syndrome (ARDS), carcinogenesis (Weitzman *et al.*, 1985) and atherosclerosis (Freeman *et al.*, 1986).

## 1.6 PRIMING AND ENHANCED CELLULAR ACTIVITY

Phagocytes circulate in the blood in a relatively latent state and react weakly to stimuli; which could limit potential damage to the vasculature and other host tissues. In order for neutrophils to function effectively in host defence they have the ability to migrate to the site of inflammation where cellular activity is enhanced and they release proteolytic enzymes, reactive oxygen intermediates and cationic proteins, collectively known as "effector" responses of neutrophils. Inappropriate release of these compounds by neutrophils may also contribute to inflammatory tissue damage and hence cause a variety of clinical disorders such as arthritis, acute respiratory distress syndrome and systemic inflammatory response syndrome. Neutrophil effector functions are under dual control by two pathways called "priming" and "activation", and this prevents inappropriate triggering (Figure 1.4) (Downey *et al.*, 1995). Priming is a process that does not immediately stimulate an effector response but results in an amplified response to later stimulation. It is triggered by engagement of cell surface receptors, that allow the recognition and response to a variety of compounds in inflammatory environment. There are at least three classes of membrane receptors on neutrophil surface: 1) the G-protein-linked seven-transmembrane-domain receptors, such as those for FMLP, PAF, complement component C5a, substance P and IL-8 (Boulay *et al.*, 1990; Murphy and Tiffany, 1991; Gerard and Gerard, 1991), 2) integrin and Fc receptors that require immobilisation and crosslinking rather than occupancy for activation (Ng-Sikorski *et al.*, 1991; Metzger, 1992) and 3) receptors for growth-regulating cytokines such as TNFα and GM-CSF (see section 1.3). It has been shown that GM-CSF causes an increase in neutrophil surface membrane expression of cellular adhesion molecules (CAMs) both *in vitro* (Arnaout *et al.*, 1986) and *in vivo* use in human (Devereux *et al.*, 1989). The second step is activation in which the primed neutrophil become responsive to activating signal. The requirement of phagocytes for priming before full activation protects the host from inadvertent triggering of damaging phagocytic processes such as; degranulation, production of toxic oxygen radicals. The priming phenomenon was first investigated in oxygen metabolite production but it is now extended to other functions such as production of lipid mediators, phagocytosis (Salmon *et al.*, 1991) and degranulation (Fittschen *et al.*, 1988). Signalling pathways from the receptors for priming agents amplify both the rate of onset and the magnitude of the response of neutrophils stimulated through the activation pathway (Bourgoin *et al.*, 1990). During priming, integrins, FMLP receptors (Atkinson *et al.*, 1988; Roberts *et al.*, 1993a) and oxidase components (DeLeo *et al.*, 1998) are mobilised from storage sites inside the cell to the cell surface through degranulation.

Activation Pathway

Priming Pathway

Agonist

Growth Factors,
Cytokines

priming

second
signals:

priming

priming

Effector
Functions:
*eg.* NADPH
Oxidase

$PLA_2$
PLC
PLD
Kinases
Phosphatases

priming

soluble factors:
rac
$p67^{phox}$
$p47^{phox}$
ARF

granule
components:
Integrins
FMLP receptors
$sPLA_2$

**Figure 1.4: Schematic presentation of priming and activation pathways.** Priming is triggered by the engagement of cell surface receptors which enhance both the rate and magnitude of the response of neutrophils through the activation pathway. ARF = ADP-ribosylation factor

Priming also results in rapid mobilisation of subcellular granules to the cell surface to increase the number of some receptors on the plasma membrane. Increase in receptor number alone do not explain the enhanced activity of primed neutrophils, suggesting that receptor function is also altered during priming. This change in function alter the ligand binding properties of the receptor, or allow the receptor to become coupled to a new signalling pathway. For example, in unprimed neutrophils, the respiratory burst activated by FMLP can occur in the absence of detectable phospholipase D (PLD) or $PLA_2$ activation, whereas the primed response is dependent on both PLD activation (Watson *et al.*, 1994) and $PLA_2$ activation (Roberts *et al.*, 1996). Similarly, another study has shown that arachidonic acid does not regulate NADPH oxidase activity in unprimed neutrophils but $PLA_2$ is involved in mediating the priming of NADPH oxidase activity by GM-CSF (Roberts *et al.*, 1996). Priming may be the result of cross-talk between the serine/threonine kinases such as PKC and the tyrosine kinase signalling system. Serine phosphorylation of the component of NADPH oxidase ($p47^{phox}$) occurs during activation. Therefore, it is possible that serine kinases form the basis for crosstalk, such that their activities are regulated and dependent both on serine/threonine phosphorylation and on tyrosine phosphorylation.

Previous work in our laboratory has demonstrated that when neutrophils are primed by a single agent, the ultimate response to an agonist can be up to five-fold that of the unprimed cell, but synergistic responses far exceeding that of single cytokines are elicited when cells are primed by combinations of agents (Khwaja *et al.*, 1992; Roberts *et al.*, 1993a). In addition, current evidence suggests that different cytokines can prime the same response such as the respiratory burst, via different mechanisms. For instance, IL-8 induces rapid but very transient priming, GM-CSF has a slower onset but is sustained, whereas priming with interferon (IFN)-$\gamma$ requires protein synthesis and maximal takes 1-2 hours (Roberts *et al.*, 1993a).

The discovery of the amplifying role of the priming pathway is a major advance in the understanding of neutrophil activation, although the mechanism of priming is not fully elucidated. Until recently, studies of neutrophil activation used gross and unphysiological stimuli like phorbol ester, that bypass the priming pathways, or cytochalasin B, which again overrides physiological priming mechanisms (Bauldry *et al.*, 1991a).

## 1.7 SIGNAL TRANSDUCTION PATHWAYS USED BY CYTOKINES AND GROWTH FACTORS

### 1.7.1 Phospholipase $A_2$

The phospholipases are important for the biosynthesis and degradation of membrane lipids and the generation of lipid derived second messengers implicated in signal transduction processes (Dennis *et al.*, 1991). Phospholipases are defined by the position they attack on the phospholipid backbone (Figure 1.5). Phospholipase activity was first studied in pancreatic juice and cobra venom in early 1900s (Wittcoff, 1951). Phospholipase $A_2$s are a diverse group of enzymes (esterases) that catalyse the hydrolysis of glycerophospholipids at the *sn*-2 ester bond and liberate free fatty acid including arachidonic acid (AA) and lysophospholipid (Figure 1.5).

The activity of $PLA_2$s depends on binding to a lipid-water interface. All these enzymes are more active on an aggregated phospholipid substrate (e.g. pure or mixed micelles, vesicles, monolayers or bilayers) compared with monomeric soluble substrates. This phenomenon has been called "interfacial recognition" (Heinrikson and Kezdy, 1990). $PLA_2$s are well known interfacial enzymes (an enzyme that operate at an organised interface such as lipid aggregates in contact with the aqueous phase). Such enzymes can access the substrate only at the interface as the concentration of the monomers of the substrate in the aqueous phase is low. $PLA_2$ acts at the membrane-water interface therefore it needs to bind to the membrane prior to formation of enzyme-substrate complex with subsequent catalytic activity yielding products (Berg *et al.*, 1991). Analysis of $PLA_2$ on model phospholipid bilayers is a useful tool for studying the kinetic properties of interfacial enzymes. The best characterised interfacial enzymes are members of the 14-kDa secretory $PLA_2$ (Gelb *et al.*, 1995). There are two different modes of interfacial catalysis on vesicles. In "hopping" mode, the enzyme is readily dissociated from the membrane interface, thus excess vesicles are accessible for hydrolysis (Figure 1.6 A). In "scooting" mode the enzyme bound to the interface does not dissociate during the process of catalysis and the excess vesicles which do not contain enzyme are not hydrolysed (Figure 1.6 B) (Ramirez and Jain, 1991; Ghomashchi *et al.*, 1992). The scooting mode of analysis has been applied to other interfacial enzymes such as the 85-kDa cytosolic $PLA_2$ (Ghomashchi *et al.*, 1992; Bayburt and Gelb, 1997) and PLC from *Bacillus cereus* (Volwerk *et al.*, 1994).

According to an updated classification, $PLA_2$s can be subdivided into several groups based on their structures and enzymatic characteristics (Table 1.4).

Phospholipase $A_1$

$H_2CO$ — C — $R_1$

Phospholipase $A_2$

$R_2$ — C — OCH

Phospholipase D

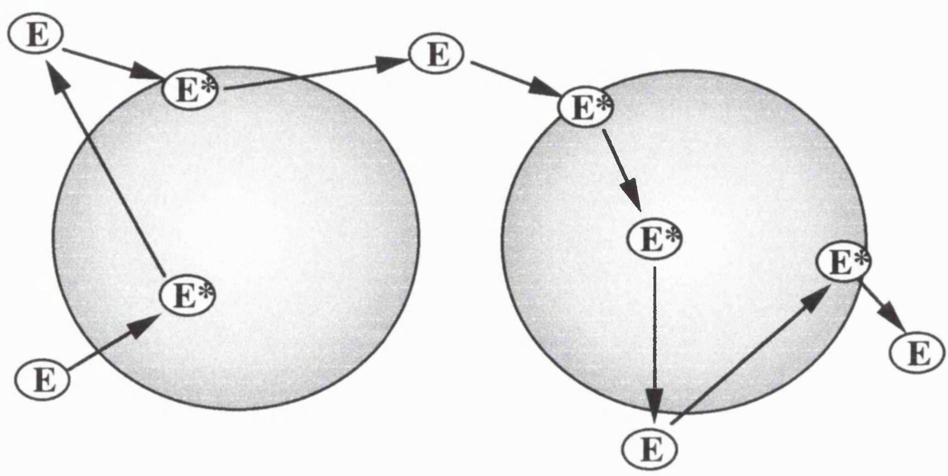
Choline
Serine
Inositol
Ethanolamine

$H_2CO$ — P — $R_3$

Phospholipase C $O^-$

**Figure 1.5: Sites of action of phospholipases on phospholipids.** $PLA_2$ cleaves phosphatidylcholine at *sn*-2 position which releases a free fatty acid such as arachidonic acid and lysophospholipid. PLC hydrolysis phosphatidyl 4,5-bisphosphate and produces inositol trisphosphate and diacylglycerol, PLD hydrolysis phospholipids and produces phosphatidic acid and free polar head group. $R_1$=saturated fatty acid, $R_2$=unsaturated fatty acid

A) Hopping through aqueous phase




B) Scooting without dissociation

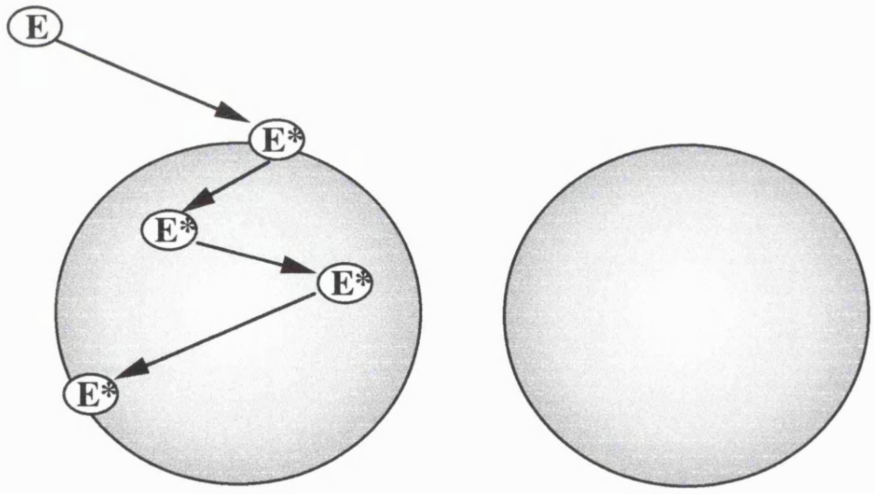



**Figure 1.6: Schematic presentation of interfacial catalysis on phospholipid vesicles in the (A) scooting and (B) hopping mode.** In scooting mode, the bound enzyme does not leave the vesicle even when all of the substrate in the outer monolayer of the target vesicle is hydrolyzed. Therefore excess vesicles are not hydrolysed by the enzyme added initially unless the vesicles are allowed to fuse. During catalysis in the hopping mode, the enzyme desorbs from the vesicle surface between the catalytic cycles, and thus all vesicles are ultimately hydrolysed. E= Enzyme in the aqueous phase, E*= Enzyme in the interface. (Adapted from Jain, 1995)

**Table 1.4: Charateristics of different isoforms of phospholipase A2 ($PLA_2$)**

| $PLA_2$s | Source | Size (kDa) | Substrate | Catalytic Centre | $Ca^{2+}$ Requir. | Disulphide bonds |
|---|---|---|---|---|---|---|
| **Secretory $PLA_2$s** (sPLA2) | | | | | | |
| I A | Cobras/Kraits | 13-15 | PE=PC | His-Asp | mM | 7 |
| B | Porcine/Human pancreas | 13-15 | | His-Asp,E.loop | mM | 7 |
| II A | Rattlesnakes/Vipers/Human synovial fluid/Platelets | 13-15 | PE,PS>PC | His-Asp,CE | mM | 7 |
| B | Gaboon viper | 13-15 | | His-Asp,CE | mM | 6 |
| C (C-16) | Rat/Mouse testes | 15 | PI>PC,PE | His-Asp,CE | mM | 8 |
| III | Bees/Lizards | 16-18 | | His-Asp | mM | 5 |
| V | Heart/Lung/$P388D_1$ macrophages | 14 | | His-Asp,no CE or E.loop | mM | 6 |
| IX | Marine snail | 14 | | His-Asp | <mM | 6 |
| **PAF-acetylhydrolases** | | | | | | |
| VII (secretory) | Human plasma/Macrophages | 45 | PAF, Oxidised PLs | GXSXG consensus sequence,Ser273, Asp296, His351 | None | None |
| VIII (cytosolic) | Bovine brain/Kidney | 29,30,45 heterotrimer | PAF | Ser47 | None | None |
| **Cytosolic $PLA_2$s** (cPLA2) IV(C-12) | Raw 264.7, Rat kidney/Human U937/Platelets | 85 | Arachidonyl PE or PC | GXSGL active site, Ser228, Arg200, Asp549, Ser505, CaL B domain * | μM | None |
| **$Ca^{2+}$-independent $PLA_2$s** ($iPLA_2$) | | | | | | |
| VI | Macrophages/CHO cells | 80-85 | PC | Ankyrin repeats | None | None |
| $iPLA_2$ | Myocardium | 40 | Plasmalogen | N.D. | None | None |
| $iPLA_2$ | Brush-border | 120 | PLs,TG,DG, MG | Ser | None | None |

PC=Phosphatidylcholine, PE=Phosphatidylethanolamine, PS=Phosphatidylserine, E.loop=Elapid loop, CE=Carboxyl extension PL=phospholipid, TG=Triacylglycerol, DG=Diacylglycerol, MG=Monoacylglycerol, *Requires micromolar concentrations of calcium for enzyme regulation not catalysis

### 1.7.1.1 Secretory $PLA_2$ ($sPLA_2$)

$sPLA_2$s are divided into groups I, II and III and also several subgroups as shown in Table 1.4 (Davidson and Dennis, 1990). Based on the presence or absence of short sequences flanking an internal cysteine and the C-terminus (i.e. the carboxyl extension, the elapid loop and the pancreatic loop), the enzymes were placed into groups I and II, each subdivided into subgroups A and B. Group I enzymes are mainly found in mammalian pancreatic juice which is a digestive enzyme for dietary phospholipids. The type I enzyme is also present in non-digestive organs such as kidney (Hara *et al.*, 1995), spleen (Tojo *et al.*, 1988) and lung (Seilhamer *et al.*, 1986). It regulates cellular functions via specific receptors such as N-type receptors, which show high affinity for neurotoxic $sPLA_2$s (Lambeau *et al.*, 1989), and M-type receptors, initially identified in skeletal muscle for myotoxic $sPLA_2$s (Lambeau *et al.*, 1990). Group I $sPLA_2$s are also present in the venoms from *Elapidae* and *Hydrophidae* snakes, whereas group II enzymes are found mostly in *Crotalidae* and *Viperidae* snakes (Waite, 1988). Enzymes of the IIA subgroup of class II enzymes were originally isolated from inflammatory fluids and cells (Kramer *et al.*, 1989). The anti-tumorigenic property of this group of $sPLA_2$ has been shown in mouse strains genetically lacking $sPLA_2$-IIA (MacPhee *et al.*, 1995). Group III $sPLA_2$s have been isolated from lizard and bee venoms. Apart from their primary catalytic function, snake venom $sPLA_2$s display myotoxic (Mebs, 1986; Gutiérrez and Lomonte, 1995), haemolytic (Condrea *et al.*, 1981), neurotoxic (Bon *et al.*, 1979) and cardiotoxic activities (Fletcher *et al.*, 1981). Group V $sPLA_2$ is expressed in heart, lung and also in mast cells and macrophages where it functions in inflammation and signal transduction (Chen *et al.*, 1994).

$sPLA_2$s are enzymes composed of a single polypeptide chain of about 119-143 amino acid with molecular mass between 12-15 kDa. $sPLA_2$s require millimolar concentrations of calcium for effective catalytic function and are thus active in the extracellular mileau where such high concentrations are found. The rigid tertiary structure of this group of enzymes is due to stabilisation by 6-8 disulphide bonds (12-14 cysteine residues) and they are relatively thermostable, however they are sensitive to reducing agents such as dithiothreitol (DTT) which break disulphide bonds. Despite these similarities, there are certain structural differences between group I and II $sPLA_2$. The critical structural differences between these two groups is the disulphide bond between cysteine residues 11 and 77 in group I, which is absent in group II enzymes (Ward and Pattabiraman, 1990). Another difference is in the region 52-65 where class I proteins display a two to three amino acid insertion, the "Elapid loop", which is extended by a further five amino acids in the case of mammalian pancreatic

PLA$_2$s (the "pancreatic loop"). Type I sPLA$_2$ hydrolyses phosphatidylcholine (PC) and phosphatidylethanolamine (PE) at the same rate (Seilhamer *et al.*, 1986), whereas type II enzymes hydrolyse PE and phosphatidylserine (PS) in preference to PC and phosphatidylinositol (PI) (Ono *et al.*, 1988). Class III enzymes display a low degree of homology to the class I and II PLA$_2$s. Despite the overall differences in primary sequence, secondary and tertiary structural elements involved in catalysis and calcium ion binding are conserved between classes I, II and III PLA$_2$s (Scott *et al.*, 1990)

The gene encoding type I PLA$_2$ was mapped to human chromosome 12 (Tischfield, 1997) and type II PLA$_2$ was positioned to chromosome 1q35, which contains five exons with 70%-80% homology among different animal species (Riggins *et al.*, 1995). Upstream of the mRNA initiation site, a TATA box and a sequence homologous to a CAAT box were found which are involved in binding of transcription factors.

An activator of sPLA$_2$ known as PLA$_2$-activating protein (PLAP), was detected (Clark *et al*, 1987a) in many cells such as, monocytes/macrophages, endothelial cells, as well as synovial fluid from patients with rheumatoid arthritis (Bomalaski *et al.*, 1990). PLAP stimulates human neutrophils to aggregate and release lysosomal enzymes and generate eicosanoids and superoxide (Bomalaski *et al.*, 1989).

### 1.7.1.2 Cytosolic PLA$_2$ (cPLA$_2$)

*i) Cellular distribution, roles and activation*

cPLA$_2$ is a widely distributed enzyme with the gene being expressed in almost all human cells including platelets (Kramer *et al.*, 1986), neutrophils (Ramesha and Ives, 1993), human monocytic cell line U937 (Clark *et al.*, 1990; Kramer *et al.*, 1991), macrophage cell line Raw 264.7 (Leslie *et al.*, 1988), epithelial cells (Hoeck *et al.*, 1993), fibroblasts (Lin *et al.*, 1992a), smooth muscle cells and renal mesangial cells (Gronich *et al.*, 1990), human astrocytes of the grey matter (Stephenson *et al.*, 1994), with elevated levels of mRNA expression in brain, spleen, lung and heart in both mouse and humans (Sharp and White, 1993). The absence of cPLA$_2$ from mature lymphocytes indicates that this enzyme is not required for the function of the mature lymphocytes but it is involved in maturation of immature lymphocytes (Gilbert *et al.*, 1996)

cPLA$_2$, hydrolyses the *sn*-2 fatty acyl ester bond of phosphoglycerides producing free fatty acid (arachidonic acid) and lysophospholipids. This enzyme plays an important role in metabolism of phospholipids and production of biologically active lipids, including eicosanoids and platelet-activating factor (PAF). cPLA$_2$ can be

activated by a variety of stimuli including the proinflammatory cytokines IL-1, TNFα, thrombin, ATP and LPS (Table 1.5). Studies of the subcellular localisation of $cPLA_2$ from many cells showed that upon activation this enzyme translocates to endoplasmic reticulum and nuclear membranes (Glover *et al.*, 1995; Schievell *et al.*, 1995), where cyclooxygenase 1 (COX 1) and COX 2 are localised, respectively (Goetzl *et al.*, 1995a; Morita *et al.*, 1995). It has been shown that 5-lipoxygenase, an enzyme immediately downstream of $PLA_2$ that metabolises AA to leukotrienes, is also translocated from the cytosol to the nuclear envelope and its activation is regulated by $cPLA_2$ (Woods *et al.*, 1993). $cPLA_2$ is also involved in activation of platelets. These cells generate large amounts of arachidonic acid in response to thrombin with subsequent generation of thromboxane $A_2$ which has a role in the thrombotic cascade (Kramer *et al.*, 1993; Takayama *et al.*, 1991) In addition, it has been shown that $cPLA_2$ possesses lysophospholipase activity towards 1-acyl-lysophosphocholine (Leslie *et al.*, 1991) and transacylase activity which proceeds through an acyl-enzyme intermediate (Reynolds *et al.*, 1993). Bonventre *et al.* (1997) have recently shown that, stimulation of peritoneal macrophages from 'knockout' mice that lack cytosolic $PLA_2$ ($cPLA_2^{-/-}$), did not produce prostaglandin $E_2$, leukotriene $B_4$ or $C_4$. In addition, these knockout mice had smaller infarcts and developed less brain oedema after transient occlusion of their middle cerebral artery. This was supported by another *in vivo* study using $cPLA_2^{-/-}$ mice. These mice showed a marked decrease in their production of eicosanoids and platelet activating factor in peritoneal macrophages, and also female mutant mice failed to deliver offspring, indicating that $cPLA_2$ plays a role in allergic responses and fertility (Uozumi *et al.*, 1997).

*ii) Chromosomal location*

The gene for human $cPLA_2$ has been mapped to human chromosome 1q25 (Tay *et al.*, 1995).This localisation is interesting as the gene for cyclooxygenase-2 (COX-2) is also mapped to chromosome 1q25 (Tay *et al.*, 1995).

*iii) Protein structure*

The cDNA for human $cPLA_2$ was first cloned in the human monocytic cell line, U937 (Clark *et al* ., 1991; Sharp *et al.*, 1991), and encodes a 749 amino acid protein with a molecular mass of 85.2 kDa [by SDS-PAGE the molecular weight has been shown to be 110 kDa (Leslie *et al.*, 1988)], and shows more than 95% homology to the murine and 74% homology to the zebrafish $cPLA_2$ sequence, indicating a similarity between $cPLA_2$ from different species. However, $cPLA_2$ does not show any

**Table 1.5: Stimuli involved in regulation of $cPLA_2$**

| *Cell Type* | *Stimuli* | *References* |
|---|---|---|
| Human neutrophils | TNFα | Waterman *et al.*, 1995 |
| | GM-CSF | Doerfler *et al.*, 1994 |
| | FMLP | Durstin *et al.*, 1994 |
| | LPS | Fouda *et al.*, 1995 |
| | LTB4 | Wijkander *et al.*, 1995 |
| Human monocytes | M-CSF | Nakamura *et al.*, 1992 |
| | LPS | Roshak *et al.*, 1994 |
| | β-chemokines | Locati *et al.*, 1996 |
| Human platelets | Thrombin | Kramer *et al.*, 1993, |
| | Collagen | Börsch-Haubold *et al.*, 1997 |
| HeLa cells | TNFα | Hoeck *et al.*, 1993 |
| | IFNα | Flati *et al.*, 1996 |
| Mouse peritoneal macrophages | Zymosan, PMA, A23187 | Qiu *et al.*, 1993; Qiu, 1994 |
| | Bacteria | Svensson *et al.*, 1993 |
| CHO cells | Thrombin | Winitz, 1994 |
| Mouse embryonic cells | EGF | Chepenik *et al.*, 1994 |
| Rat fibroblasts rat-2 | PDGF, EGF | Lin *et al.*, 1992a |
| Mouse Keratinocytes (HEL-30) | TGF-α | Kast *et al.*, 1993 |
| Rat mesangial cells | IL-1α | Gronich *et al.*, 1994 |

sequence homology with low molecular weight $sPLA_2$.

The overall primary structure of $cPLA_2$ is illustrated in Figure 1.7 and has several interesting features; The N-terminal region of the $cPLA_2$ sequence consists of a stretch of 126 amino acids called the $Ca^{2+}$-dependent lipid-binding (CaLB) domain which is responsible for $Ca^{2+}$-dependent translocation of $cPLA_2$ from the cytosol to nuclear or endoplasmic reticular membranes (Sharp *et al.*, 1991). The CaLB domain also contains four acidic groups, Asp37, Asp43, Asp93 and Glu100, which are important for binding calcium (Nalefski and Falke, 1996). This region shows significant homology to the second conserved (C2) regulatory domain of protein kinase C ( PKC $\alpha,\beta,\gamma$), and it is also present in phospholipase C (PLC$\gamma$), Unc-13 (a plasma membrane protein in nerve terminals), synaptic vesicle protein p65, synaptotagmin (an integral membrane protein localised to synaptic vesicles), rabphilin-3A (a synaptic vesicle protein) and the GTPase activating protein (GAP) (Clark *et al.*, 1991; Nalefski *et al.*, 1994) (Figure 1.8). All these proteins share a common feature in that they associate with membranes and this suggests that their CaLB-like domain is partly responsible for this association.

Several amino acids have been shown to play an important role in the catalytic mechanism of $cPLA_2$. Serine (Ser) 228 in the segment Gly-Leu-$Ser^{228}$-Gly-Ser (GXSXS) acts as an active site nucleophile which is required for the catalytic function of $cPLA_2$. This was demonstrated when the mutation of this serine to alanine abolished $cPLA_2$ activity (Sharp *et al.*, 1994). Aspartate (Asp) 549 which resides in the sequence motif (Ile-X-Val-Val-Asp-Ser-Gly-Leu-X-X-X-Asn) has also been shown to participate in the catalytic mechanism of $cPLA_2$ (Pickard *et al.*, 1996). Unlike serine-esterases and lipases, histidine is not involved in catalytic function of $cPLA_2$. There are nine cysteine (Cys) residues of which only Cys-331 appear to have catalytic funtion as it resides near the active site (Li *et al.*, 1996). Arginine (Arg) 200 is important for the regulation of $cPLA_2$ because it interacts with the CaLB domain and assists the binding of the enzyme to the lipid interface (Pickard *et al.*, 1996). Another characteristic of the $cPLA_2$ protein is the presence of a segment of 33 amino acids in the middle of the sequence that lacks any hydrophobic amino acids which may represent a flexible hinge region. It should be noted that although calcium is required for translocation of $cPLA_2$ to the membrane, it is not required for catalysis unlike the $sPLA_2$ family that require millimolar calcium levels for catalysis.

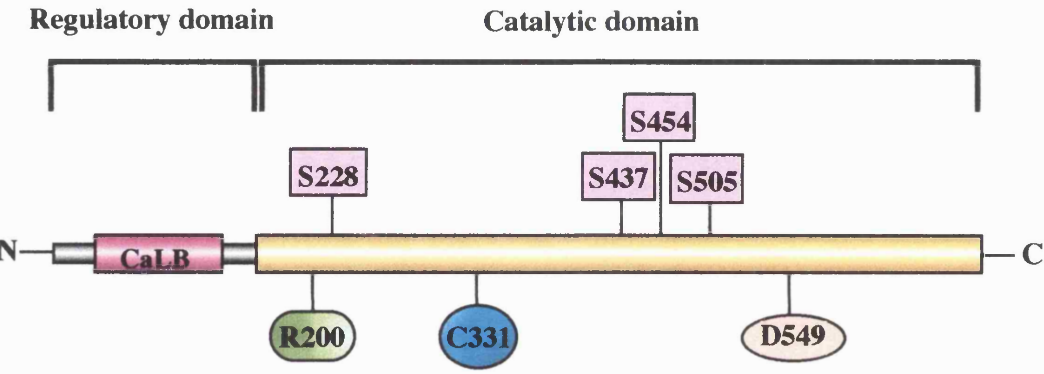



**Figure 1.7: Schematic presentation of the primary structure of $cPLA_2$**
S (Serine), R (Arginine), C (Cysteine), D (Aspartic acid).

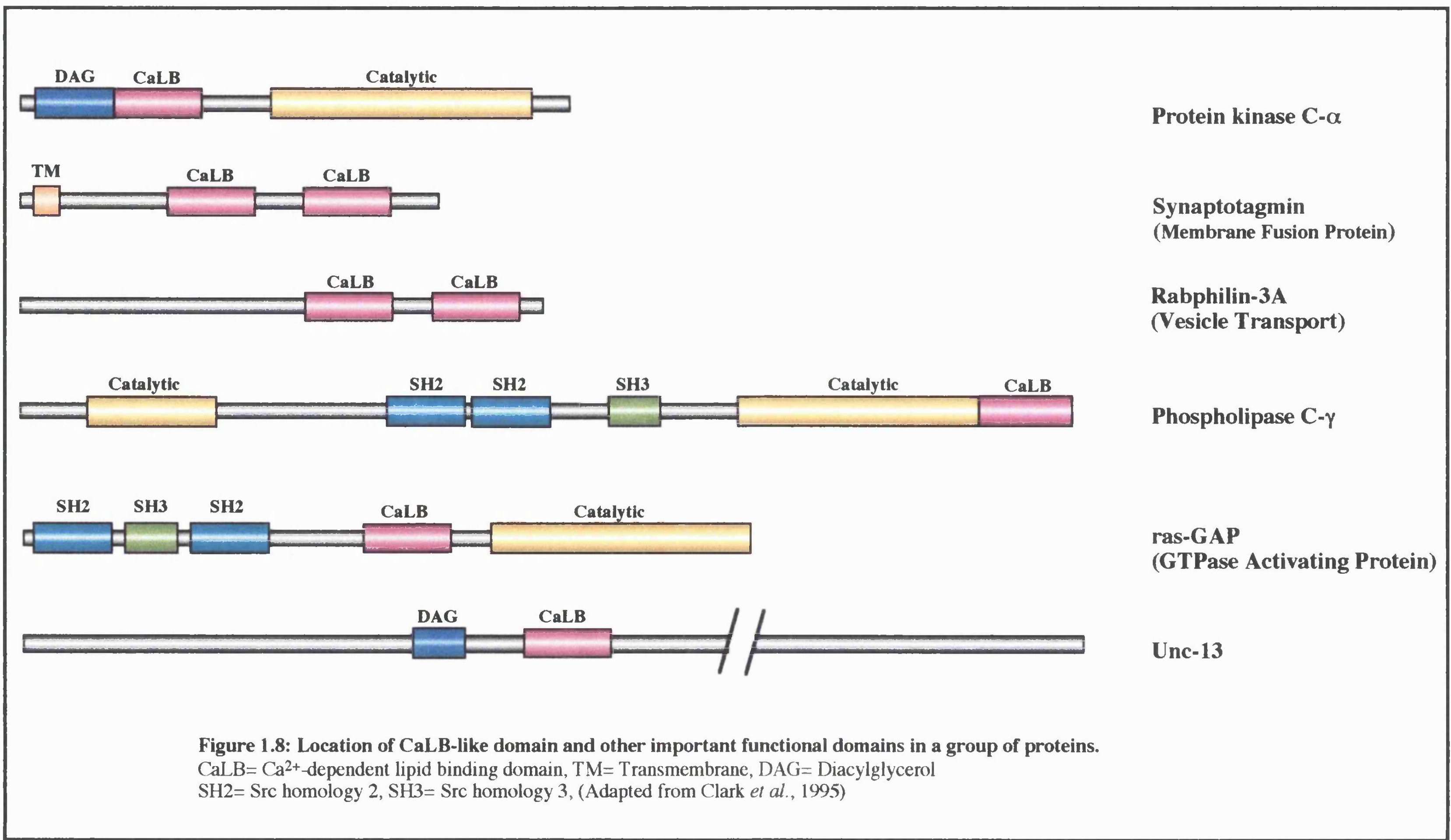



**Figure 1.8: Location of CaLB-like domain and other important functional domains in a group of proteins.**
CaLB= $Ca^{2+}$-dependent lipid binding domain, TM= Transmembrane, DAG= Diacylglycerol
SH2= Src homology 2, SH3= Src homology 3, (Adapted from Clark *et al.*, 1995)

*iv) Regulation of $cPLA_2$*

$cPLA_2$ is regulated at both post-transcriptional and post-translational levels which makes this enzyme capable of responding rapidly to signals from physiological stimuli and second messengers. It has been demonstrated that $cPLA_2$ is regulated by multiple mechanisms including:

*1)* ***Calcium dependent translocation*** which is mediated through the CaLB domain which serves to localize the catalytic site with its substrate in the membrane. $cPLA_2$ is catalytically active in the presence of submicromolar $Ca^{2+}$ concentrations (Kramer, 1994), other bivalent cations such as $Sr^{2+}$, $Ba^{2+}$ and $Mn^{2+}$ also result in full activity of $cPLA_2$ by increasing the association of the CaLB-domain of $cPLA_2$ with the membrane. However, in the absence of $Ca^{2+}$, high salt also increases the association of $cPLA_2$ with the membrane by increasing hydrophobic interactions (Reynolds *et al.*, 1993).

*2)* ***Phosphorylation*** of $cPLA_2$ has been observed in many cells stimulated with a variety of stimuli (Table 1.5). The initial evidence for the phosphorylation of $cPLA_2$ was from experiments in Chinese hamster ovary (CHO) cells overexpressing $cPLA_2$ (Lin *et al.*, 1992b). Activation of these cells with various agonists such as; thrombin, ATP, phorbol ester and A23187, stimulated the phosphorylation of $cPLA_2$ on serine residues. It has been shown that $cPLA_2$ has multiple (Ser-437, Ser-454, Ser-505, Ser-727) phosphorylation sites (de Carvalho *et al.*, 1996) of which only 2 such sites Ser-505 and Ser-727 are conserved in $cPLA_2$ from evolutionarily distant species (human, murine, chicken, zebrafish) consistent with functional roles (Clark *et al.*, 1995). The characteristic decrease in electrophoretic mobility shift of $cPLA_2$ after phosphorylation allows detection of agonist-induced phosphorylation of $cPLA_2$.

Several studies have shown that p42/p44 MAPK has a role in activation of $cPLA_2$ stimulated by various agonists such as; GM-CSF (Durstin *et al.*, 1994), thrombin (Kramer *et al.*, 1993), TPA and zymosan (Qiu and Leslie, 1994). There is also evidence that $cPLA_2$ phosphorylation in neutrophils stimulated by LPS or TNF$\alpha$ or in platelets stimulated by thrombin, occurs via p38 MAP kinase (Fouda *et al.*, 1995; Kramer *et al.*, 1995). Interestingly, in macrophages treated with calcium-ionophore, increased phosphorylation of $cPLA_2$ was not associated with MAP kinase activation (Qiu and Leslie, 1994) suggesting, involvement of additional kinases such as PKC-$\alpha$ and PKA in regulation of $cPLA_2$ (Godson *et al.*, 1993). There is also evidence that Ser-727 is phosphorylated on $cPLA_2$ (Börsch-Haubold *et al.*, 1998). Ser-727 lies within the consensus sequence which flanked by arginines (Arg-Arg-$(X)_4$-Arg-Ser-$(X)_8$-Arg-Arg) typical of sites preferred by basotrophic kinases such as PKC and PKA.

***3) GTP-binding proteins*** **(G-proteins)**: Several studies have suggested that the pertussis-toxin-sensitive G protein ($G_i$) may be involved in the regulation of $cPLA_2$, as GTPγS stimulated arachidonate release in various cell types and pertussis toxin inhibited AA release by activated cells (Silk *et al.*, 1989; Xing and Mattera, 1992). However, at present there is little evidence for the direct interaction of $cPLA_2$ with $G_i$. Because G-protein-initiated signals are coupled to PI-specific phospholipase C-β (Rhee and Choi, 1992) leading to production of inositol trisphosphate, which in turn causes an increase in cytoplasmic calcium levels, therefore the inhibitory effect of pertussis toxin on arachidonic acid release is likely to be due to the inhibition of calcium signalling which is essential for $cPLA_2$ activation. It has been shown that inhibition of thrombin or ATP-induced $cPLA_2$ activation by pertussis toxin in the $cPLA_2$-overexpressing CHO cells was due to the decreased MAPK-mediated $cPLA_2$ phosphorylation (Clark *et al.*, 1995), which is compatible with the signalling pathway in which the trimeric G-protein pathway is functionally linked to the MAP kinase pathway (Crespo *et al.*, 1994; Pumig lia *et al.*, 1995; Druey *et al.*, 1996).

***4) Transcriptional and Post-transcriptional*** regulation of $cPLA_2$ has been shown in several studies. The expression of $cPLA_2$ protein can be controlled by both transcriptional and post-transcriptional mechanisms to maintain the cellular levels of arachidonate. The 5' flanking region of the human gene has consensus binding sites for AP-1, AP-2, PEA3, glucocorticoid response element (GRE), NF-κB (Tay *et al.*, 1994). However, none of these elements have been shown experimentally to regulate expression of $cPLA_2$. In addition the 5'-flanking region has no TATA or CAAT box and it is atypical in that it is not GC rich. The 5' flanking region also contains a 27 base pair polypyrimidine sequence which is important for transcriptional activity of the $cPLA_2$ gene and it is also responsible for the basal expression of $cPLA_2$ (Miyashita *et al.*, 1995). In addition, the 5' flanking region contains a long run of CA repeats (48 base purine/pyrimidine repeats) which has an inhibitory effect on $cPLA_2$ gene transcription (Tay *et al.*, 1994; Miyashita *et al.*, 1995). M-CSF treatment of monocytes for 24 hours was shown to increase the half-life of $cPLA_2$ mRNA from 30 minutes to 2 hours, suggesting that post-transcriptional control is mediated through regulation of mRNA half-life via multiple conserved AUUUA sequences in the 3' untranslated region of $cPLA_2$ gene in mitogen stimulated cells (Nakamura *et al.*, 1992; Clark *et al.*, 1995).

*V) $cPLA_2$ substrates*

Activation of $cPLA_2$ provides a direct pathway of AA release specifically from the *sn*-2 position of the fatty acyl ester bond of the membrane phospholipids (Clark *et al.*, 1991; Diez *et al.*, 1994). In contrast, $sPLA_2$s do not show fatty acid specificity (Murakami *et al.*, 1991; Hara *et al.*, 1995). $cPLA_2$ hydrolyses phospholipids in the order of phosphatidylcholine (PC) = phosphatidylinositol (PI) > phosphatidyl-ethanolamine (PE) > phosphatidic acid = phosphatidylserine (PS) (Hanel *et al.*, 1993). In contrast to the above *in vitro* studies which used experimental phospholipid mixes, studies on "natural membranes" demonstrated no selectivity among PC, PI and PE, and the rate of hydrolysis was based on the content of arachidonic acid in each phospholipid subclass (Diez *et al.*, 1994). $cPLA_2$ also has a preference for polyunsaturated fatty acids, especially those with three *cis* double bonds between carbon 5 and 6, 8 and 9, 11 and 12, therefore phospholipids containing linolenate (C18:3) or eicosapentaenoic acid (C20:5) are also good substrates for $cPLA_2$.
In addition, phosphatidic acid through the action of PA-specific $PLA_2$ can enhance release of free AA (Billah *et al.*, 1981; Thomson and Clark, 1995; Buckland and Wilton, 1998).

### 1.7.1.3 Calcium-independent $PLA_2$

*i) $iPLA_2$*

Another group of $PLA_2$ enzymes are the calcium independent isoforms of which three major classes have been cloned.

**1) Myocardial $iPLA_2$:** has been identified (Hazen *et al.*, 1991) with a molecular mass of 40 kDa. The interaction between this 40 kDa catalytic subunit and an 85 kDa polypeptide known as phosphofructokinase which is modulated by ATP is thought to form an ATP-sensitive regulatory complex which has the ability to hydrolyse plasmalogen, a major phospholipid constituent of myocardial tissue.

**2) Macrophage $iPLA_2$:** was first purified from the cytosol of the $P388D_1$ macrophage cell line (Ackermann *et al.*, 1994) and it is also present in neutrophils (Larsson *et al.*, 1998). The catalytic subunit of this enzyme with a molecular mass of 80 kDa interacts with ATP for activation. It does not show preference for *sn*-2 arachidonic acid or *sn*-1 alkenyl-ether phospholipids (Ackermann *et al.*, 1994), and displays 5% lysophospholipase activity.

**3) Brain $iPLA_2$:** was first identified in bovine brain (Yang *et al.*, 1994; Farooqui *et al.*, 1995). It has been shown that bovine brain cytosol contained two

$iPLA_2s$, one with molecular mass of 110 kDa and the other 39 kDa with substrate preferences for 1,2-diacyl-PE and 1-alkenyl-2-acyl-(plasmenyl)-PE, respectively. The plasmalogen-selective $iPLA_2$ in brain showed a molecular weight close to that of myocardial $iPLA_2$. However, brain $iPLA_2$ does not require ATP for stimulation.

*ii) Brush-Border membrane $iPLA_2$*

This is an enzyme with a molecular mass of 120-140 kDa and is found in the brush-borders of the small intestine. It has a broad substrate specificity, and has $Ca^{2+}$-independent $PLA_2$ activity as well as *sn*-1 lysophospholipase and glycerol lipase activity (Pind *et al.*, 1989). It also hydrolyses mono-, di- and triacylglycerols (Gassama-Diagne *et al.*, 1992)

*iii) Platelet activating factor-acetyl hydrolases (PAF-AHs)*

This class of calcium-independent $PLA_2$ are divided into two groups, extracellular and intracellular PAF-AHs. Their main funtion is the removal of the acetyl moiety from the *sn*-2 position of the phospholipid (Bazan, 1995). Extracellular PAF-AH is important in inflammation and the regulation of circulating PAF levels. It has been shown that PAF-AH is a $PLA_2$ with characteristics of neutral lipases and esterases. An interesting PAF-AH was isolated from human erythrocytes with a molecular mass of 25 kDa which appeared to be a homodimer. Erythrocytes are not capable of synthesising PAF, therefore the function of this enzyme is probably to scavenge oxidatively damaged phospholipids generated in the erythrocyte (Stafforini *et al.*, 1993).

### 1.7.1.4 Effects of arachidonic acid generation in cells

AA is a long chain, polyunsaturated fatty acid (C20:4), the bulk of which is esterified in the fatty acyl chains of glycerophospholipids at the *sn*-2 position. Stimulation of leukocytes with a heterogeneous group of mediators such as opsonised particles and cytokines, induces the production of AA (Duyster *et al.*, 1992; Robinson *et al.*, 1996). After release, free AA can diffuse out of the cell. Alternatively, it can be either reincorporated into membrane phospholipids which requires conversion of the fatty acid into arachidonoyl-coenzyme A (AA-CoA) by AA-CoA synthase, and its subsequent esterification by arachidonoyl-lysophospholipid transferase, or it can metabolised into proinflammatory substances, called eicosanoids by one of the three pathways shown in Figure 1.9. 1) Cyclooxygenase (prostaglandin G/H synthase,

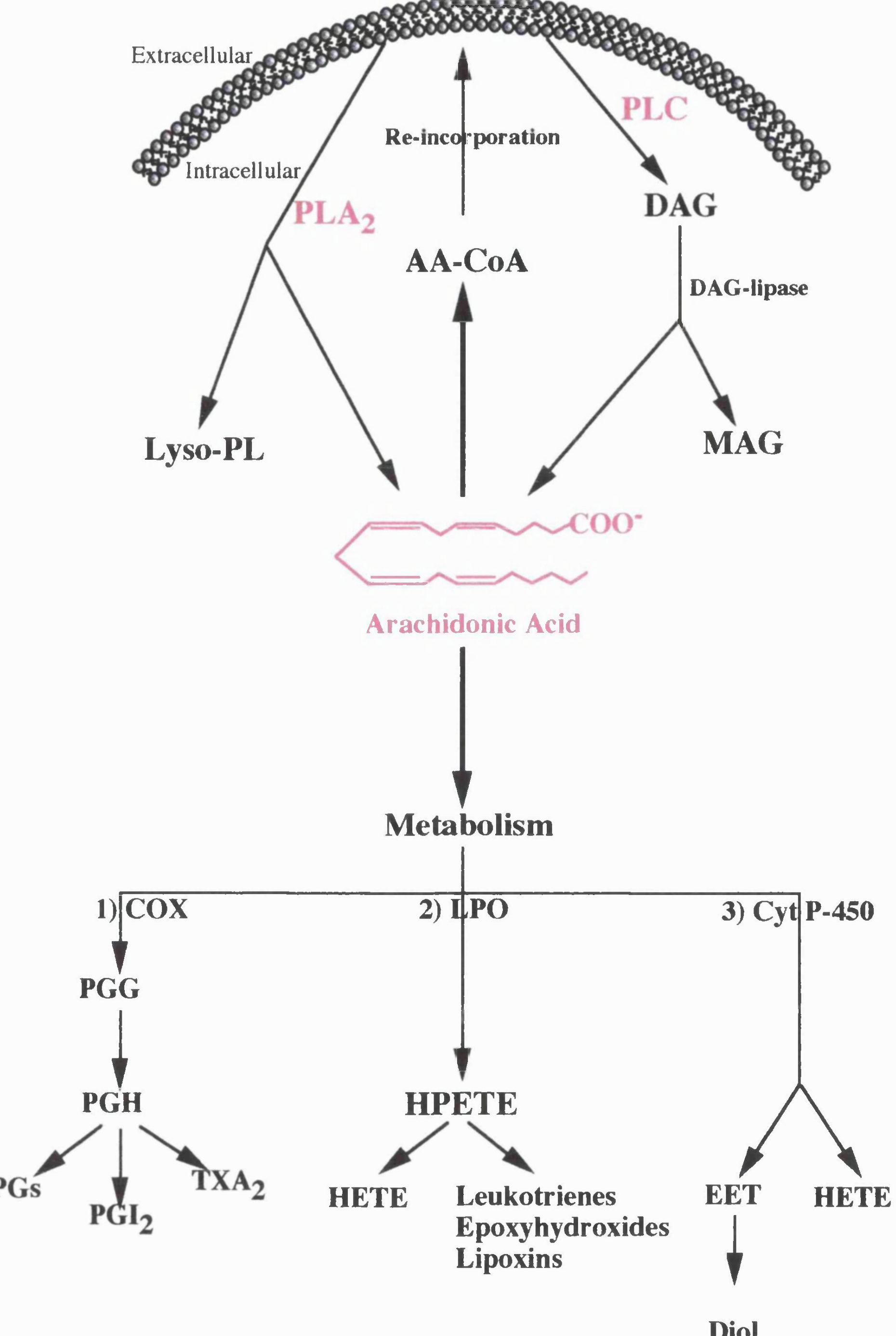



**Figure 1.9: The arachidonate cascade.** Pathways involved in metabolism of arachidonic acid leading to the production of eicosanoids are 1) cyclo-oxygenase (COX), 2) Lipoxygenase (LPO) and 3) Cytochrome P-450 (Cyto p-450). AA-CoA (arachidonyl-Coenzyme A), PLC (Phospholipase C), $PLA_2$ (Phospholipase $A_2$), DAG (Diacylglycerol), MAG (Monoacylglycerol), Lyso-PL (Lysophospholipase), PGH/PGG (Prostaglandins), $PGI_2$ (Prostacyclin), HPETE (Hydroperoxyeicosatetraenoic acid), HETE (Hydroxyacid), $TXA_2$ (Thromboxane), EET (Epoxyeicosatrienoic acid)

PGHS) catalyses the conversion of AA into the reactive intermediates PGG and PGH, which are the precursors of prostaglandins, prostacyclin and thromboxanes ($TXA_2$). 2) Lipoxygenases form hydroperoxyeicosatetraenoic acids (HPETE) as primary products, which can either undergo reduction to corresponding hydroxyacids (HETE), or conversion into leukotrienes and epoxyhydroxides. 3) P-450 catalyses the conversion of AA into epoxyeicosatrienoic acids (EET) which are hydrolysed to corresponding diols by epoxide hydrolase (reviewed by Sigal, 1991). AA is the rate limiting precursor in the biosynthesis of eicosanoid lipid mediators and may itself act as second messenger in many cellular functions (reviewed by Piomelli and Greengard, 1990; Nishizuka, 1992). The concentration of of free AA in resting cells is maintained at submicromolar levels by an AA-selective reacylation pathway.

AA and its metabolites modulate diverse physiological and pathological responses, including growth and invasiveness of tumor cells as well as immune surveillance (Ara and Teicher, 1996; Tang *et al.*, 1995; Young, 1994). In addition to their role in regulating mitogenesis, various eicosanoids can either trigger or inhibit apoptosis. For example, production of PGE2 plays an important role in the apoptosis required for the release of the eggs during ovulation (Lim *et al.*, 1997). In contrast, PGE2 blocks activation-induced apoptosis in $CD4^+/CD8^+$ T lymphocytes (Goetzl *et al.*, 1995b). It has been shown that AA suppresses ceramide-induced cell death in prostate cancer cells and that this suppression depends on the formation of lipoxygenase products (Herrmann *et al.*, 1997). Recently, it has been shown that inhibition of 5-lipoxygenase triggers apoptosis in human prostate cancer cells (Ghosh and Myers, 1998).

AA and related fatty acids are involved in a series of biological events that amplify/regulate cell activation and inflammatory responses. For example, AA has been shown to induce degranulation (Bates *et al.*, 1993), regulate chemotaxis (Ferrante *et al.*, 1994), increase expression of CR3 receptors (Bates *et al.*, 1993) and activate the oxygen-dependent respiratory burst in neutrophils (Badwey *et al.*, 1984; Steinbeck *et al.*, 1991; Hardy *et al.*, 1991; Poulos *et al.*, 1991), macrophages (Bromberg and Pick, 1983) and the monocytic U937 cell line (Sellmayer *et al.*, 1996). AA stimulates the activity of NADPH oxidase by increasing the number of active enzyme complexes and its affinity for the NADPH (Rubinek and Levy, 1993; Sumomoto *et al.*, 1994). This activation may depend on the activation of leukocyte G-proteins by AA. It has been shown by Chuang *et al.* (1993) that AA and other lipid second messengers such as phosphatidic acid regulate complex formation between Rac and GDI (GDP dissociation inhibitor). In addition, several studies have demonstrated that AA exerts direct effects on phagocyte $Ca^{2+}$ and $H^+$ ion flux (Kapus *et al.*, 1994; Leaver *et al.*, 1992; Henderson and Chappell, 1992; DeCoursey and Cherny, 1993).

Thus free AA, with a limited range owing to enzymes of esterification and metabolism, activates phagocyte NADPH oxidase over short distances (Sakata *et al.*, 1987; Forehand *et al.*, 1993).

It has also been proposed that signal-induced responses of AA are mediated by activation of PKC (McPhail *et al.*, 1984; Dennis *et al.*, 1991), indicating a second messenger role for AA in cell function. Several studies have shown the importance of AA as the regulator of various genes such as c-fos and *c-jun* (Haliday *et al.*, 1991; Jurivich *et al.*, 1994; Rizzo and Boswell, 1994). It has been shown that AA induces *c-jun* gene expression in stromal cells stimulated by IL-1 and TNF-$\alpha$ (Rizzo *et al.*, 1995). AA can also stimulate sphingomyelinase activity, which in turn catalyses the hydrolysis of sphingomyelin to generate choline phosphate and the second messenger, ceramide (Jayadev *et al.*, 1994).

#### 1.7.1.5 Pathological & physiological roles of $PLA_2$

There is evidence to indicate that $PLA_2$ has beneficial physiological roles as well as being involved in the production of pro-inflammatory mediators which are involved in inflammatory processes and degenerative disease. Table 1.6 summarises some of these roles.

### 1.7.2 Phospholipase C and PKC

To date ten mammalian phospholipase C (PLC) isoforms have been recognised which are all single polypeptides. The major isoforms of PLC are $\alpha$, $\beta$, $\gamma$ and $\delta$. PLC is present in most mammalian cells, plants and microorganisms. PLC hydrolyses the membrane phospholipid, phosphatidylinositol 4,5-bisphosphate ($PIP_2$) which yields inositol trisphosphate ($IP_3$), which regulates the release of $Ca^{2+}$ from intracellular stores and diacylglycerol (DAG), which allows the translocation of protein kinase C (PKC) to the plasma membrane (Rhee and Choi, 1992; Berridge, 1993). Activation of PLC and PKC has been shown to be important for mitogenic signals and growth regulation. Stimulation of the immature cell lines, U937, HL-60 by GM-CSF has been shown to cause an increase in intracellular DAG and hence, translocation of PKC to the plasma membrane (Adunyah *et al.*, 1991; Rajotte *et al.*, 1992), which was not the case in neutrophils (Sullivan *et al.*, 1987; Mege *et al.*, 1989). In another study, PKC inhibitors caused a significant enhancement of GM-CSF stimulated responses in neutrophils and reduced GM-CSF receptor internalisation (Khwaja *et al.*, 1990), suggesting that PKC plays a role in down regulation of neutrophil responses to GM-CSF. PKC has been impilcated in the mitogen-activated protein kinase (MAPK)

**Table 1.6: Physiological and pathological roles of phospholipase $A_2$**

| *$PLA_2s$* | *Roles* | *References* |
|---|---|---|
| **Secretory $PLA_2$** | | |
| Type I | Digestion of dietary fat | Waite, 1988 |
| | Acute pancreatitis | Nevalainen, 1980 |
| | | Nevalainen&Gronroos, 1995 |
| | Acute chest syndrome | Rae *et al.*, 1994 |
| Type II | | |
| | Inflammation | Kramer *et al.*, 1989 |
| | Ischaemia | Saevanian *et al.*, 1985 |
| | Eicosanoid generation | Schalkwijk *et al.*, 1991 |
| | Antimicrobial activity | Elsbach *et al.*, 1988 |
| | Anticoagulation | Kini *et al.*, 1987 |
| | Degranulation | Murakami *et al.*, 1992 |
| | Cell adhesion | Lefkowith *et al.*, 1992 |
| | T-cell activation | Asaoka *et al.*, 1993 |
| | Bone resorption | Ellies *et al.*, 1991 |
| **Cytosolic $PLA_2$** | | |
| Type IV | Signal transduction | Dennis *et al.*, 1991 |
| | Eicosanoid generation | Bonventre *et al.*, 1992 |
| | PAF generation | Dennis *et al.*, 1997 |
| | Reproduction | Sugimoto *et al.*, 1997 |
| | Neurotoxicity | Bonventre&Koroshetz, 1993 |
| **iPLA2** | Phospholipid remodelling | Balsinde *et al*, 1995 |
| | Arachidonate release | Lehman *et al*, 1993 |
| | Endocytosis | Mayorga *et al.*,1993 |
| | Ischaemia | Hazen *et al.*, 1991 |

pathway and its activation also results in phosphorylation of Raf both *in vitro* and in stimulated NIH3T3 cells (Kolch *et al.*, 1993; Sozeri *et al.*, 1992). The precise role of PKC in cytokine-mediated activation of the MAP kinase pathway still remains to be defined.

### 1.7.3 Phospholipase D

PLD catalyses the hydrolysis of terminal phosphodiester bond of the phospholipid leading to the formation of phosphatidic acid (PA) and the free polar head group such as choline, in the case of phosphatidylcholine (Figure 1.5), in response to variety of growth factors and hormones. PA can be further metabolised to DAG by phosphatidate phosphohydrolase, and to lysophosphatidic acid (LPA) and AA by a PA-specific $PLA_2$ (Billah *et al.*, 1981; Thomson and Clark, 1995). Most mammalian PLD activity is associated with the membranes and appears to be specific for PC. Mammalian PLD is activated via multiple pathways involving G-proteins, $Ca^{2+}$, unsaturated fatty acids and PKC. PLD activity can be stimulated by phorbol esters in many cells such as human granulocytes, human monocytes, human B lymphocytes (infected by Epstein-Barr virus), human platelets, suggesting that PKC plays an important role in PLD activation (Gelas *et al.*, 1989; Kinsky *et al.*, 1989; Randall *et al.*, 1990). It has been proposed that both PA and DAG act as second messengers to link cell stimulation to cellular responses (reviewed by Thompson *et al.*, 1993).

PA is a potent biologically active lipid which is involved in induction of platelet aggregation (Gerrard *et al.*, 1979; Tokumura *et al.*, 1981), smooth muscle contraction (Tokumura *et al.*, 1982) and chemotaxis (Jalink *et al.*, 1993). PA is also a unique phospholipid required for the docking of Raf-1 kinase to the cell membrane (Ghosh *et al.*, 1996). Neutrophils contain PLD which is involved in cleavage of both PC and PI in response to physiological stimuli (English, 1992; Bauldry *et al.*, 1991b). It has been shown that priming of human neutrophils *in vitro* with GM-CSF involves upregulation of of PLD activity leading to enhanced generation of PA and DAG in FMLP-stimulated neutrophils ( Bourgoin *et al.*, 1990). Generation of PA by the action of PLD plays a role in the activation of the NADPH oxidase in neutrophils (Rossi *et al.*, 1990). The induction of $cPLA_2$ activity by PLD-generated PA has been demonstrated in human neutrophils (Bauldry and Wooten, 1997).

## 1.7.4 JAK/STATS pathway

The Janus kinases (JAK) are a group of tyrosine kinases which have a role in the signal transduction pathways of many cytokines. The members of JAK family are JAK1, JAK2, JAK3 and Tyk2, with molecular weights ranging from 120-140 kDa. All these kinases are expressed in many cells except JAK3 which is predominantly expressed in myeloid natural killer cells, activated T-lymphocytes and endothelial cells (Rane and Reddy, 1994; Johnston *et al.*, 1994; Verbsky *et al.*, 1996) The JAKs share an overall structure with seven conserved domains with 35-45% sequence homology. One of the structural characteristics of JAKs is the absence of src homology 2 (SH2) and SH3 domains which characterise the SRC family tyrosine kinases and have been shown to be essential for their function. Growth hormone receptors (Goujon *et al.*, 1994), Epo (Witthuhn *et al.*, 1993) and members of cytokine receptors such as IL-3, IL-5 and GM-CSF (Quelle *et al.*, 1994) upon multimerisation activate JAK kinases and initiate intracellular signal transduction. The JAK kinases have been shown to activate another group of proteins known as signal transducers and activators of transcription (STATs). These proteins are recognised by their dual functions in signal transduction in the cytoplasm and activation of transcription of the nucleus. The first STAT family members were identified as part of the transcription complex, termed ISGF3 which binds to the interferon regulatory response element (ISRE) and activates transcription following IFN-$\alpha/\beta$ stimulation (Schindler *et al.*, 1992). To date, six members of the STAT family have been cloned, STATs 1-6 (Fu *et al.*, 1992; Zhong *et al.*, 1994; Wakao *et al.*, 1994; Hou *et al.*, 1994).

## 1.7.5 MAP kinase pathways

### 1.7.5.1 Ras/Raf/MEK/ERK pathway

Mitogen-Activated Protein kinases (MAPK) were first discovered by Ray and Sturgill in 1987. MAP kinases are family of serine/threonine kinases whose primary role is to modify protein function through post-translational serine/threonine phosphorylation which is reversible through the action of serine/threonine phosphatases. The MAPK signalling cascade consists of multiple isoforms at each level of the cascade. They have as their "core" a three-component protein kinase cascade consisting of a serine/threonine protein kinase (MAPKKK, eg: Raf) which phosphorylates and activates a dual-specificity protein kinase (MAPKK, eg: MEK), which in turn phosphorylates and activates another serine/threonine protein kinase (MAPK, eg: ERK) (reviewed by Marshall, 1995). These pathways serve to link

signals from the cell surface to cytoplasmic and nuclear events. Cytokine binding results in tyrosine phosphorylation of the receptor and the recruitment of the Shc-Grb2-Sos complex. As shown in Figure 1.10 the exchange factor Sos activates Ras through the promotion of guanine nucleotide exchange on Ras, leading to activation of Raf, MEK and ERK (Marshall, 1994).

Once activated, MAPKs phosphorylate regulatory molecules, including transcription factors, therefore effecting changes in gene expression and proliferative activity. In addition MAPK pathways are involved in mediating cell shape, stress responses in mammalian cells (Han *et al.*, 1994) and cytokine signalling (Freshney *et al.*, 1994). Although the components of the Ras/Raf/MEK/ERK pathway are now well established, the role of each individual signalling molecule is still unclear. Ras and Raf are known to be involved in proliferation, whereas the roles of MEK and ERK are not fully understood.

### 1.7.5.2 Properties of components of the MAPK cascade

*i) Ras*

Ras was first identified as the transforming agent of the murine sarcoma virus (Harvey, 1964; Kirsten and Mayer, 1967). The viral ras oncogene (v-Ras) is capable of transforming fibroblasts in culture and causes sarcomas and erythroleukaemias in mice. There are three mammalian *ras* genes; H-*ras*, N-*ras* and K-*ras* and all encode 21-kDa products. Their structures are similar with approximately 90% homology in the first 164 amino acids but little homology in the C-terminal region between amino acids 165-185, apart from a CAAX box and positively charged region which includes six lysine residues which are required for membrane attachment (Hancock *et al.*, 1990).

Ras has an intrinsic GTPase and is found in both active GTP-bound and inactive GDP-bound forms (Bourne *et al.*, 1991) (Figure 1.11). It has been shown that the activity of Ras guanine nucleotide exchange factors such as Sos (Bar-Sagi, 1994) accelerate following stimulation of cells with ligand and hence, increase the activity of Ras. Point mutations in the effector region of Ras (amino acid residues 32-40) can prevent its interaction with Raf. Other Ras effector proteins are, p120 $ras^{GAP}$, the p110 subunit of phosphoinositide 3'- kinase (PI3K) and Ral-GDS.

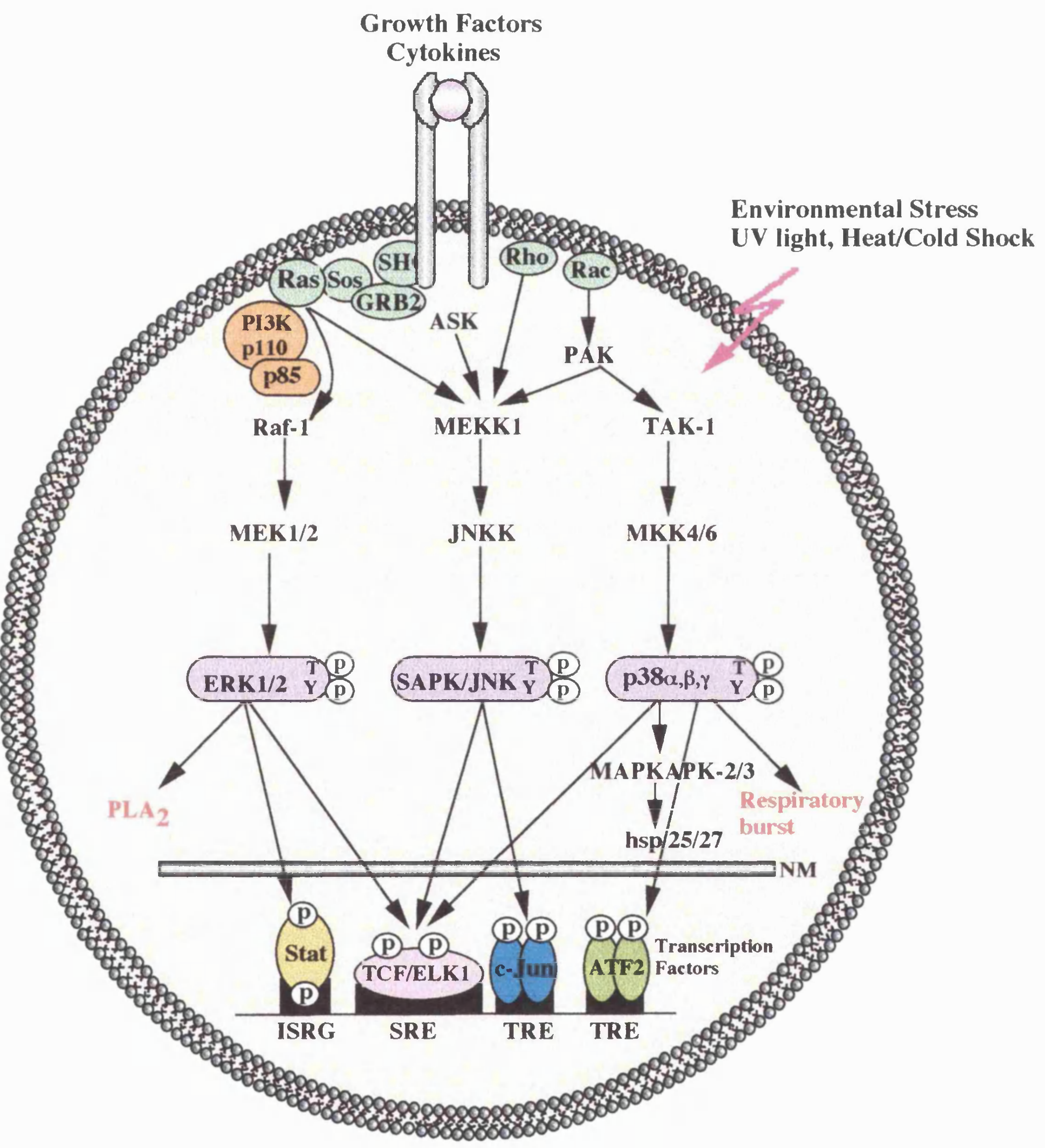



**Figure 1.9: The MAP Kinase pathways.**
PAK (p21-Activated Kinase), ASK (Apoptosis Signal regulating Kinase), PI3K (Phosphatidylinositol 3-kinase), ATF2 (Activating Transcription Factor 2), SRE (Serum Response Element), TRE (TPA Response Element), ISGF (Interferon Stimulated Gene Factor), TAK-1 (TGFβ-Activated protein Kinase-1), NM (Nuclear Membrane)

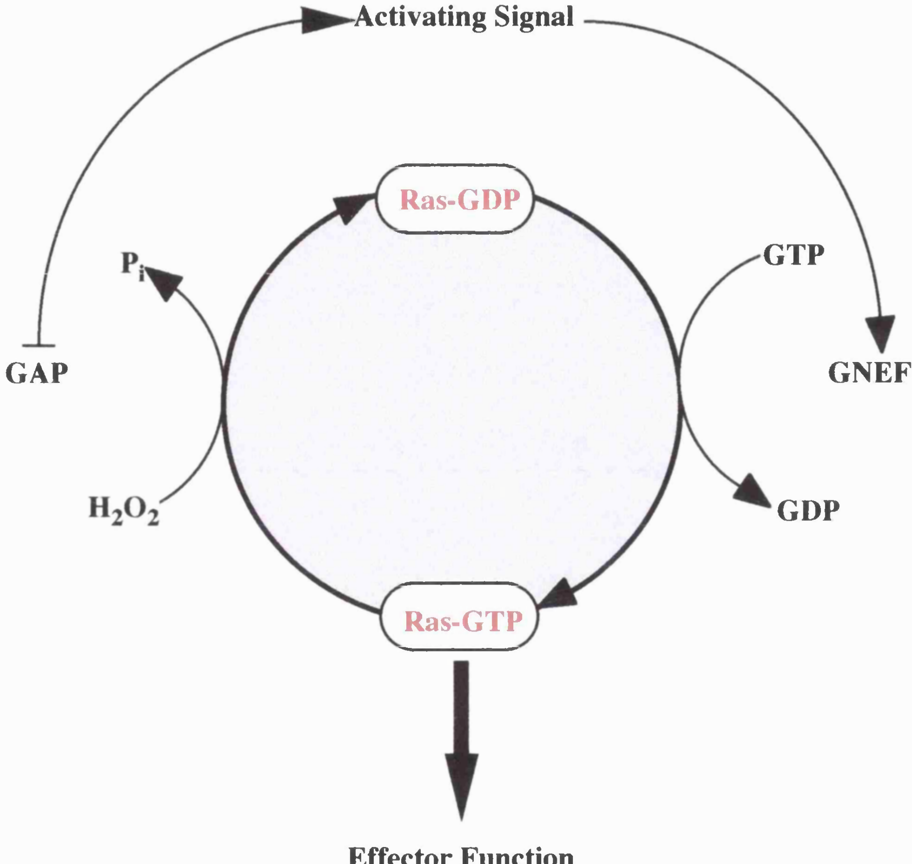



**Figure 1.11: The Ras cycle.** This model shows how Ras can be regulated by the opposing activities of guanine nucleotide exchange factors (GNEFs) and GTPase activating proteins (GAPs). GNEFs catalyse the activation of Ras via exchange of Ras-bound GDP for GTP, whereas GAPs accelerate the slow intrinsic GTPase activity of Ras proteins, therefore inactivating them. (Heldin and Purton, 1996)

*ii) Raf-1*

Raf-1 is a 70-75 kDa, serine/threonine kinase that connects Ras to the MAPK cascade. It contains a kinase domain in its carboxy-terminal segment and a putative regulatory domain occupies the remaining amino-terminal region. Other members of the Raf family are A-Raf and B-Raf (Rapp *et al.*, 1988) which all share a conserved regions known as CR1 and CR2 (serine/threonine rich region) in their regulatory domain and CR3 which falls into the kinase domain. It has been shown that interaction of Raf with Ras is not sufficient for its activation but is responsible for recruitment of Raf to the plasma membrane where it becomes activated (Stokoe *et al.*, 1994; Leevers *et al.*, 1994). Activation of Raf is accompanied by phosphorylation of serine-43, serine-259 and serine-621 as well as Tyrosine 340 and 341 which are required for the catalytic activity of Raf (Fabian *et al.*, 1993). The downstream substrate of Raf is MEK (also known as MAPK kinase).

*iii) MEK*

MEKs constitute an evolutionarily conserved family of protein serine/threonine kinases which includes the highly homologous mammalian isoforms, MEK1 and MEK2. MEK is activated by Raf upon phosphorylation of serine-217 and serine-221 which have been shown to be important for full activation (Alessi *et al.*, 1994). These serine residues are situated within the kinase subdomains VII and VIII which are located in the kinase's catalytic pocket and the phosphorylation of catalytic residues in this region keep the catalytic pocket open for substrate binding. Other residues in MEK are threonine-292 and threonine-386, the phosphorylation of which may play a key role in MEK's inactivation process (Brudet *et al.*, 1994). It has been shown that MEKs are highly specific for their downstream substrates ERK-1 (p44 MAPK) and ERK-2 (p42 MAPK).

*iv) The MAP kinase family*

The MAP kinases (MAPK) constitute a superfamily of proline-directed serine/threonine protein kinases that are regulated by extracellular signals including growth factors, mitogens and cellular stresses (Davis, 1993). MAPK family members are divided into: extracellular signal regulated kinase (ERK), JNK (c-*jun* $NH_2$-terminal kinase), p38 MAP kinase (a mammalian homologue of HOG1, also known as CSBP, reactivating kinase or *mpk2*), and Big MAPK (BMK).

ERKs are proline directed protein kinases, that are identified by their dual phosphorylation motif $Thr^{183}$-Glu-$Tyr^{185}$ in the regulatory lip of the enzyme (Zhang *et al.*, 1994). Activation of ERKs requires phosphorylation of both threonine and tyrosine residues (Johnson *et al.*, 1996) by a dual specificity kinase (MEK). It has been shown that stimulation of ERK1 and ERK2 by growth factors results in their translocation to the nucleus and inactivation can be achieved by dephosphorylation of either threonine or tyrosine using PP2A or CD45, respectively (Anderson *et al.*, 1990; Zheng and Guan, 1993), or by dual-specificity protein phosphatases such as CL-100 or PAC-1 (reviewed by Keyes, 1995). Agonists such as; epidermal growth factor (EGF), platelet derived growth factor (PDGF), TPA, insulin (Alessi *et al.*, 1995) and thrombin (Kramer *et al.*, 1995) can activate ERKs. Lin *et al.* (1993) have shown that phosphorylation of cytosolic $PLA_2$ on serine-505 by ERK enhances the activity of $cPLA_2$.

The JNK group of stress-activated MAP kinases (also known as SAPK) consists of ten protein kinases that phosphorylate the NH2-terminal domain of c-*jun* on serine-63 and serine-73 causing increased transcriptional activity. JNK is activated by dual phosphorylation on threonine and tyrosine within the tripeptide motif Thr-Pro-Tyr located in kinase sub-domain VIII. This phosphorylation is mediated by the dual specificity protein kinases MKK4 and MKK7. A variety of extracellular stimuli activate the JNK pathway including inflammatory cytokines, UV light, osmotic stress and protein synthesis inhibitors. Inflammatory cytokines such as TNFα and IL-1 activate this pathway via the Rac/Rho family of small GTP binding proteins, whereas inhibitors of protein synthesis and UV light act independently of Rho and Rac (Sluss *et al.*, 1994; Coso *et al.*, 1995).

The third MAPK signalling pathway is designated as p38 which possesses considerable homology to the HOG1 gene of yeast (*Saccharomyces cerevisiae*) which forms part of an osmosensing pathway. Members of this pathway are p38, Mpk2 (from Xenopus) and RK. This family of MAP kinases are characterised by a $Thr^{180}$-Gly-$Tyr^{182}$ sequence in the regulatory lip which is phosphorylated by MKK4, leading to activation of p38 (Lin *et al.*, 1995). Activators of p38 include proinflammatory cytokines, bacterial toxin and physical-chemical stimuli. It has been shown that the activity of p38 can be inhibited by a group of bicyclic imidazole compounds including, SB 203580, which do not inhibit the activity of ERK1/ERK2, JNK or ERK5 (Cuenda *et al.*, 1995). To date three isoforms of p38 has been identified, p38α, p38β (Jiang *et al.*, 1996) and p38γ. The mRNA for p38α, p38β is expressed in many tissues while p38γ is predominantly expressed in skeletal muscle but not in macrophages.

*v) MAPKAP kinase*

This group of MAPK-activated protein kinases (MAPKAPK) consist of various kinases which are phosphorylated by the MAPKs. The first substrate for MAPKAPK to be identified was ribosomal protein S6. RSKs, $p90^{rsk}$ and $p70^{rsk}$, are activated by phosphorylation on threonine-396 located between subdomain VII and VIII. Substrates for RSK are the transcription factors, c-*fos* and c-*jun*.

MAPKAP-2, a serine/threonine kinase, has been shown to be phosphorylated and activated by MAP kinases both *in vivo* and *in vitro* (Stokoe *et al.*, 1992a). It is phosphorylated in response to GM-CSF, IL-3 (Ahlers *et al.*, 1994) and FMLP (Zu *et al.*, 1996; Krump *et al.*, 1997). MAPKAPK-2 can be activated in various cells (neutrophils, macrophages, monocytes, PC12, HeLa cells), and it can phosphorylate small heat shock proteins hsp25/hsp27 (Stokoe *et al.*, 1992b; Stokoe *et al.*, 1993), glycogen synthase (Stokoe *et al.*, 1992a), and tyrosine hydroxylase (Sutherland *et al.*, 1993). It has been shown that phosphorylation of hsp27 enhances actin polymerisation, contributing to the repair of stress damaged microfilaments (Lavoie *et al.*, 1995). Further studies have demonstrated that MAPKAPK-2 is not activated *in vivo* by growth factors which are potent activators of p42/p44 MAP kinases but instead by chemical or osmotic stress and heat shock (Lee *et al.*, 1994). It has been shown that inhibition of the enzymatic activity of MAPKAPK-2, by the competitive inhibitory peptide to hsp27, reduced both PMA and FMLP-stimulated superoxide anion generation in human neutrophils. This suggests that MAPKAPK-2 is involved in the activation of the human neutrophils (Zu *et al.*, 1996).

*vi) Phosphoinositide 3-kinase*

Phosphoinositide 3-kinase (PI3-K) was first purified from bovine brain and its cDNA was cloned (Auger, *et al.*, 1989). This enzyme has been implicated in several signalling pathways. The activity of PI3-K is rapidly and transiently stimulated by growth factors and cytokines in many cell types. PI3-K phosphorylates the 3'-hydroxyl group at the D-3 position of the inositol ring of phosphatidylinositol (PtdIns). The PI3-K activity is rapidly and transiently stimulated by growth factors and cytokines in many cell types. It has been shown that in growth factor activated cells contain elevated levels of PtdIns-(3,4)-$P_2$ and PtdIns-(3,4,5)-$P_3$, two of the products resulting from PI3-K metabolism of PI (Parker and Waterfield, 1992; Hu *et al.*, 1993). PI3-K exists as a complex of two proteins consisting of a 85 kDa (p85) regulatory subunit, in the amino terminal half, which contains an SH3 domain as well as proline-rich motifs which interacts with the SH3 domain of Grb2 suggesting an

alternative route for activation of MAP kinase pathway following stimulation by cytokines (Wang *et al.*, 1995). The 110 kDa (p110) catalytic subunit has both protein serine/threonine kinase and PI3-K activity (Brown *et al.*, 1995) (Figure 1.10). The p110 has been shown to interact with Grb-2 and GTP-bound Ras (p21 Ras) resulting in stimulation of the lipid kinase activity (Harrison-Findik and Varticovski, 1994; Rodriguez-Viciana *et al.*, 1994). Inactivation of PI3-K with either a dominant negative enzyme or the use of pharmacological inhibitors has shown that PI3-K plays an important role in many cellular processes such as; DNA synthesis, cell proliferation (Fantl *et al.*, 1993; Roche *et al.*, 1994), prevention of apoptosis (Scheid *et al.*, 1995; Minshall *et al.*, 1996), membrane ruffling (Wennstrom *et al.*, 1994; Kotani *et al.*, 1994) and receptor internalisation (Joly *et al.*, 1994). Treatment of phagocytes with wortmannin, an inhibitor of PI3-K revealed that PI3-K activity is required for the stimulation of the respiratory burst, exocytosis and phagocytosis (Dewald *et al.*, 1988; Thelen *et al.*, 1994; Araki *et al.*, 1996). However, PI3-K is not involved in chemotaxis and shape change of neutrophils stimulated by agonists of G-protein-coupled receptors (Thelen *et al.*, 1995; Baggiolini and Kernen, 1992). The stimuli that activate PI3-K in the cell, range from growth factors, non-receptor tyrosine kinases, GTP-binding proteins, thrombin and FMLP (Fry, 1994). It has been shown that p21-activated kinase (PAK) and $p47^{phox}$ are downstream targets of PI3-K in neutrophils (Knaus *et al.*, 1995; Didichenko *et al.*, 1996). In addition other protein kinases such as; PKC isoforms ($\delta$,$\varepsilon$ and $\eta$) (Toker *et al.*, 1994) and protein kinase B (PKB) (Franke *et al.*, 1995) have been shown as downstream targets of PI3-K.

*vii) Protein kinase B*

The serine/threonine protein kinase B (PKB) was first identified as an oncogene. The kinase shows similarity to protein kinase A (PKA) and PKC and thus has been termed Related to A- and C-kinase (RAC-PK) (Jones *et al.*, 1991) as well as AKT (Bellacosa *et al.*, 1991). In addition, PKB contains a pleckstrin-homology (PH) domain at its N-terminal end. PH domains have been implicated in interactions with G-protein $\beta\gamma$ subunits, and with lipids such as phosphoinositides (Haslam *et al.*, 1993). PKB is activated by a wide variety of stimuli including IL-2, -3, -4, -5 and -8, FMLP, heat shock, integrins and nerve growth factor (Konishi *et al.*, 1996; King *et al.*, 1997; Ahmed *et al.*, 1997; Mazure *et al.*, 1997; Tilton *et al.*, 1997; Songyand *et al.*, 1997; Andjelkovic *et al.*, 1998). It has been shown that PKB is a major target of PI3-K-dependent signalling as growth factor-induced PKB activation was completely blocked by addition of wortmannin (Cross *et al.*, 1995). Alessi *et al.* (1997) have identified and purified a kinase, PtdIns-(3,4,5)-$P_3$-dependent kinase 1 (PDK1), that

phosphorylates PKB resulting in its activation. Activated PKB provides a survival signal to cells that protects them from apoptosis (Khwaja *et al.*, 1997).

Analysis of the signal transduction pathways activated in response to cytokines indicate that both immature and mature cells signal via similar pathways, namely JAK-STAT and Ras-MAP kinase pathways. It has been shown that cells can use transient and sustained activation of MAP kinase which could lead to nuclear translocation to alter gene expression (Dikic *et al.*, 1994; Traverse *et al.*, 1994).

## 1.8 AIMS OF STUDY

The aim of this thesis was to investigate the regulation of phospholipase $A_2$ by growth factors in both immature and mature phagocytic cells. *In vitro* priming of both neutrophil respiratory burst activity and phospholipase $A_2$ activity has been well documented, however there are relatively few examples of *in vivo* priming to date. Therefore a study was carried out in patients with sickle cell disease. There is evidence of inflammation of the endothelium in this disease due to adherence of sickled erythrocytes and in addition circulating cytokines have been detected in the peripheral blood, both of which might lead to activation of neutrophils in the circulation. Therefore experiments were undertaken to investigate whether there was any evidence for *in vivo* neutrophil priming in sickle cell disease and whether priming *in vivo* might subsequently render the patients' neutrophils less likely to be primed *ex vivo*. In Chapter 3 and 4 *ex vivo* priming by GM-CSF and TNF$\alpha$ of neutrophil phospholipase $A_2$ as well as NADPH oxidase was studied. The activity of both these enzymes in neutrophils may be intimately linked, as there is evidence that priming of NADPH oxidase may require functional phospholipase $A_2$ activity and arachidonate itself activates a proton pump situated in the gp91$^{phox}$ component of the NADPH oxidase. In addition, there is evidence that priming of both NADPH oxidase and phospholipase $A_2$ activity may occur via the activation of p42/44 MAP kinase. Therefore in Chapters 5 and 6 the relative dependence of the priming and activation signal transduction pathways on MAP kinase was studied in order to investigate whether the activation pathways of $PLA_2$ and NADPH oxidase could be dissociated. Finally, in Chapter 7 I turned to study immature myeloid cells to ask whether there was any evidence of phospholipase $A_2$ activity in these cells and if so whether the activity could be primed by growth factors known to regulate proliferation and differentiation of haemopoietic cells.

# CHAPTER 2

# GENERAL MATERIALS AND METHODS

## 2.1 Separation of neutrophils from whole blood

Materials

- 100 mM EDTA pH 7.4 (in distilled water)
- 10% Dextran (in 0.9% NaCl) (Pharmacia Biotech)
- Ficoll-Paque (density 1.077 g/ml) (Pharmacia Biotech, Uppsala, Sweden)
- Dulbecco's Phosphate Buffered Saline ($PBS^-$) (Gibco BRL, Paisley, UK)
- PBS containing 0.9 mM calcium and 0.5 mM magnesium ($PBS^+$) (Gibco BRL)
- Glucose (BDH, UK)
- 0.4% Trypan blue (solution in 0.81% sodium chloride and 0.06% potassium phosphate) (Sigma, Poole, Dorset, UK)

Method

Peripheral blood from healthy volunteers was obtained and granulocytes were isolated as described previously by Roberts *et al.* (1990), using sterile conditions to minimize the contact of cells with endotoxin hence reduce priming. Venous blood was collected into 2.5 mM EDTA to prevent coagulation and then processed immediately. Dextran (1% wt/vol, final concentration) was added to the blood which was allowed to stand for 20 minutes at room temperature until the erythrocytes had sedimented. The leukocyte-rich supernatant was gently layered onto 15 ml of Ficoll-paque and mononuclear cells were separated by centrifugation at 800 x g for 20 minutes. The residual erythrocytes present in the neutrophil pellet were lysed by hypotonic lysis as follows: The pellet was resuspended in 2 ml $PBS^-$ and 5 ml of ice cold distilled water was added for 30 seconds with gentle mixing. Then 2 ml of 3.5 % (wt/vol) sodium chloride was added to correct the tonicity and the volume was made up to 50 ml with $PBS^-$ and the cell suspension was washed twice in $PBS^-$ by centrifugation at 180 x g for 7 minutes. Finally, the pellet was resuspended in $PBS^+$ supplemented with 5 mM glucose. Neutrophils isolated by this method were shown to be of 99% viable by trypan blue exclusion and more than 95% pure by Leishman staining.

## 2.2 Cytospin preparation of neutrophils and Leishman staining

Cell suspension at concentration of 1-2 x $10^5$ cells/ml were prepared. Each sample chamber was filled with 0.2 ml of the cell suspension and centrifuged at 60 x g for 7 minutes. Slides were allowed to air dry for 30-60 minutes at room temperature.

Cytocentrifuge preparations of cells were stained for 2 minutes at room temperature with Leishman's stain and for 7 minutes after 2 fold dilution of the stain with phosphate buffer (0.1 M, pH 6.8). Morphology was assessed by counting a minimum of 250 cells in each preparation.

## 2.3 Measurement of superoxide anion production

Materials

- Ferri-cytochrome c (Horse heart type IV) (Sigma, Poole, Dorset, UK)
- Superoxide dismutase (EC 1.15.1.1. from bovine erythrocytes) (Sigma)
- n-ethyl maleimide (NEM) (Sigma)
- Disposable spectrophotometer cuvettes (1 ml volume) (Starstedt, Leicester, England)
- FCS (Gibco BRL)
- *rh*GM-CSF at concentration of 1 μg/ml stock solution was prepared in 1% (v/v) FCS and was stored at -20°C. (recombinant human GM-CSF, expressed in *E.coli*) (Hoechst, UK/Behringwerke, Marburg, Germany)
- Tumor Necrosis Factor α (TNFα) (10000 U/ml stock solution in 1% FCS, stored at -20°C) (A kind gift from Dr Anthony R Mire-Sluis: Department of Immunology, National Institute for Biological Standards and Control, South Mimms, Hertfordshire, UK)
- Calcium ionophore (A23187) (100 μM stock solution in PBS was prepared immediately prior to use) (Sigma)
- N-Formyl-Met-Leu-Phe (FMLP) (100 μM stock solution in PBS) (Sigma)
- Phorbol myristate acetate (PMA) (500 μg/ml stock solution in DMSO, diluted 1000 fold to final concentration of 500 ng/ml on the day of experiment) (Sigma)

Method

Release of superoxide anions was measured by superoxide-dismutase inhibitable reduction of cytochrome c as previously described by Roberts *et al.*, (1990). Purified cells in duplicate samples at $1x10^6$/ml were added into spectrophotometer cuvettes. Then cells were pre-warmed at 37°C for 5 minutes prior to addition of 100 μM (final concentration) ferri-cytochrome c with matched control sample containing 50 μg/ml superoxide dismutase in addition to ferri-cytochrome c for 5 minutes at 37°C. Then cells were mixed and stimulated with FMLP (1μM) or PMA (500 ng/ml final concentration)

and incubated for 5 minutes. The reaction was terminated by addition of 2mM NEM. The total amount of superoxide produced were calculated from the change in absorbance at 550 nm in a dual-beam spectrophotometer (Unicam UV/Vis UV2) using extinction coefficient (E):

$$E_{550\ nm} = 21.0\ mM^{-1}\ cm^{-1}$$

### 2.3.1 Priming of superoxide production

Purified cells in duplicate samples ($1x10^6$ cells/ml) were incubated with either cytokines or diluent control (0.01% FCS) for 30 minutes at 37°C. Cells were then incubated with 100 μM ferri-cytochrome c with matched control sample containing superoxide dismutase (50 μg/ml) in addition to ferri-cytochrome c. Cells were then stimulated with 1 μM FMLP for 5 minutes prior to termination of the reaction by addition of NEM. Changes in the absorbance was measured as described above.

## 2.4 Assay of phospholipase $A_2$ activity

Materials

- [5,6,8,9,11,12,14,15-$^3$H]Arachidonic Acid (specific activity 7.33 TBq/ml; 202 Ci/mmol, Amersham International, Amersham, Bucks., UK)
- PBS containing 0.9 mM calcium and 0.5 mM magnesium ($PBS^+$) (Gibco BRL, Paisley, UK)
- $PBS^+$ supplemented with 5 mM glucose (PBSG)
- MK886 (200μM stock solution in dimethyl sulphoxide was prepared immediately prior to use) (A gift from Merck-Forsst Canada Inc., Pointe Claire-Dorval, Quebec, Canada)
- Calcium ionophore (A23187) (100 μM stock solution in PBS) (Sigma, Poole, Dorset, UK)
- N-Formyl-Met-Leu-Phe (FMLP) (100 μM stock solution in PBS) (Sigma)
- Fatty acid-free bovine serum albumin (BSA) (100 mg/ml stock solution in PBS (Sigma)
- Foetal Calf Serum (FCS) (Gibco BRL)
- *rh*GM-CSF (1 μg/ml stock solution in 1% FCS) (Recombinant human GM-CSF, expressed in *E.coli*; Hoechst, UK/Behringwerke, Marburg, Germany)
- Tumor Necrosis Factor α (TNFα) (10000 U/ml stock solution in 1% FCS) (A kind gift from Dr Anthony R Mire-Sluis: Department of Immunology, National Institute for Biological Standards and Control, South Mimms, Hertfordshire, UK)

- Liquid scintillation cocktail (Optiphase 'Hisafe' 2, Wallac scintillation products, Wallac OY, Turku, Finland)

Method

### 2.4.1 Radiolabelling

In a radiometric assay the labelling and release of $^3$H-AA was carried out by the method previously described by Roberts *et al.*, in 1996. Purified cells were washed twice in PBS$^+$ and the pellet was resuspended at 5x$10^6$ cells/ml in PBSG, 0.1% FCS and incubated with 0.5 μCi/ml of $^3$H-AA for 2 hours at room temperature with gentle mixing every 20 minutes. This allowed the incorporation of 60% isotope into the endogenous phospholipid pool as confirmed by thin layer chromatography (Roberts *et al.*, 1996). The radiolabelled cells were centrifuged at 180 x g for 7 minutes and the supernatant was discarded. The cell pellet was washed 4 times in PBS$^+$ to remove excess unincorporated radioisotope and finally resuspended to 2x$10^6$ cells/ml in PBSG.

### 2.4.2 Cell activation

Duplicate aliquots (0.5 ml) of cell suspension at 1x$10^6$ cells/sample (as prepared in section 2.6.1) were incubated with 200 nM MK886 at 37°C for 5 minutes in order to inhibit metabolism of AA by the 5-lipoxygenase pathway (Gillard *et al.*, 1989), therefore maximising the recovery of AA in the samples. Then cells were stimulated with 1μM FMLP, 1μM calcium ionophore, A23187, or PBS (as a control to measure unstimulated levels of activity) for 15 minutes. In order to trap AA which was released to the extracellular medium BSA (1 mg/ml final concentration) was added to the cells with the stimulus (see Figure 2.1 for the principle of radiometric $PLA_2$ assay). The reaction was terminated by placing the samples on ice for 10 minutes. Cells were centrifuged at 12000 x g for 4 minutes and 0.4 ml aliquots of the supernatant were mixed with scintillation fluid and assayed for radioactivity using a β emission counter (1212 Rack Beta 'Compact', LKB Wallac OY, Turku, Finland).

### 2.4.3 Cell priming

Aliquots (0.5 ml) of cell suspension at 1x$10^6$ cells/sample were incubated with 200 nM MK886 at 37°C for 5 minutes. Then cells were preincubated with either GM-CSF (10 ng/ml), TNFα (500 U/ml) or diluent (0.01% FCS) for 20 minutes at 37°C prior to stimulation with 1μM FMLP, 1μM calcium ionophore, A23187 or diluent

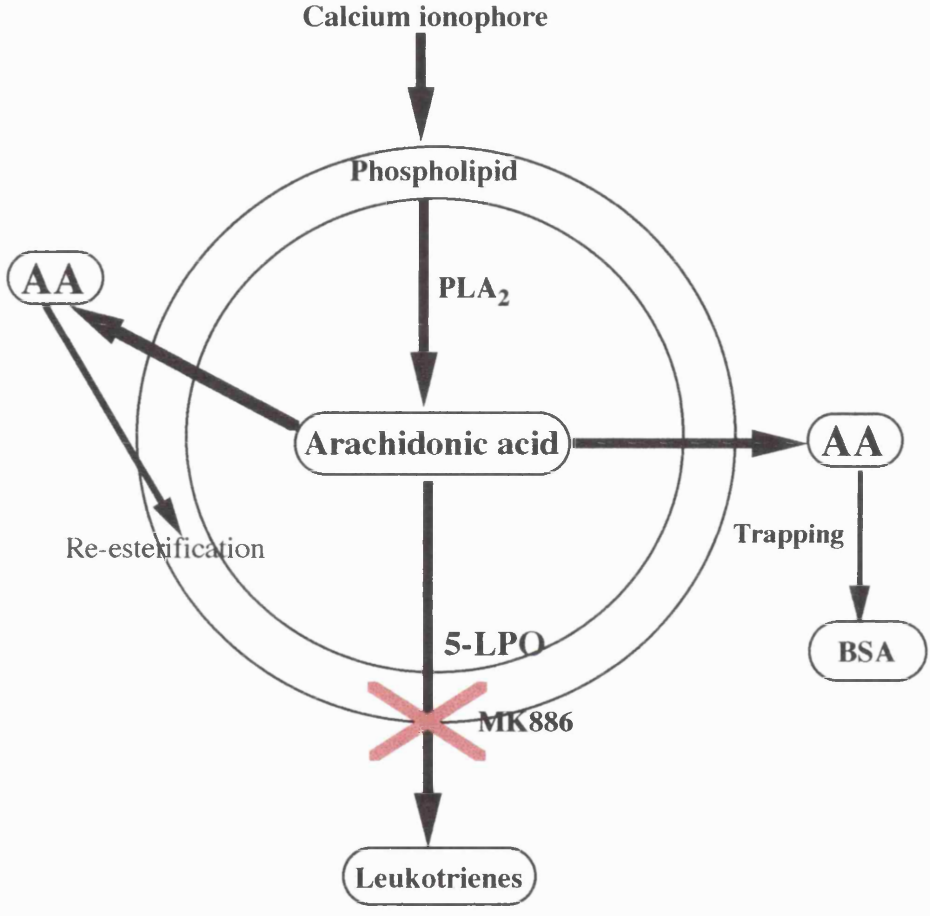



**Figure 2.1: Principle of radiometric $PLA_2$ assay.**
AA (arachidonic acid), BSA (bovine serum albumin), 5-LPO (5-lipoxygenase)

control (PBS) for 15 minutes at 37°C. The released AA was trapped by addition of 1 mg/ml BSA to the cells with the stimulus. The reaction was terminated by placing the samples on ice for 10 minutes. The radioactivity was measured as described above.

## 2.5 Immunoblotting

Materials

- n-ethyl maleimide (NEM) (Sigma, Poole,Dorset UK)
- Diisopropyl fluorophosphate (DIFP) (Sigma)
- Phenylmethylsulfonylfluoride (PMSF) (Sigma)
- Nonidet-40 (NP-40) (Sigma)
- Aprotinin (Sigma)
- Pepstatin A (Sigma)
- Leupeptin (Sigma)

- **Lysis buffer:-** 137 mM NaCl, 20 mM Tris-HCl pH 8.0, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% NP-40, 10% glycerol and phosphatase inhibitors: 1mM Na orthovanadate, 1 mM β glycerophosphate, 1 mM NaF, 5mM pyrophosphate, 2mM EDTA.

- **Protease inhibitors:-** 1 mM PMSF, 10μg/ml leupeptin 10μg/ml aprotinin, 10μg/ml pepstatin A, 1mM DIFP.

- **Laemmli's SDS sample buffer (reducing):-** 60 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 100 mM dithiotreitol (DTT), 0.001% bromophenol blue

Methods

### 2.5.1 Preparation of cell lysates

Cells were washed and resuspended at $1x10^6$ cells/ml in PBS/5 mM glucose. Aliquots of cell suspension were incubated at 37°C for 5 minutes prior to stimulation with diluent (0.01% FCS) or cytokines (GM-CSF or TNFα). The reaction was terminated by addition of 2 mM NEM. Cells were then centrifuged at 12000 x g for 30 seconds and the pellet was lysed in 50 μl of lysis buffer containing protease and phosphatase inhibitors, for 15-30 minutes on ice followed by addition of 50 μl of 2x

concentrated Laemmli's sample buffer. Then the samples were heated at 95°C for 10 minutes and stored at -20°C.

### 2.5.2 Estimation of protein concentration

To measure the concentration of protein in each sample standard solutions were prepared using bovine serum albumin (0, 1, 5, 10, 15, 20 μg/ml) with distilled water in final volume of 0.8 ml followed by addition of 1μl 2x concentrated Laemmli's sample buffer and 0.2 ml of Bio-Rad dye. Measurement of proteins in each sample was carried out by diluting 1μl of protein with 0.8 ml of distilled water followed by addition of 0.2 ml of the Bio-Rad dye. The absorbances at 595 nm were measured spectrophotometrically (Unicam UV/Vis UV2) and the samples calibrated against the standard curve.

## 2.6 SDS-polyacrylamide gel electrophoresis (PAGE) and western blotting

Materials

- Acrylamide/N'N'-bis-methylene (bis)-acrylamide ratio of 37.5:1 (30% monomer:2.67% cross-linking monomer concentration) (Boehringer, Mannheim, Germany).
- N'N'N'N'-Tetra-methylethylenediamine (TEMED) (Biorad, CA, USA)
- Ammonium persulphate (BDH, UK)
- Sodium dodecyl sulphate (SDS) (BDH)
- Prestained molecular weight rainbow markers (Amersham International, Amersham, UK)
- Nitrocellulose membrane, Hybond-C-Extra (Amersham International)
- 5% non-fat dried milk (Marvel)
- Peroxidase-conjugated antisera (Dako Ltd., High Wycombe, Bucks, UK)
- Enhanced chemiluminescence kit (Amersham International)
- Hyperfilm™ MP (High Performance Autoradiography film) (Amersham Life Sciences)

- **Electrode running buffer pH 8.3:-** 25 mM Tris, 200 mM glycine, 0.1% (wt/vol)SDS

- **Western transfer buffer:-** 25 mM Tris 200 mM glycine, 20% (vol/vol) methanol

- **Tris buffered saline (TBS):-** 10 mM Tris/HCl pH 8.0, 150 mM NaCl with 0.05% Tween 20 (Sigma)

- **Preparation of Separating gel (12.5%)** :- 2 ml distilled water, 3.47 ml 1 M Tris-HCl pH 8.8, 4.2 ml acrylamide/bis (30%:0.8%), 0.1 ml 10% SDS, 0.075 ml 10% (wt/vol) ammonium persulphate, 0.009 ml TEMED

- **Preparation of Separating gel (15%)** :- 1.2 ml distilled water, 3.74 ml 1 M Tris-HCl pH 8.8, 5 ml acrylamide/bis (30%:0.8%), 0.1 ml 10% SDS, 0.075 ml 10% (wt/vol) ammonium persulphate, 0.009 ml TEMED

- **Preparation of Separating gel (4%)**:- 6.1 ml distilled water, 2.5 ml 0.5 M Tris-HCl pH 6.8, 0.1 ml 10% SDS, 1.3 ml acrylamide/bis (30%:0.8%), 0.075 ml 10% (wt/vol) ammonium persulphate, 0.015 ml TEMED

Method

The separating gel was prepared and poured into a minigel apparatus (Mini-Protean II, BioRad, CA, USA) set up according to the manufacturers instructions. Top of the gel was overlayed with water saturated butanol and left for 30 minutes at room temperature to allow the gel to set. The butanol was gently washed from top of the gel with distilled water and the stacking gel was poured on top of the separating gel, then a 10 well comb was inserted into place. The gel was allowed to polymerise for 20 minutes and once set, the comb was removed and wells were washed with electrode running buffer to remove unpolymerised acrylamide.

**Western blot analysis:** Typically 10 μl of sample was loaded into each lane and electrophoresis carried out at a constant 120 V for 1-2 hours. The proteins were transferred onto a nitrocellulose membrane using a semi-dry transfer apparatus (Milliblot, Millipore, USA) at a constant current of 0.45 Amps for 45 minutes. The non-specific binding sites were blocked by 2 hours incubation of the membrane in TBST/5% non-fat dried milk. Then the blot was probed with appropriate antiserum (diluted in TBST/5% non-fat dried milk) for 1 hour. The blot was then washed three times in TBST and incubated with a 1:2000 dilution (in TBST/5% non-fat dried milk) of peroxidase-conjugated secondary antibody for 1 hour at room temperature. The blot was washed twice for 10 minutes in TBST and once in TBS. Detection of the protein bands was carried out by using enhanced chemiluminescence method according to the

manufacturers instructions (Amersham International, Amersham Place, Bukinghamshire, UK) prior to analysis by autoradiography.

# CHAPTER 3

# RAISED NEUTROPHIL PHOSPHOLIPASE $A_2$ ACTIVITY AND DEFECTIVE PRIMING OF NADPH OXIDASE AND PHOSPHOLIPASE $A_2$ IN SICKLE CELL DISEASE

## 3.1 INTRODUCTION

Sickle cell disease is an inherited disorder which is characterised by chronic haemolytic anaemia and recurrent painful episodes due to the presence of an abnormal haemoglobin S (HbS). The adult haemoglobin A (HbA) composed of a tetramer of globin chains, of which two molecular forms are present: $\alpha$ and $\beta$ chains ($\alpha_2\beta_2$) each with a haem group. Each haem group consists of a protoporphyrin ring structure bound to a single iron atom. Reduction or abnormal synthesis of normal globin chain results in the formation of an abnormal Hb and hence group of diseases such as; sickle cell anaemia and thalassaemias.

It was not until 1910 that James Herrick first reported the presence of abnormal elongated, sickle shaped red blood cells in the blood of a 20 year old student of African descent who was suffering from recurrent pain and anaemia. Then in 1922 Mason gave the name Sickle Cell Disease (SCD) to this disorder. In 1949 Pauling and colleagues reported that the electrophoretic mobilities of haemoglobin S (HbS) and normal adult haemoglobin (HbA) were different and postulated that this resulted from a change in the amino acid sequence of the haemoglobin molecule. Ingram (1956) described the molecular abnormality of HbS. It is now known that SCD is an inherited disorder which is caused by single base, point mutation at the sixth codon with substitution of valine for glutamic acid in the $\beta$ globin chain (Figure 3.1) which gives rise to the formation of unstable HbS within red blood cells. If both normal $\beta$ globin chains are substituted by $\beta^S$ chains, a condition is created known as homozygous SS disease or sickle cell disease.

Occasionally, the $\beta^S$ gene on one chromosome is associated with another abnormal $\beta$ gene on the opposite locus, which gives rise to a double heterozygous condition with apparently mild sickle cell anaemia. The Hb variants that coexist with HbS are shown in Table 3.1. The combination of HbS with normal HbA will give rise to the condition known as sickle cell trait, where the carrier produces both HbA and HbS with the concentration of HbS varying from 25% to 45% of the total haemoglobin, and these carriers are usually asymptomatic (Embury, 1986).

HbS has an altered charge and configuration with increased surface hydrophobicity due to the substitution of valine for glutamic acid at the sixth codon. The mechanism by which HbS turns into a polymer was explained by Eaton and Hofrichter in 1987. Deoxygenated HbS tetramers polymerise into long fibers, each consisting of seven intertwind double strands held together by cross-linkers. The early stage of polymer formation involves the aggregation of tetramers into a single nucleus in a process known as homogeneous nucleation, which provides a surface for more polymer formation (Ferrone *et al.*, 1985). This leads to spicule formation, red cell

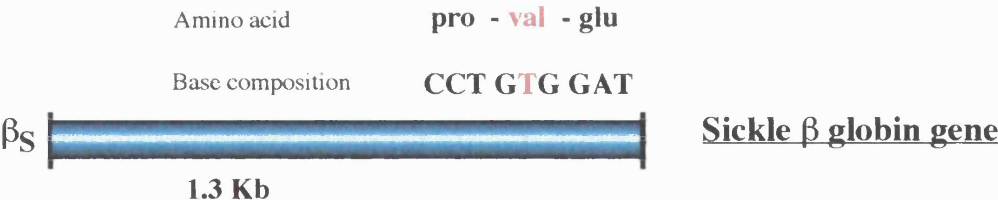



**Figure 3.1: Molecular pathology of sickle cell anaemia.** A single base change in the DNA coding for the amino acid in the sixth position of the β globin chain (adenine is replaced by thymine). This leads to substitution of valine for glutamic acid. pro:(proline), glu:(glutamic acid), val:(valine), $\beta_A$:(Normal β globin chain), $\beta_S$: (Sickle β globin chain)

**Table 3.1: Haemoglobin variants that may coexist with HbS**

| Syndrome | Haemoglobin | Mutation |
|---|---|---|
| | C | β 6 glu lys |
| Haemolysis | D | β 121 glu gln |
| | E | β 26 glu lys |
| | O | β 121 glu lys |
| Thalassaemia: | | |
| 1) α | – | |
| 2) β: | – | Reduced globin chain synthesis |
| minor | | |
| intermedia | | |
| major | | |

lys = lysine, glu = glutamic acid, gln = glutamine

rigidity and distortion of red blood cells (RBC). Deoxygenated HbS is insoluble and blockage of the vessel creates a local region of low oxygen concentration which causes more HbS to crystallize and hence more sickling occurs.

The lipid bilayer that comprises the red cell membrane is normally arranged asymmetrically so that 75-80% of the phospholipids containing phosphatidylcholine (PC) and sphingomyelin are found in the outer monolayer while the inner membrane leaflet retains most of the phosphatidylserine (PS) and phosphatidylethanolamine (PE). Deoxygenation-induced sickling causes an alteration of phospholipid organisation allowing PS to be exposed to the outer membrane leaflet (Lubin *et al.*, 1981; Kuypers *et al.*, 1996). This disorganisation of sickle RBC phospholipids is responsible for increased adherence of sickle RBC to macrophages and other cells. The increased adherence of sickle RBC to cells of the reticuloendothelial system plays a significant role in shortening the life span of the sickle RBC from 120 days to 10-12 days which results in increased haemolysis with haemoglobin levels reduced to 60-90 g/L compared with the normal levels of 115-155 g/L (female) and 135-175 g/L (male). Approximately 80-90% of normal RBC destruction occurs within macrophages of the spleen but most haemolytic anaemias are characterised by predominantly extravascular destruction of RBC. Free haemoglobin in plasma is readily oxidised to methaemoglobin S (MetHbS) which is rapidly converted into haem. This process contributes to the increased generation of oxidative radicals by sickle red blood cells. Abnormal deposition of iron at the cytoplasm/membrane interface promotes oxidative damage to the proteins and lipids of the sickle RBC membrane possibly by conversion of $Fe^{3+}$ to $Fe^{2+}$ which in turn can reduce $H_2O_2$ to a highly toxic hydroxyl radical (Reaction 1), which can result in the loss of bilayer asymmetry.

Reaction 1) $$Hb\text{-}Fe^{2+} + H_2O_2 \longrightarrow Hb\text{-}Fe^{3+} + HO^- + OH\cdot$$

The gene for HbS is carried by of black Americans and black Africans with frequency of the gene being as high as 40% in some parts of Africa. The gene is most common in the West and Central African populations, the Mediterranean, Middle East and some parts of Indian subcontinent (Luzzatto, 1981).

Hebbel *et al.* (1980) was first to hypothesize that the abnormal shape of the sickled erythrocytes is not the only factor involved in microvascular occlusion, and by using a static culture system of endothelial cells obtained from human umbilical cord veins, they have been able to show that an increase in adherence of sickle cells to the vascular endothelium with an increase in sickle red blood cell density is an additional factor responsible for vascular occlusion, and later this was confirmed by others using endothelial cells of other mammalian origin (Kaul *et al.*, 1989).

The mechanisms underlying the adherence of sickle RBC suggest the involvement of plasma factors as well as endothelial and RBC membrane adhesion molecules. Several studies indicated that the integrin adhesion receptor $\alpha_4\beta_1$ can be found on sickle reticulocytes in much greater numbers than normal reticulocytes (Swerlick *et al.*, 1993; Joneckis *et al.*, 1993; Gee and Platt, 1995; Kumar *et al.*, 1996). Vascular-cell adhesion molecule-1 (VCAM-1) is the ligand for $\alpha_4\beta_1$ and is expressed on vascular endothelium in response to a variety of cytokines ( Vordermeier *et al.*, 1992; Croizat, 1994; Kasschau *et al.*, 1996; Natarajan *et al.*, 1996). Adherence of sickle RBC to endothelial cells increases with exposure to either interleukin-1 (IL-1), IL-4, IL-6, GM-CSF or necrosis factor-$\alpha$ (TNF-$\alpha$), providing a mechanistic link between vaso-occlusive events and infection, a known trigger for vaso-occlusive crises of SCD. $\alpha_4\beta_1$ may participate in sickle RBC adherence to the vascular endothelium via additional mechanisms such as its binding with fibronectin. This binding requires activation of $\alpha_4\beta_1$ which occurs with IL-8 and phorbol ester stimulation (kumar *et al.*, 1996). Other membrane adhesive molecules reported to be involved in sickle cell adhesion to endothelium are E-selectin, intercellular adhesion molecule-1 (ICAM-1) (Blei *et al.*, 1994) and vitronectin receptors ($\alpha_v\beta_3$ adhesion molecules) which are also elevated in inflammatory disorders such as vasculitis and rheumatoid arthritis (Gearing and Newman, 1993). Upregulation of these molecules accelerates the adhesion cascade (Natarajan *et al.*, 1996) and hence, vaso-occlusive episodes with obstruction of small blood vessels (Figure 3.2). It has been suggested that endothelial cells are damaged by adhesion of sickle erythrocytes which inhibits endothelial cell DNA synthesis *in vitro* (Weinstein *et al.*, 1990).

Several plasma adhesive elements have been shown to act as potential adhesogens; fibrinogen (Smith and La Celle, 1986; Wautier *et al.*, 1983), fibronectin (Wautier *et al.*, 1983; Wick *et al.*, 1987), high molecular weight von Willebrand factor multimers (Kaul *et al.*, 1993) and thrombospondin being released from activated platelets which acts through an interaction with the CD36 receptors on reticulocytes (Sugihara *et al.*, 1992; Parise *et al.*, 1993; Brittain *et al.*, 1993; Browne *et al.*, 1996).

The risk of obstruction of microcirculation and tissue damage in patients with SCD is further exacerbated by the release of vasoconstrictors including; thromboxane $A_2$, endothelin (Yanagisawa *et al.*, 1988) and prostaglandin $G_2/H_2$ (Kato *et al.*, 1990) from stimulated endothelium and reduced production of the endothelial relaxation factor, nitric oxide, via inhibition of the expression of nitric oxide synthase (Furchgott and Zawadski, 1980).

It has also been shown that sickle RBC have an enhanced adherence tendency when suspended in autologous platelet-rich plasma which release their granule contents and therefore interaction with platelets further contribute to vascular occlusion

and hence painful crises (Platt *et al.*, 1993; Antonucci *et al.*, 1990) (Figure 3.2). The evidence for this activity includes increased levels of thrombin generation (Ofuso *et al.*, 1991; Peters *et al.*, 1994), plasma β-thromboglobulin, thromboxane $B_2$ (Westwick *et al.*, 1983), platelet factor 4 (Westwick *et al.*, 1983), decrease in anticoagulant factors such as protein C and S (Karayalcin and Lanzowsky, 1989; Francis, 1988; Nsiri *et al.*, 1996), antithrombin III (Caccioloa *et al.*, 1989) and heparin cofactor II, a plasma protease inhibitor, (Porter *et al.*, 1993). Activation of the thrombin cascade leads to organ infarction such as spleen, kidney, heart and retina, the latter being the most vulnerable to vaso-occlusion. Brittain (1993) showed that thrombospondin released from the α granules of activated platelets, increases adhesion by acting as a bridge between receptors on endothelial cells and sickle red blood cell membrane under flow condition which can further promote vascular occlusion.

Activated platelets express P-selectin which use this adhesion molecule to adhere to other cells such as neutrophils (Hamburger and McEver, 1990; Kuijper *et al.*, 1996). Activation of neutrophils during the cascade of vaso-occlusive events (Kasschau *et al.*, 1996), can damage endothelial cells by oxygen radical dependent and protease dependent mechanisms. It has been shown that endothelial cell injury by neutrophils is initiated by production of oxygen radicals in the early phase of injury which act synergistically with neutrophil proteases such as trypsin, elastase and cathepsin G which bring about endothelial cell killing (Varani *et al.*, 1989). A recent *in vitro* study demonstrated that when normal neutrophils were incubated with sickle red blood cells in the presence of autologous plasma, a significant increase in respiratory burst of neutrophils was induced compared with normal red blood cells, indicating direct activation of neutrophils by sickle RBC (Hofstra *et al.*, 1996) and this emphasizes that neutrophil activation may play an important role in SCD.

Activation of the endothelial cells by cell-cell interaction results in increased metabolism of endothelial cell phospholipids, with release of eicosanoids and downstream pro-inflammatory products of both lipoxygenase and cyclo-oxygenase enzymes (Setty *et al.*, 1996; Ibe *et al.*, 1994) (see Figure 1.9 for details).

In patients with sickle cell disease, long asymptomatic periods known as "steady state" occur, which depend on an equilibrium in which the excessive destruction of red blood cells (especially in the circulation rather than in macrophages of the reticuloendothelial system) is balanced by an increased rate of erythropoiesis. Disruption of this equilibrium by factors such as; dehydration, infection, deoxygenation, trauma, exposure to cold and vigorous exercise, can lead to intermittent episodes of painful crisis which is characterised by rapid fall in blood haemoglobin accompanied by vaso-occlusion and intense pain in the extremities, chest, abdomen or spine and symptoms of shock. Infarcts may also occur in a variety

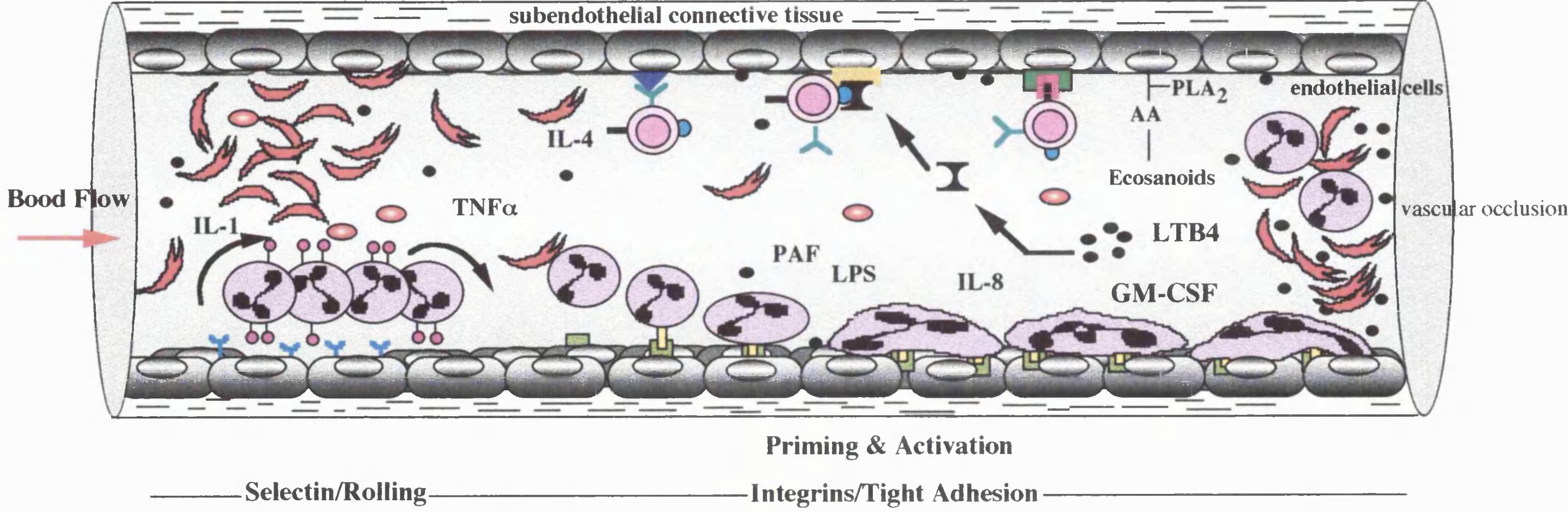



**Figure 3.2: Mechanism of neutrophils and sickled reticulocyte activation and adhesion to vasuclar endothelial cells during inflammatory reaction.** Reticulocytes in patients with SCD adhere directly to vascular adhesion molecule-1 (VCAM-1) expressed on endothelial cells which is induced by IL-1, IL-4 and TNFα. GPIIb/IIIa expressed on reticulocytes bind to vWF and this complex bind to endothelial cells by the vitronectin receptor (VNR). CD36 on reticulocytes bind to endothelial cells via thrombospondin (Tsp). Migration of leukocytes from the circulation is initiated by rolling motion mediated by members of selectin family. The leukocytes become activated by proinflammatory mediators which cause changes in expression of intergins followed by their interaction with intracellular adhesion molecule-1 (ICAM-1) and hence firm adhesion of leukocyte to the endothelium. Finally, Activated neutrophils, sickle RBC and platelets in association with plasma factors enhance vasoocclusive events of SCD.

of organs including spleen, lung, brain and bones. The involvement of multiple factors underlies the complexity of the pathophysiology of SCD and makes developing strategies for therapy very difficult. The acute painful episode in SCD patients is treated by administration of analgesics and maintaining optimal hydration by oral or intravenous fluid administration. Pilot studies of the ability of magnesium and clotrimazole to decrease intracellular dehydration have been successful, and nitric oxide therapy for changing oxygen affinity and inhibition of sickle RBC adhesion to the endothelium becomes a potentially promising therapy for SCD (McMahon *et al.*, 1997). Another potential therapeutic strategy for SCD based on increasing HbF has led to the successful clinical trials with hydroxyurea, which is an S-phase specific cytotxic agent that inhibits enzyme ribonucleoside diphosphate reductase (Charache, 1997).

Patients with SCD also have an increased tendency to infection (Francis *et al.*, 1991). The type of infection caused by particular organisms are shown in Table 3.2. Despite the use of antibiotics, infection is still one of the most frequent complications of SCD which accounts for about 90% of mortality, particularly in young children. *Streptococcus pneumoniae* is the most common cause of pneumonia and meningitis with an overall mortality of 5-13% and 30%, respectively (Finch, 1988). The second most common bacteraemia in SCD is caused by *Haemophilus influenzae* type b. The use of vaccination against *H. influenzae* type b, and prophylactic penicillin has greatly ameliorated the risks of infection. In older children and adults bacteraemia is more likely due to *Escherichia coli* and other Gram-negative organisms. As a result of infarction or infection of bone, osteomyelitis occurs more commonly in patients with SCD than normal individuals (Givner *et al.*, 1981). Factors which may contribute to an increased susceptibility of patients with SCD to infection are; defective splenic function (Pearson, 1977), defective serum opsonisation (Johnston *et al.*, 1973), altered complement activity (Mohamed *et al.*, 1993), low levels of immunoglobulin G (IgG) (Orda *et al.*, 1981) and impaired IgM antibody response (Ballester *et al.*, 1985). The presence of high concentrations of iron in the serum as a result of the haemolytic condition of SCD and consequent reduction in concentration of unsaturated transferrin has been suggested by Masawe and Nsanzumuhire, (1973) to increase growth and virulence of certain microorganisms in the blood of patients with SCD. But infection may also be due to an alteration of neutrophil function in patients with SCD.

Neutrophils are the major host defence against life-threatening microbial invasion, and their function may be altered by on-going events of SCD. Neutrophils are the first cells to be recruited to the site of infection or injury and to participate in the host defence response neutrophils must adhere to the vascular endothelium at an infective focus by means of adhesion molecules such as integrins, selectins (for firm

**Table 3.2: List of bacteria and viruses that cause infection in patients with SCD**

| *Micro-organism* | *Infection* | *Reference* |
|---|---|---|
| *Streptococcus pneumoniae* | Pneumonia/Meningitis/Septicaemia | Wong *et al.*, 1992 |
| *Haemophilus influenzae*-b | Pneumonia/Meningitis/Septicaemia | Ward & Smith, 1976 |
| *Salmonella* sp. | Osteomyelitis/Septicaemia | Givner *et al.*, 1981 |
| *Staphylococcus aures* | Osteomyelitis | Givner *et al.*, 1981 |
| *Mycoplasma pneumonia* | Pneumonia | Poncz *et al.*, 1985 |
| *Chlamydia pneumonia* | Pneumonia | Poncz *et al.*, 1985 |
| *Escherichia coli* | Septicaemia/Osteomyelitis/ Urinary tract infection | Zarkowsky*et al.*, 1986 |
| Hepatitis viruses (A, B, C) *Parvovirus B19* | Hepatitis Bone marrow suppression | Serjeant *et al.*, 1981 |

adhesion) and immunoglobulin superfamily of adhesion molecules (ICAM-1, ICAM-2 and VCAM-1). They transmigrate into the tissues and are primed by inflammatory cytokines to partake in the processes of phagocytosis and killing of microorganisms (Figure 3.2). The binding of a pathogen to the neutrophil via surface receptors activates its bactericidal arsenal. Exposure of neutrophils to cytokines *in vitro* greatly increases the capacity of the phagocytic enzyme NADPH-dependent oxidase in a respiratory burst which generates oxygen metabolites and free-radicals (singlet oxygen, hydrogen peroxide, hypochlorous acid and chloramines) (Chapter 1). These processes may be affected by physiological events in SCD and several investigators have reported impairment of phagocytic functional activity. Neutrophils from patients with SCD in steady state are defective in their adhesion to glass and migration into skin windows (Boghossian *et al.*, 1985), which is indicative of failure of the primary response of neutrophils which involves attachment, movement and shape change (Boghossian *et al.*, 1983; Addison *et al.*, 1982). Another report has shown that the presence of large numbers of $CD64^+$ (high affinity Fc receptor) PMN from SCD pateints in crisis were highly adherent to cultures of vascular endothelial monolayer trated with or without $TNF\alpha$ (Fadlon *et al.*, 1998). There is also evidence for decreased chemotaxis of neutrophils during painful crises (Akenzua and Amiengheme, 1981). For effective function phagocytes require priming for an enhanced production of oxygen metabolites.

Activation of primed neutrophils also results in the stimulation of phospholipase $A_2$ ($PLA_2$) with the generation of a range of arachidonic acid (AA) derivatives including the leukotrienes (Palmblad *et al.*, 1990) and prostaglandins which have potent chemotactic properties (Chapter 1, Figure 1.9). The priming events induced by the inflammatory cytokines are central to these processes (Chapter 1). In neutrophils an increase in $PLA_2$ activity and release of AA have been reported to occur in association with stimuli that also activate the oxidase (Lu and Grinstein, 1990). Arachidonic acid is also essential for upregulation of adhesion molecules and activation of kinases involved in signal transduction pathways. It has been shown that exposure of neutrophils to cytokines *in vitro* greatly increases the capacity of the phagocytic NADPH oxidase and phospholipase $A_2$ to respond to activation via cell surface receptors for chemotactic peptide (Roberts *et al.*, 1996; Gasson, 1991) or IgG (Roberts *et al.*, 1990) - a process referred to as "priming" (see Chapter 1 for details). Priming of neutrophils by GM-CSF and granulocyte-colony stimulating factor (G-CSF) has been demonstrated *in vivo* (Jaswon *et al.*, 1990; Khwaja *et al.*, 1992).

The aim of this study was to look for neutrophil activation and altered neutrophil inflammatory responses in SCD, and therefore the activation state of the neutrophil respiratory burst and phospholipase $A_2$ both in steady state SCD and during painful

vaso-occlusive crises. This study further investigated the effects of cytokine mediated priming on these enzymes which has not previously been studied in SCD.

## 3.2 MATERIALS AND METHODS

Materials

- Preservative free heparin (1000 Units/ml) (Monoparin, CP Pharmaceuticals, Wrexham, Clwyd, UK)
- 100 mM 2'7'dichlorodihydrofluorescein diacetate (DCFH-DA) in DMSO (Molecular Probes, Eugene, OR)
- Phorbol 12-myristate 13-acetate (PMA) reconstituted in DMSO (200 μg/ml stock solution was diluted 10 fold in DMSO immediately before use) (Sigma Chemical Co,
  Poole, Dorset, UK)
- Calcium ionophore (A23187) and N-Formyl-Met-Leu-Phe (FMLP) (stock solutions prepared in DMSO at 5 mg/ml and stored at -20°C, were diluted to 100 μM in PBS immediately prior to use) (Sigma)
- *rh*GM-CSF [1 μg/ml stock solution in 1% (v/v) FCS] (Recombinant human GM-CSF, expressed in *E.coli*; Hoechst, UK/Behringwerke, Marburg, Germany)
- Tumor Necrosis Factor α (TNFα) (10000 U/ml stock solution in 1% FCS) (A kind gift from Dr Anthony R Mire-Sluis: Department of Immunology, National Institute for Biological Standards and Control, South Mimms, Hertfordshire, UK)
- MULTI-Q-PREP (erythrocyte lytic agent for use on the coulter, consisting of 3 reagents: A, B, C) (Coulter Immunology, Hialeah, FL 33010 USA)
  Immunoprep A: (formic acid, stabiliser)
  Immunoprep B: (sodium carbonate, sodium chloride, sodium sulphate, stabiliser)
  Immunoprep C: (paraformaldehyde, stabiliser)
- Ficoll-Histopaque® (density 1.119 g/ml) (Sigma)
- Ficoll-Histopaque (density 1.077 g/ml) (Pharmacia Biotech, Uppsala, Sweden)
- Dulbecco's Phosphate buffered Saline ($PBS^-$) (Gibco BRL, Paisley, UK)
- PBS containing 0.9 mM calcium and 0.5 mM magnesium ($PBS^+$) (Gibco BRL)
- Glucose (BDH, UK)

### 3.2.1 Patients studied

Twenty-three patients from University College London and North Middlesex Hospitals who were homozygous for the sickle gene (HbSS) were studied:
a) 17 patients (age 17-50 years, 12 male) in steady state between painful vaso-occlusive crises were tested on 20 occasions (10 for NADPH oxidase and 10 for phospholipase $A_2$). The mean interval since the previous crisis requiring hospital

admission was 40 ± 10 weeks (range 3-132 weeks). All these patients were asymptomatic and non-infected at the time of study and were receiving prophylactic antibiotic therapy (penicillin V 250 mg b.d.) and folic acid 5 mg o.d. None were receiving repeated blood transfusion, opiate analgesics or hydroxyurea. b) 6 hospitalized HbSS patients (age 17-31 years, 3 male) during vaso-occlusive crisis treated by prophylactic antibiotics and analgesia. Two of these patients had received a blood transfusion within 6 days of being tested and their levels of HbS were 47% and 24 %, respectively, but their neutrophil functional responses were not different from those of the non-transfused patients in crisis. Further details of blood samples tested from SCD patients in steady state or crisis are given in Tables 3.3 and 3.4 respectively. c) 23 healthy laboratory personnel (age 24-62 years, 9 males, tested on 26 occasions) one of whom was tested each time that blood from the above categories was tested (same day controls). In all cases peripheral blood was obtained with the informed consent of the donor.

Methods

### 3.2.2 $H_2O_2$ Production Assay

Intracellular $H_2O_2$ production was measured in a whole blood flow cytometric assay of the oxidation of dichlorodihydrofluorescein diacetate (DCFH-DA), using the method previously described by Jaswon *et al.* in 1990. DCFH-DA is a non-polar compound that diffuses into the cells where the acetyl groups are cleaved leaving the polar molecule DCFH by the action of intracellular esterases. This prevents the subsequent release of DCFH from the cells. The non-fluorescent DCFH is oxidised by the $H_2O_2$, produced during the phagocyte oxidative burst, to DCF which is a fluorescent compound (Figure 3.3).

The mean cell fluorescence due to oxidation of DCF was measured by a Coulter Elite flow cytometer (Coulter, Luton, UK). Cells were excited with an air-cooled 488 nm argon laser at 15mW. The emission filter on the fluorescence detector was a 520-530 nm band pass filter. At least 10,000 cells were analysed per sample. The ratio of forward scatter area:peak height was used to discriminate between cell doublets and single cells. The mean fluorescence and percentage of positive cells was determined by manual gating as described below (Figure 3.4).

$H_2O_2$ production by resting cells was determined from the mean cell fluorescence (MCF) of the ungated neutrophil population measured on a linear scale. From this distribution a 'positive' gate was set to include 5% of the brightest cells in the control population. $H_2O_2$ production stimulated by FMLP was determined as the

## Table 3.3: Haematological indices of patients with SCD in steady state

| Patients(SEX) | AGE (years) | Hb (g/L) | Retic (%) | Neut (%) | Mono (%) | Lymph (%) | Eos (%) | Baso (%) | WBC $x10^9/L$ | Hb(%) S | A | F |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 (M) | 30 | 84 | - | 69 | 9 | 21 | 1 | 0 | 13 | - | - | - |
| 2 (F) | 30 | 80 | 18 | 75 | 4 | 18 | 2 | 1 | 12 | p | a | 4 |
| 3 (M) | 25 | 76 | 18 | 62 | 14 | 22 | 2 | 0 | 12 | - | - | - |
| 4 (M) | 33 | 86 | - | 68 | 6 | 25 | 1 | 0 | 13 | p | a | a |
| 5 (F) | 42 | 74 | 20 | 47 | 6 | 34 | 13 | 0 | 20 | 80 | 20 | a |
| 6 (F) | 27 | 110 | 15 | 81 | 13 | 39 | 0 | 2 | 8 | p | a | a |
| 7 (F) | 40 | 79 | 10 | 46 | 13 | 39 | 2 | 0 | 11 | p | a | 8 |
| 8 (M) | 50 | 64 | 6 | 51 | 6 | 42 | 1 | 0 | 12 | p | a | 9 |
| 9 (M) | 17 | 68 | 11 | - | - | - | - | 0 | - | - | - | - |
| 10 (F) | 30 | 80 | 18 | 71 | 4 | 19 | 6 | 0 | 12 | p | a | a |
| 11 (M) | 31 | 75 | 12 | 65 | 8 | 23 | 3 | 1 | 16 | p | a | a |
| 12 (M) | 31 | 81 | 10 | 70 | 5 | 20 | 5 | 0 | 10 | - | - | - |
| 13 (M) | 38 | 78 | 8 | - | - | - | - | - | | - | - | - |
| 14 (M) | 18 | 89 | - | 70 | 4 | 25 | 1 | 0 | 9 | p | a | a |
| 15 (M) | 38 | 83 | 10 | 69 | 3 | 24 | 3 | 1 | 14 | p | a | 4 |
| 16 (M) | 18 | 66 | 9 | 58 | 1 | 40 | 1 | 0 | 15 | p | a | a |
| 17 (M) | 22 | 80 | 14 | 60 | 5 | 33 | 2 | 0 | 8 | p | a | a |
| Mean ± SE | 30 ± 2 | 85 ± 6(F)<br>78 ± 2(M) | 13 ± 1 | 64 ± 3 | 7 ± 1 | 28 ± 2 | 3 ± 1 | 0.3±0.1 | 12 ± 1 | - | | |
| Normal Range | | 115-155(F)<br>135-175(M) | 0.2 - 2 | 40 - 75 | 2 - 10 | 20 - 45 | 1 - 6 | <2 | 3 - 10 | - | | |

F(Female), M(Male), Hb(Haemoglobin), Retic.(Reticulocyte), Neut.(Neutrophil), Mono.(Monocyte), Lymph.(Lymphocyte), Eos.(Eosinophil), Baso.(Basophil), SE(Standard Error), WBC(White Blood Cell), p (predominant), a (absent), HbF (foetal Hb)

**Table 3.4: Haematological indices of patients with SCD during painful crises**

| *Patients(SEX)* | *AGE* *(years)* | *Hb* *(g/L)* | *Retic* *(%)* | *Neut* *(%)* | *Mono* *(%)* | *Lymph* *(%)* | *Eos* *(%)* | *Bas* *(%)* | *WBC* *x* $10^9$*/L* | *Hb(%)* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | *S* | *A* | *F* |
| 1 * (M) | 21 | 114 | - | 37 | 12 | 47 | 3 | 1 | 5.8 | 47 | 53 | a |
| 2 (F) | 41 | 74 | 11 | 55 | 12 | 33 | 0 | 0 | 5.1 | p | a | 12 |
| 3 * (M) | 31 | 98 | - | 61 | 10 | 26 | 3 | 0 | 15.1 | 24 | 76 | a |
| 4 (F) | 27 | 98 | - | 45 | 11 | 31 | 11 | 1 | 15.9 | p | a | a |
| 5 (M) | 17 | 77 | 7 | 48 | 4 | 44 | 4 | 0 | 7.7 | p | a | 10 |
| 6 (F) | 48 | 80 | - | 69 | 13 | 15 | 3 | 0 | 18.6 | p | a | a |
| Mean ± SE | 31 ± 5 | 84 ± 7(F)<br>96 ± 11(M) | 9 ± 2 | 52 ± 5 | 10 ± 1 | 33 ± 5 | 4 ± 2 | 0.3±0.2 | 11 ± 2 | - | | |
| Normal Range | | 115-155(F)<br>135-175(M) | 0.2 - 2 | 40 - 75 | 2 - 10 | 20 - 45 | 1 - 6 | <2 | 3 - 10 | - | | |

F(Female), M(Male), Hb(Haemoglobin), Retic.(Reticulocyte), Neut.(Neutrophil), Mono.(Monocyte), Lymph.(Lymphocyte), Eos.(Eosinophil), Baso.(Basophil), SE(Standard Error), WBC(White Blood Cell), HbF (foetal haemoglobin), p (predominant), a (absent), * Recent blood transfusion

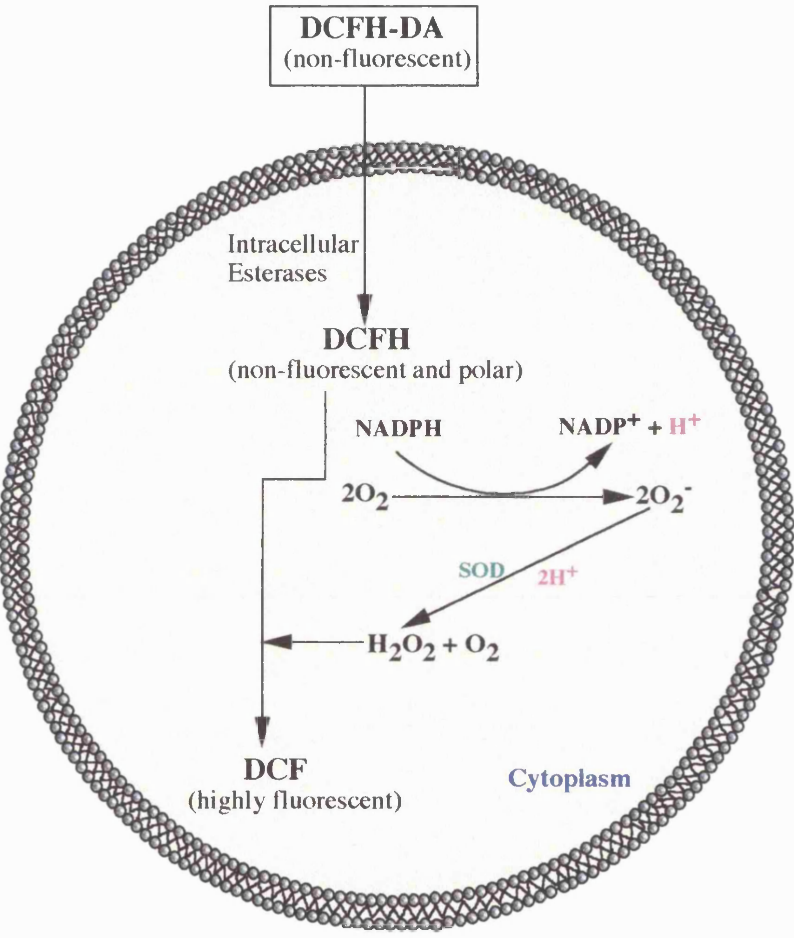



**Figure 3.3: The DCFH-DA chemical reaction.**
2'7'-dichlorodihydrofluorescin diacetate (DCFH-DA), a non-fluorescent compound diffuses readily into the cell where it hydrolyses by intracellular esterases into DCFH, which is polar, non-fluorescent and becomes trapped inside the cell. When exposed to $H_2O_2$ , DCFH is oxidised into a highly fluorescent 2'7'-dichlorofluorescein (DCF). SOD=Superoxide dismutase

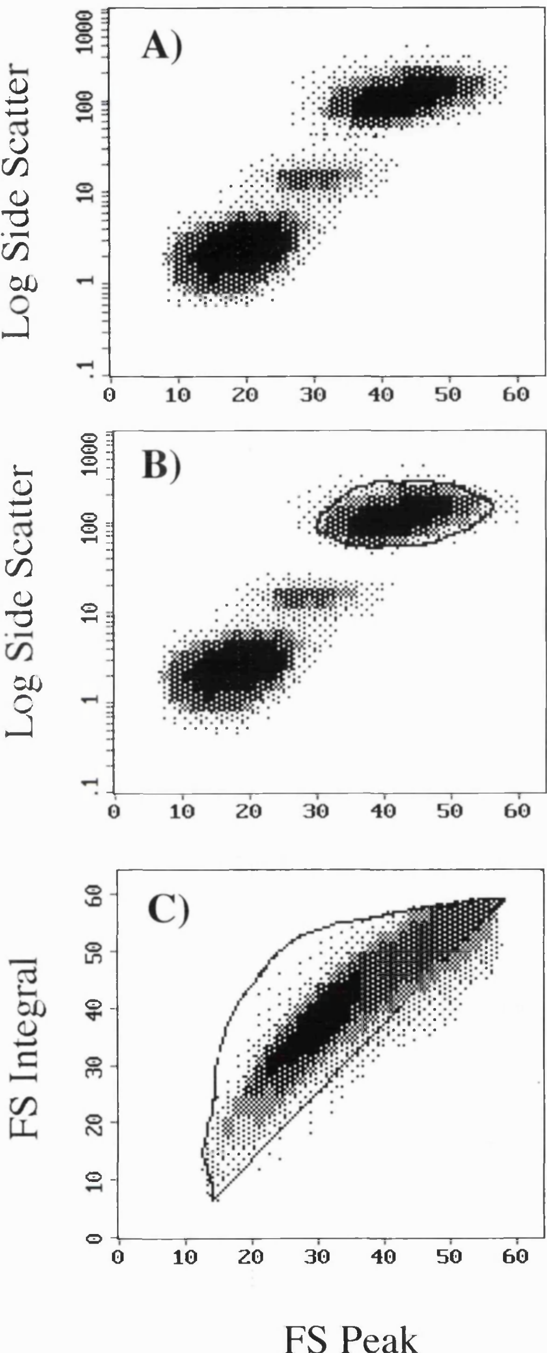



**Figure 3.4: Flow cytometric profile of whole blood after lysis of red blood cells.**
A=Overall profile
B=Gated neutrophils
C=Doublet discriminator
FS=Forward scatter

product of the percentage and MCF of cells entering the preset positive gate measured in arbitrary fluorescence units. In the cytokine-priming experiments, these MCF values were normalised by expressing the MCF of cytokine-treated cells as a percentage of the MCF of the diluent control. An estimate of the total $H_2O_2$ production in a given sample was made by multiplying the percentage of positive cells by the normalised MCF, and the data expressed in arbitrary fluorescence units.

Samples of venous blood were anti-coagulated with 10 IU/ml heparin. Care was taken to ensure that the patients and their same-day controls were venesected within 30 minutes of each other (when blood was taken from patients at the North Middlesex Hospital control blood was taken at the same time and both samples were sent to UCL together), as the capacity of neutrophils to produce $H_2O_2$ diminished if blood was stored for more than 90 minutes at room temperature (Figure 3.5). Aliquots of blood were incubated at 37°C for 30 minutes with 100 μM DCFH-DA. Replicate samples were prepared and incubated for 30 minutes with either GM-CSF or TNFα (at various doses stated in the text) to induce priming, or FCS diluent (0.01% v/v, final concentration). Stimulation of NADPH oxidase was for 15 minutes at 37°C with either 1 μM FMLP or 20 μg/ml phorbol-myristate-acetate (PMA) as this was the dose that gave the maximal response. The solvent, DMSO, had no effect on $H_2O_2$ production at the concentration used in the assay (for PMA dose response see Figure 3.6). The control samples were stimulated with PBS. After stimulation samples were then taken onto ice and processed using the Coulter Immunoprep reagents: (the blood sample was mixed whilst adding reagent A for 10 seconds, followed by 5 seconds mixing with reagent B, and finally 5 seconds with reagent C) in order to lyse the red blood cells and fix the white blood cells. Following this fixation procedure the fluorescence in the samples remained stable for at least 6 hours (Jaswon *et al.*, 1990).

### 3.2.3 Phospholipase $A_2$ Assay

#### *3.2.3.1 Discontinuous gradient purification of neutrophils*

Purification of neutrophils from the peripheral blood of patients with SCD with the method routinely used in this laboratory (the single density gradient centrifugation method as described in Chapter 2) was not effective due to; 1) presence of a heterogeneous population of red blood cells with an inability for effective sedimentation with dextran, therefore there was contamination of the leukocyte rich supernatant with great number of red blood cells and 2) the presence of a large number of reticulocytes (nucleated red blood cells) which is associated with SCD (as shown in Table 3.5) which were less susceptible to hypotonic lysis hence there was a poor

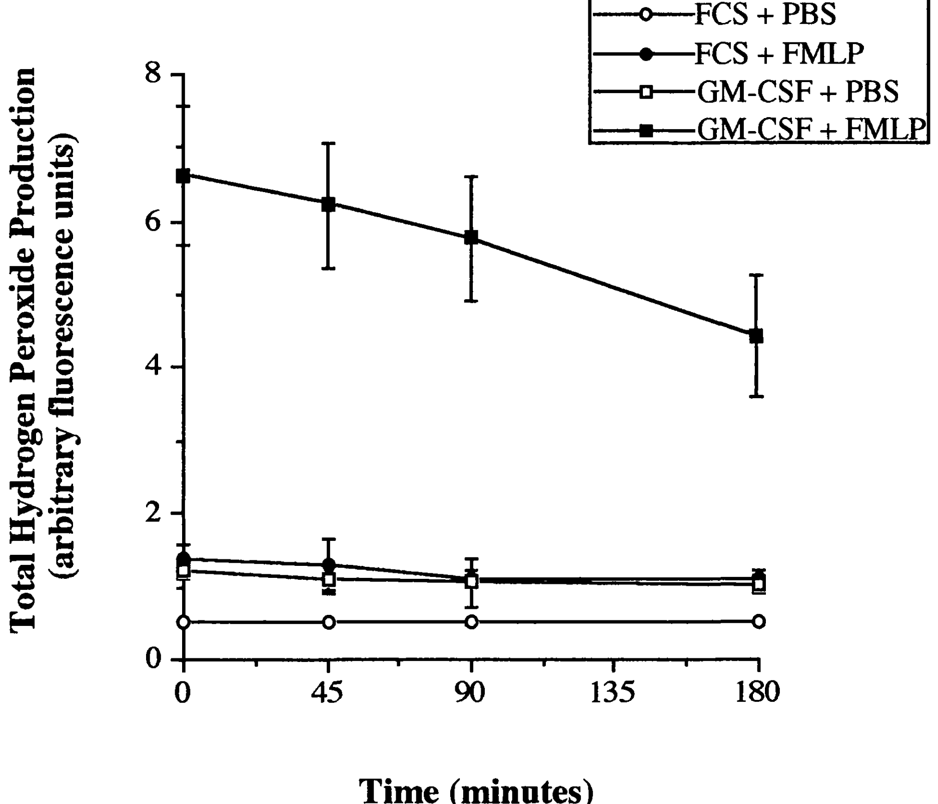



**Figure 3.5: Effect of blood storage on $H_2O_2$ production at room.** Venous blood was taken on the day of experiment and at each time interval, 0, 45, 90 and 180 minutes, aliquots were taken and examined for the neutrophil's capacity to produce hydrogen peroxide. Data shown are mean ± range of 2 experiments.

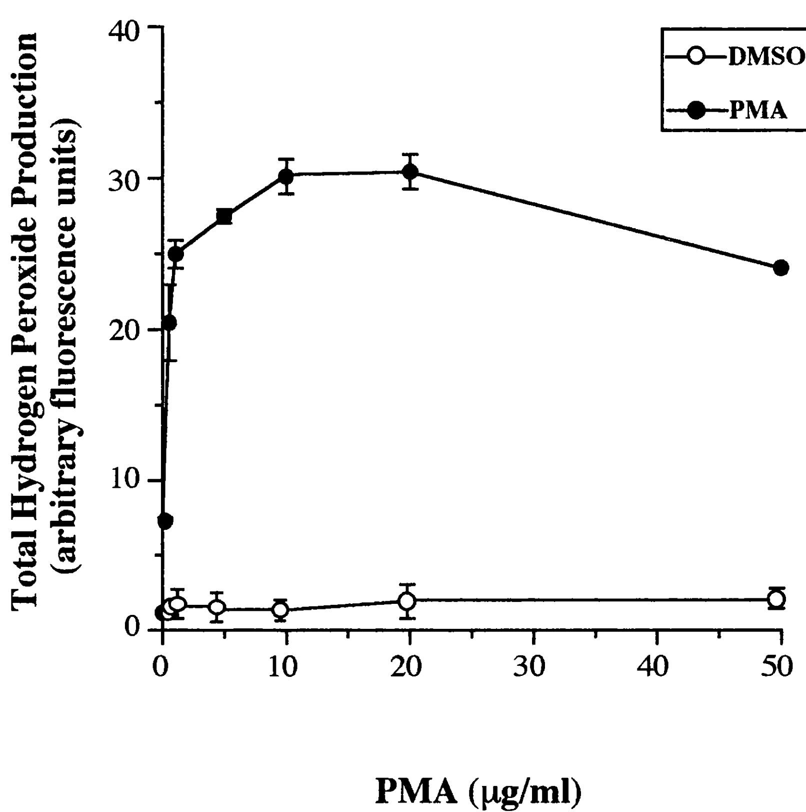



**Figure 3.6: PMA dose response curve**
Neutrophils were loaded with DCFH-DA prior to incubation with different doses of PMA. The hydrogen peroxide production was measured by flow cytometry. Data shown are the mean ± range of 2 experiments

**Table 3.5: Cellular composition and purity of neutrophils isolated by Ficoll-Histopaque discontinuous gradient centrifugation**

| *Subjects (n)* | *Neutrophils (%)* | *Monocytes (%)* | *Lymphocytes (%)* | *Erythrocytes (%)* |
|---|---|---|---|---|
| Patient (10) | 89 ± 2 | 3 ± 1 | 3 ± 1 | 5 ± 1 |
| Control (10) | 93 ± 2 | 2 ± 1 | 2 ± 0 | 3 ± 1 |

Cytospin preparations of neutrophils purified by discontinuous gradient centrifugation were stained with Leishman's stain as described in Chapter 2. Data shown are the mean ± 1 SE of 10 experiments.

recovery of neutrophils. The following methodology allowed simultaneously, the higher recovery of neutrophils, with reduced contamination by red blood cells.

Peripheral blood (20 ml) from patients with SCD in steady state and healthy controls was taken into EDTA (2 mM final concentration, pH 7.4) and neutrophils were isolated through a discontinuous gradient of Histopaque using the method previously described by English and Anderson (1974), and in sterile conditions to minimise the contact of cells with endotoxin and hence reduce priming. The discontinuous gradients were prepared using two Ficoll-Histopaque solutions. The upper separating solution (10 ml) with density of 1.077 g/ml was layered onto 10 ml of the second solution with density of 1.119 g/ml. Blood (5 ml) was diluted with 5 ml of $PBS^-$ and layered onto the Ficoll-Histopaque gradients and centrifuged at 1400 x g for 30 minutes at room temperature. Three distinct layers of cells were observed. The first top layer consisted of plasma, with platelets and mononuclear cells at the first interface. The second layer contained a neutrophil rich interface, and at the bottom the pellet consisted of erythrocytes. Neutrophils were collected from the interface between the second and third layer.

Neutrophils isolated by this method were transferred into a fresh tube and washed twice in $PBS^-$ by centrifugation at 170 x g for 7 minutes at room temperature and finally resuspended in $PBS^+$ supplemented with 5 mM glucose. The cellular composition and the relative number of contaminating cells present in this suspension was assessed by Leishman's stain (Table 3.5) and neutrophils were 99% ± 2% viable by trypan blue exclusion.

*3.2.3.2 Measurement of $PLA_2$ Activity*

$PLA_2$ activity was measured by the release of tritiated arachidonic acid (AA) from radiolabelled intracellular phospholipid stores, as previously described in the Methods Sections 2.6.1 and 2.6.2.

For priming experiments, neutrophils ($1x10^6$ cells in 0.5 ml samples in duplicate) were incubated with diluent (0.01% vol/vol FCS) or primed with either GM-CSF (1, 10 ng/ml) or TNFα (50, 500 U/ml) for 20 minutes before stimulation with 1μM calcium ionophore (A23187), for 15 minutes. Identical control samples were prepared either unstimulated or stimulated with PBS. The AA release was measured as described in Chapter 2.

### 3.2.4 Statistical Analysis

Unless otherwise stated the data are the mean ± 1SE of the number of experiments given in the text. Statistical analysis of the data was performed by Wilcoxon's matched-pairs signed-ranks test (Siegel, 1956).

## 3.3 RESULTS

### 3.3.1 Haematological indices of patients with SCD

As shown in Tables 3.3 and 3.4 patients with SCD showed reduced haemoglobin levels for both female and male patients in steady state (n=17) and during crisis (n=6) of SCD, indicative of anaemia and chronic haemolysis associated with the disease. The presence of large number of reticulocytes in the circulation, 13% ± 1% and 9% ± 2% for patients in steady state and during painful crises, respectively, indicated an increase in erythropoiesis due to haemolytic conditions. In all patients HbS was predominant with HbA and HbF being very low or completely absent. The higher concentration of HbF is normally associated with a milder condition. All other white cell parameters fell within normal range. The total white cell count was slightly higher than normal in SCD patients in steady state and during painful crisis. None of the patients were infected on the day of experiment.

### 3.3.2 NADPH oxidase activity of neutrophils in patients with SCD

Activation of the neutrophil NADPH-dependent oxidase was measured by a fluorescent assay of $H_2O_2$ production (Jaswon *et al.*, 1990) both in the resting state, after activation with the agonist FMLP, and following priming of the cells by incubation with cytokines with subsequent stimulation with FMLP. Measurements of $H_2O_2$ production were made in whole blood samples from patients with SCD in parallel with samples from a same day control.

#### *3.3.2.1 Resting levels of $H_2O_2$ production*

Activation of the neutrophil NADPH-dependent oxidase was measured by a fluorescent assay of $H_2O_2$ production (Jaswon *et al.*, 1990). The resting rate of neutrophil $H_2O_2$ production was slightly higher in SCD patients in steady state [mean cell fluorescence of the ungated neutrophil population (MCF), 59 ± 10, n=9] than their respective same day controls (MCF 53 ± 8, n=9, $P>0.05$), which was not significantly different. Six patients in painful crisis of SCD were also studied, and again the resting values for neutrophil $H_2O_2$ production were not different from control (MCF for patients in crisis, 62 ± 6 and same day controls, 60 ± 8, $P>0.05$, n=6).

*3.3.2.2 Stimulation of unprimed neutrophils with FMLP and PMA*

Stimulation of neutrophil NADPH oxidase with the chemotactic peptide, FMLP, increased $H_2O_2$ production to between two and four times the resting level (Table 3.6). The mean FMLP responses in both steady state and crisis SCD patients were greater than control but the values were not significantly different (in all cases $P>0.05$).

The PMA responses of neutrophils from patients in the steady state were the same magnitude as control, but the PMA responses of neutrophils from patients in crisis were on average only 89% ± 3% of their same-day controls. This small difference was statistically significant ($P=0.014$, n=6), which may be due to the analgesic drugs administered in SCD patients in crisis.

*3.3.2.3 Cytokine mediated priming of NADPH oxidase in neutrophils*

Pre-incubation with varying concentrations of GM-CSF (0-10 ng/ml) and TNFα (0-500 U/ml) prior to stimulation with FMLP, caused a dose-dependent enhancement of neutrophil FMLP-stimulated $H_2O_2$ responses in both patient and control samples when compared with samples primed with diluent (control) (Figure 3.7). Neutrophils from SCD patients in steady state produced significantly less $H_2O_2$ after priming with either cytokines than their same-day controls (Figure 3.7 A and B). At the maximal doses of GM-CSF and TNFα used, $H_2O_2$ production was 65% ± 11% ($P=0.03$, n=7), and 57% ± 7% ($P=0.007$, n=10) of same day controls, respectively. Similar experiments with SCD patients in crisis (Figure 3.7 C and D) showed a more marked defect in neutrophil $H_2O_2$ production after priming with cytokines. At maximal doses of GM-CSF and TNFα, $H_2O_2$ production was 34% ± 9% ($P=0.03$, n=6) and 25% ± 3% ($P=0.03$, n=6) of same day controls, respectively.

Representative fluorescence distributions of neutrophils from patients and controls from which these data derived are shown in Figure 3.8. In confirmation of previous studies of cytokine-mediated priming of the respiratory burst measured in whole blood (Jaswon *et al.*, 1990; Khwaja *et al.*, 1992), the increase in FMLP-stimulated fluorescence after TNFα (Table 3.7) and GM-CSF (Table 3.8) priming was due to an increase in both the percentage of 'positive' cells and their mean cell fluorescence. In comparison with control samples, patients with SCD had fewer neutrophils recruited into the 'positive population' and the mean cell fluorescence of these positive cells was also lower than the controls.

**Table 3.6: NADPH oxidase activity of unprimed neutrophils from SCD patients and healthy controls tested on the same day.**

| | *Total $H_2O_2$ Production* [(% positive) x (MCF)] | | |
|---|---|---|---|
| **Subjects** | **PBS** | **FMLP** | **PMA** |
| **Steady State** | 1454 ± 209 (9) | 6497 ± 2180 (9) | 86976 ± 4145 (3) |
| Control | 1138 ± 226 (9) | 4107 ± 725 (9) | 88512 ± 3634 (3) |
| **Crisis** | 2201 ± 458 (6) | 4118 ±1087 (6) | 75190 ± 3484* (6) |
| Control | 1642 ± 333 (6) | 3770 ± 511 (6) | 84906 ± 1353 (6) |

The data shown are the total cellular $H_2O_2$ production in arbitrary fluorescence units and are the mean ± 1SE of the number of experiments shown in parantheses.*$P$<0.02 versus control

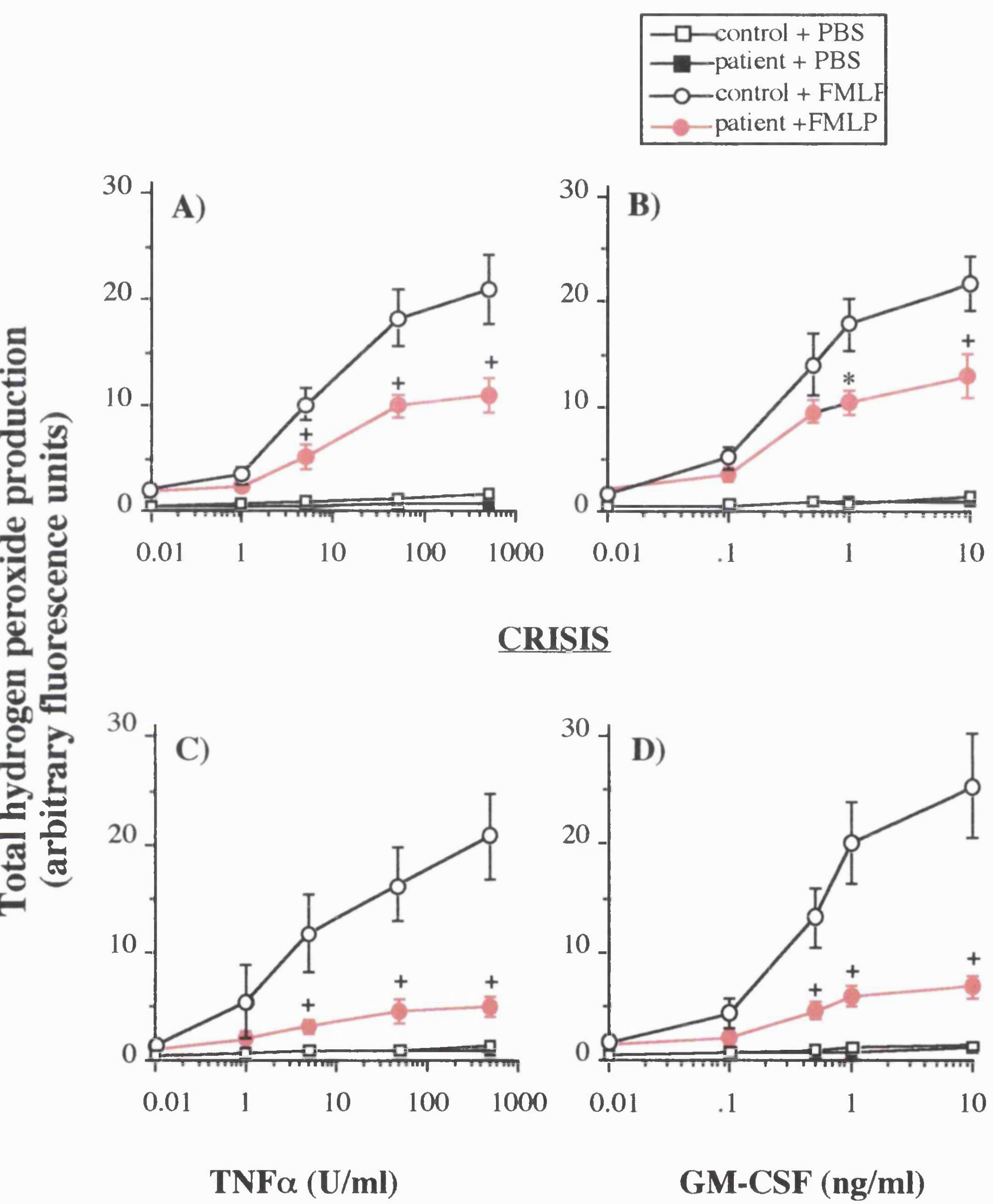
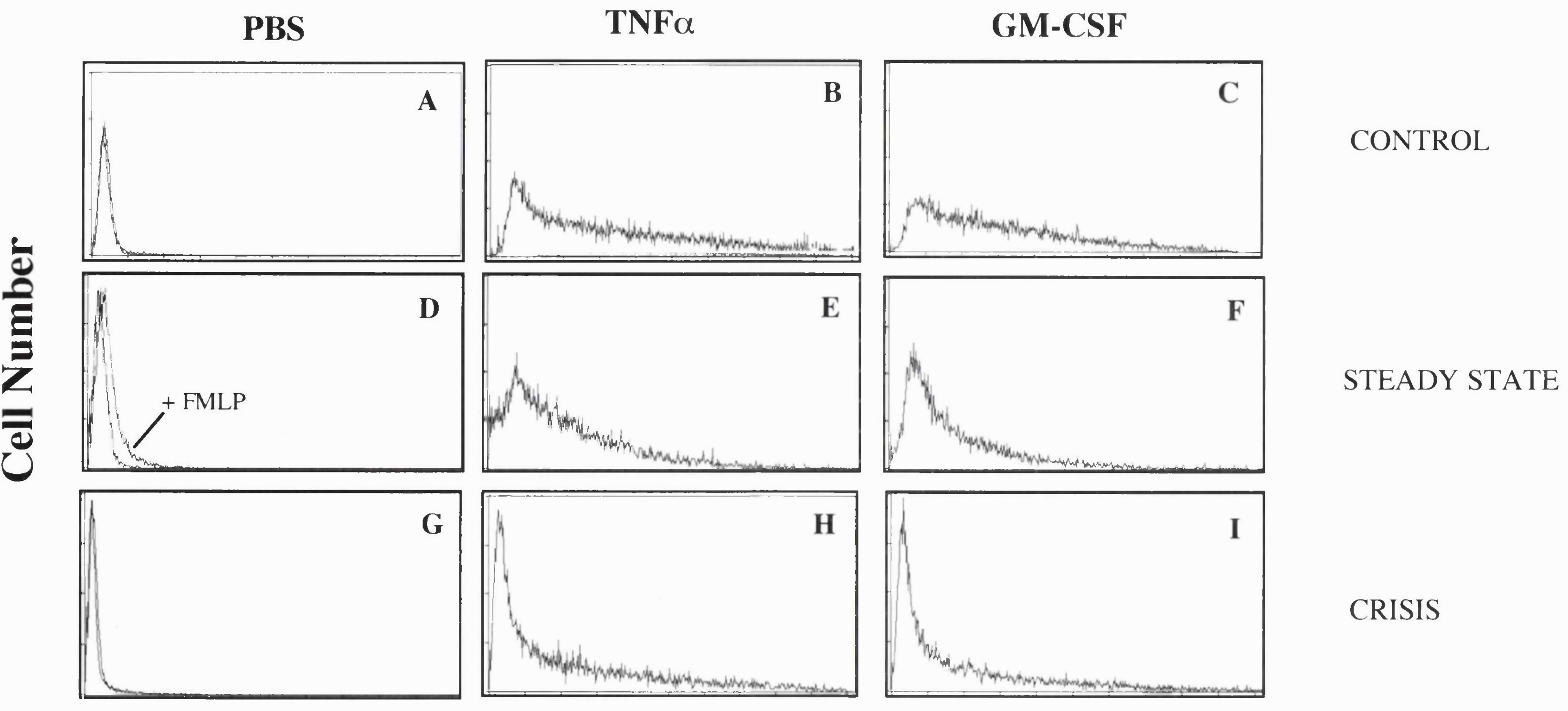



**Figure 3.7: Cytokine mediated priming of neutrophil NADPH oxidase in SCD.** SCD patients in steady state (A and B) or patients during crisis (C and D) compared with controls tested on the same day. Total $H_2O_2$ production was calculated as the product of the percent of positive cells and their standardised MCF. The data shown are the mean ± 1 SE of (A) 10 experiments, (B) 7 experiments, and (C and D) 6 experiments. Significant differences between patients and controls (Wilcoxon's matched pairs signed-ranks test) are shown as: *$P$<0.01, $^{+}P$<0.005.




Fluorescence Intensity

**Figure 3.8: Representative flow cytometric histograms show the effect of priming on the FMLP-stimulated neutrophil respiratory burst.** In histogram A, D and G the overlaid distribution of unprimed samples stimulated with and without FMLP are shown. In D the stimulated distribution is marked.

A,D,G = (unprimed)
B,E,H = TNF(500 U/ml)+FMLP
C,F,I = GM-CSF(10 ng/ml)+FMLP

| $H_2O_2$ Production | | | | |
|---|---|---|---|---|
| ***Stimulus*** | ***Steady state*** | *Control* | ***Crisis*** | *Control* |
| **FCS +PBS** | | | | |
| %+ | 5 | 5 | 5 | 5 |
| (Absolute value) MCF | 278 ± 48 | 225 ± 31 | 446 ± 99 | 348 ± 69 |
| %MCF | 100 | 100 | 100 | 100 |
| **FCS + FMLP** | | | | |
| %+ | 20 ± 4 | 17 ± 2 | 10 ± 1 | 17 ± 3 |
| (Absolute value) MCF | 326 ± 65 | 258 ± 34 | 421 ± 109 | 289 ± 70 |
| %MCF | 100 | 100 | 100 | 100 |
| **TNF + PBS** | | | | |
| (1 U/ml) %+ | 4 ± 1* | 7 ± 1 | 6 ± 3 | 7 ± 1 |
| %MCF | 99 ± 2 | 99 ± 4 | 96 ± 3 | 98 ± 3 |
| **TNF + FMLP** | | | | |
| (1 U/ml) %+ | 23 ± 4 | 30 ± 7 | 18 ± 3 | 25 ± 8 |
| %MCF | 103 ± 2 | 112 ± 4 | 114 ± 9 | 151 ± 40 |
| **TNF + PBS** | | | | |
| (5 U/ml) %+ | 5 ± 1* | 10 ± 2 | 8 ± 1 | 9 ± 3 |
| %MCF | 93 ± 4 | 101 ± 5 | 104 ± 4 | 100 ± 4 |
| **TNF + FMLP** | | | | |
| (5 U/ml) %+ | 42 ± 9* | 65 ± 6 | 25 ± 4* | 61 ± 5 |
| %MCF | 117 ± 4+ | 155 ± 13 | 126 ± 14 | 178 ± 38 |
| **TNF + PBS** | | | | |
| (50 U/ml) %+ | 7 ± 2* | 12 ± 1 | 8 ± 1* | 10 ± 3 |
| %MCF | 97 ± 6 | 103 ± 4 | 101 ± 3 | 105 ± 6 |
| **TNF + FMLP** | | | | |
| (50 U/ml) %+ | 67 ± 5* | 83 ± 4 | 32 ± 4* | 76 ± 5 |
| %MCF | 148 ± 10+ | 217 ± 27 | 135 ± 18 | 210±37 |
| **TNF + PBS** | | | | |
| (500 U/ml) %+ | 7 ± 1* | 19 ± 5 | 8 ± 2* | 12 ± 1 |
| %MCF | 99 ± 4 | 102 ± 6 | 99 ± 5 | 109 ± 8 |
| **TNF + FMLP** | | | | |
| (500 U/ml) %+ | 70 ± 6+ | 88 ± 4 | 36 ± 3‡ | 84 ± 5 |
| %MCF | 151 ± 17* | 234 ± 34 | 136 ± 17+ | 240 ± 40 |

**Table 3.7: Effect of TNFα on FMLP-stimulated superoxide production in patients with SCD compared with healthy controls.** Data shown are the mean ± 1 SE of 9 experiments in steady state of SCD and 6 experiments during crisis of SCD. The percentage positive responding cells stimulated with PBS was set at 5%. The MCF of TNFα-treated cells stimulated with or without FMLP was expressed as a percentage of the MCF of their respective FCS diluent controls (the absolute values in arbitrary fluorescent units for these are shown). Statistical significance of the difference between patients and controls was tested by Wilcoxon matched-pairs signed-ranks test. $*0.05>P>0.01$, $^{+}0.01>P>0.001$, $‡P<0.001$

| $H_2O_2$ Production | | | | |
|---|---|---|---|---|
| ***Stimulus*** | ***Steady state*** | *Control* | ***Crisis*** | *Control* |
| **FCS +PBS** | | | | |
| %+ | 5 | 5 | 5 | 5 |
| (Absolute value) MCF | 278 ± 48 | 225 ± 31 | 446 ± 99 | 348 ± 69 |
| %MCF | 100 | 100 | 100 | 100 |
| **FCS + FMLP** | | | | |
| %+ | 20 ± 4 | 17 ± 2 | 10 ± 1 | 17 ± 3 |
| (Absolute value) MCF | 326 ± 65 | 258 ± 34 | 421 ± 109 | 289 ± 70 |
| %MCF | 100 | 100 | 100 | 100 |
| **GM-CSF + PBS** | | | | |
| (0.1 ng/ml) %+ | 5 ± 1 | 5 ± 1 | 6 ± 1 | 9 ± 1 |
| %MCF | 98 ± 2 | 106 ± 14 | 102 ± 1 | 96 ± 5 |
| **GM-CSF + FMLP** | | | | |
| (0.1 ng/ml) %+ | 33 ± 7 | 38 ± 5 | 19 ± 4 | 29 ± 3 |
| %MCF | 109 ± 2 | 130 ± 12 | 114 ± 8 | 142 ± 25 |
| **GM-CSF + PBS** | | | | |
| (0.5 ng/ml) %+ | 8 ± 1 | 8 ± 1 | 8 ± 1 | 10 ± 1 |
| %MCF | 99 ± 2 | 109 ± 3 | 103 ± 3 | 100 ± 4 |
| **GM-CSF + FMLP** | | | | |
| (0.5 ng/ml) %+ | 66 ± 6 | 69 ± 6 | 34 ± 4 | 65 ± 3 |
| %MCF | 144 ±12 | 192 ± 28 | 130 ± 13 | 197 ± 35 |
| **GM-CSF + PBS** | | | | |
| (1 ng/ml) %+ | 9 ± 1 | 7 ± 1 | 8 ± 1 | 12 ± 1 |
| %MCF | 99 ± 3 | 105 ±7 | 101 ± 5 | 103 ± 6 |
| **GM-CSF + FMLP** | | | | |
| (1 ng/ml) %+ | 68 ± 4 | 82 ± 5 | 40 ± 2 | 81 ± 7 |
| %MCF | 151 ± 15 | 215 ± 26 | 143 ± 18 | 238 ±39 |
| **GM-CSF + PBS** | | | | |
| (10 ng/ml) %+ | 8 ± 1 | 9 ± 1 | 10 ± 2 | 13 ± 1 |
| %MCF | 104 ± 4 | 110 ± 6 | 107 ± 7 | 106 ± 8 |
| **GM-CSF + FMLP** | | | | |
| (10 ng/ml) %+ | 75 ± 2‡ | 90 ± 3 | 45 ± 1+ | 87 ± 7 |
| %MCF | 173 ± 20 | 239 ± 26 | 147 ± 18* | 279 ± 46 |

**Table 3.8: Effect of GM-CSF on FMLP-stimulated superoxide production in patients with SCD compared with healthy controls.** Data shown are the mean ± 1 SE of 7 experiments in steady state of SCD and 6 experiments during crisis of SCD. The percentage positive responding cells stimulated with PBS was set at 5%. The MCF of GM-CSF-treated cells stimulated with or without FMLP was expressed as a percentage of the MCF of their respective FCS diluent controls (the absolute values in arbitrary fluorescent units for these are shown). Statistical significance of the difference between patients and controls was tested by Wilcoxon matched-pairs signed-ranks test. *0.05>$P$>0.01, +0.01>$P$>0.001, ‡$P$<0.001

### 3.3.3 Phospholipase $A_2$ activity

#### *3.3.3.1 Resting level of $PLA_2$ activity*

Resting levels of neutrophil phospholipase $A_2$ activity, as measured by the release of arachidonate from radiolabelled phospholipid stores, were two-fold greater in patients with steady state SCD than their same day controls (Table 3.9). The mean resting rate of arachidonate release in neutrophils from steady state patients was 4.0% ± 0.5% of total cell radioactivity, and in controls was 2.0% ± 0.2% ($P$=0.008, n=10) (the absolute values are given in the legend to Table 3.9). Resting levels of phospholipase $A_2$ activity did not alter when neutrophils were incubated with either GM-CSF (1 and 10 ng/ml) or TNFα (50 and 500 U/ml) and the differential between patient and control values remained significantly different as shown in Table 3.9.

#### *3.3.3.2 Effect of cytokine mediated priming of A23187 stimulated arachidonic acid release by neutrophils*

Stimulation of neutrophils with calcium ionophore (A23187) resulted in the activation of phospholipase $A_2$ above resting levels and this increment is shown in Figures 3.9 a and b. The increment due to calcium dependent stimulation was determined by subtracting the resting values from the stimulated values. A23187-stimulated arachidonate release from unprimed neutrophils was not significantly different in the steady state patients compared to their respective same day controls (SCD patients 2.6 % ± 0.5 % of total cell radioactivity; controls 3.9 % ± 0.8%, $P$=0.06, n=10). Priming of neutrophils with either GM-CSF (1 and 10 ng/ml) or TNFα (50 and 500 U/ml) for 20 minutes prior to stimulation with A23187 resulted in a dose-dependent increase in phospholipase $A_2$ activity which was significantly smaller in neutrophils from patients with steady state SCD than their respective controls (Figure 3.9 a and b). It was noted that TNFα was a much weaker priming agent for A23187-stimulated arachidonate release than GM-CSF.

**Table 3.9: $PLA_2$ activity in neutrophils from patients with SCD in steady state**

| *Arachidonic Acid Release* (% of total radioactivity) | | | | | |
|---|---|---|---|---|---|
| *Subjects* (n) | *FCS* (control) | *GM-CSF* (ng/ml) | | *TNF-α* (U/ml) | |
| | | **1** | **10** | **50** | **500** |
| **Patients** (10) | 4.0 ± 0.5 | 3.9 ± 0.5 | 4.4 ± 0.7 | 3.8 ± 0.5 | 4.3 ± 0.6 |
| **Controls** (10) | 2.0 ± 0.2 | 2.1 ± 0.2 | 2.0 ± 0.2 | 1.9 ± 0.2 | 2.1 ± 0.4 |
| ***P* values** | 0.008 | 0.010 | 0.005 | 0.003 | 0.002 |

The data shown are the mean ± 1 SE for 10 patients, compared with controls tested on the same day. The absolute values for the resting levels (FCS) of arachidonate release are 3204 ± 116 cpm/$10^6$ neutrophils and 1404 ± 211 cpm/$10^6$ neutrophils for SCD patients and their same-day controls, respectively. ($P<0.01$, n=10)

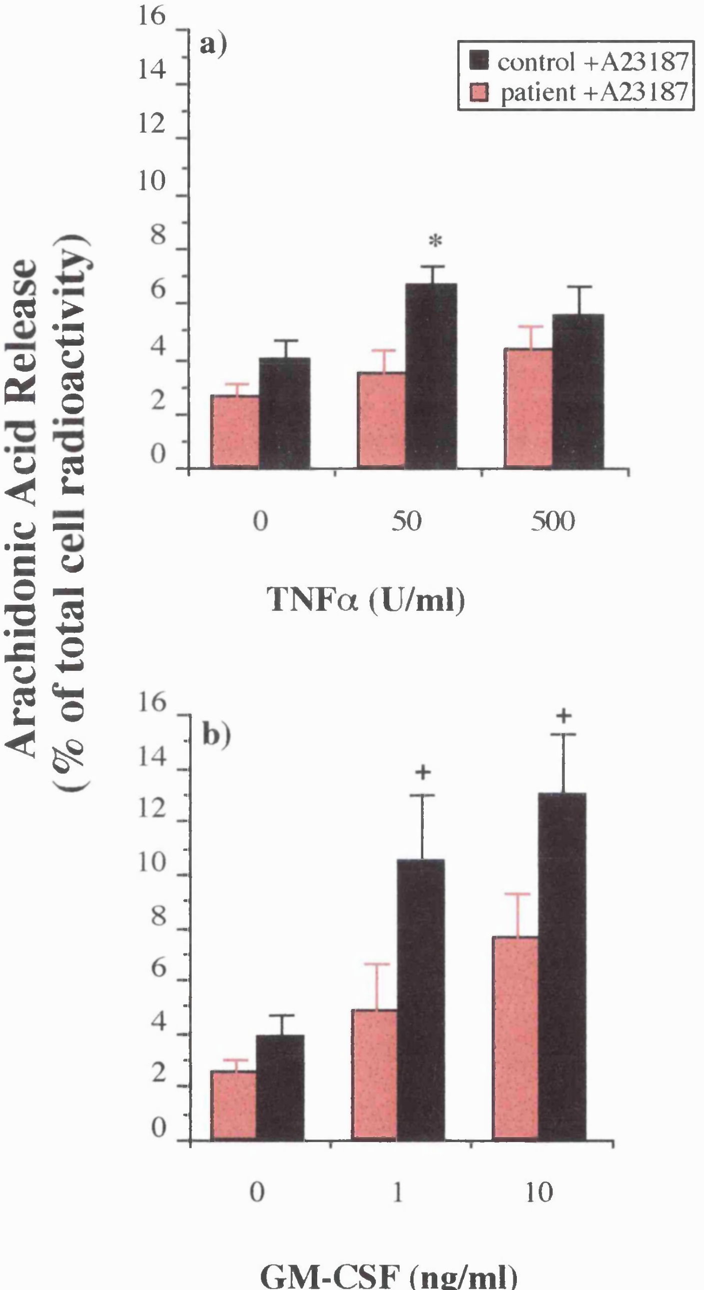



**Figure 3.9: Neutrophil phospholipase $A_2$ activity in response to priming with cytokines in patients with SCD.** The data shown are the mean ± 1 SE for 10 patients compared with controls tested on the same day. (a) and (b) are increments in arachidonate release above resting level when cells primed with 500 U/ml TNF-α or 10 ng/ml GM-CSF followed by stimulation with calcium ionophore (A23187). Significant differences between patients and controls (Wilcoxon's matched pairs signed-ranks test) are shown as: $^{+}P<0.01$, $^{*}P<0.05$.

## 3.4 DISCUSSION

Activation of neutrophils as evidenced by enhanced neutrophil aggregation was previously noted during episodes of vaso-occlusive crisis in SCD (Lachant and Oseas, 1987), but there is little published evidence of neutrophil activation during the steady state. If there is an ongoing interaction between damaged endothelium and activated neutrophils in the steady state, it is conceivable that this may contribute to the initiation of vaso-occlusive crises. Agents that decrease such interactions might therefore be of value to reduce the frequency of painful crises. This study shows that neutrophil phospholipase $A_2$ activity in steady state SCD is raised approximately two-fold compared to control, demonstrating that neutrophils are activated in steady state of SCD. The mechanism of this activation is unclear at the present. However, there was no evidence for parallel basal activation of NADPH oxidase activity of neutrophils in SCD when tested in the whole blood mileau. A recent study showed that the neutrophil NADPH oxidase could be activated by contact with sickled RBC *in vitro* (Hofstra *et al.*, 1996) and neutrophil phospholipase $A_2$ may also be activated in this way. Release of phospholipase $A_2$ from activated neutrophils might contribute to the threefold increase above control in the plasma levels of the secreted isoform of phospholipase $A_2$ that were recently detected in patients with steady state SCD (Styles *et al.*, 1996).

This study also demonstrates that neutrophils from patients in the steady state of SCD have reduced NADPH oxidase and phospholipase $A_2$ responses to the agonists FMLP and calcium ionophore after *in vitro* priming with the cytokines GM-CSF and TNF$\alpha$. The amounts of $H_2O_2$ and arachidonate produced were about 50% of that produced by control cells and in addition the defect in oxidase activity was more severe in patients in crises.

Priming of the NADPH oxidase by GM-CSF and endotoxin has been attributed to increased production of arachidonate (Roberts *et al.*, 1996; Forehand *et al.*, 1993) thus the inability of SCD neutrophils to fully upregulate NADPH oxidase activity could be a sequelae of the suboptimal activation of phospholipase $A_2$ by agonist after cytokine-mediated priming. Un-primed neutrophils from steady-state SCD patients produced equivalent amounts of $H_2O_2$ as control cells in response to stimulation with FMLP and the more potent stimulus, PMA (elicits maximal production of $H_2O_2$ in control cells), suggesting that the defect in priming was not explained by an intrinsic problem with the NADPH oxidase itself. This is in accord with previous studies demonstrating a normal respiratory burst in unprimed phagocytes from patients with SCD (Strauss *et al.*, 1976; Dias-Da-Motta *et al.*, 1996). However there was a small

but statistically significant defect of $H_2O_2$ production stimulated with PMA in patients with SCD during crisis which could possibly be to analgesic drugs administered.

Assays of phospholipase $A_2$ used washed and purified neutrophils unlike the NADPH oxidase assay, which was performed in whole blood, suggesting that the reduced reponses to cytokine-mediated priming were an inherent cellular defect. The raised resting levels of AA release in steady state of SCD could be associated with raised plasma levels of the secreted isoform of $PLA_2$ which may attack the membrane phospholipid of neutrophils. It could also reflect increased basal activity of the cytosolic $PLA_2$ isoform.

Spicule formation in sickled red cells with concomitant destabilisation of membrane organisation promotes adherence of RBC to endothelium resulting in stimulation of endothelial cells. These cells when activated can secrete a wide range of biological response modifiers which may contribute to the raised levels of GM-CSF, IL-1, IL-3, IL-6, and TNFα which have been detected in the peripheral blood of some SCD patients (Croizat, 1994; Kasschua *et al.*, 1996; Thomson and Dick, 1988; Francis and Haywood, 1992).This raises the possibility that the defective *in vitro* cytokine-mediated priming of neutrophils might be due to prior exposure to cytokines in the circulation. This would leave the cells refactory to further priming, either because of downregulation of cytokine receptors or post-receptor signal transduction mechanisms. Previous work has shown that neutrophils primed *in vivo* by infusion of GM-CSF are not responsive to subsequent *in vitro* priming with GM-CSF, and after a period of two hours have a priming capacity that is reduced to about 50% of pre-infusion values (Jaswon *et al.*, 1990; Khwaja *et al.*, 1992) confirming that this is a possible mechanism.

Patients with SCD have an increased susceptibility to infection especially with encapsulated bacteria which could be due to several abnormalities of host defence mechanisms. Phagocytic cells are capable of phagocytosis and destruction of invading microorganisms by a combination of oxygen-dependent and independent mechanisms. Evidence for involvement of oxygen and its metabolites in the microbicidal activity by neutrophils is provided by the observations that killing of certain microorganisms is impaired under anaerobic conditions (Klebanoff 1980). In addition, the absence of oxidative metabolic activity in Chronic Granulomatous Disease has been found to be associated with both recurrent infection and impaired killing by neutrophils *in vitro* (see Chapter 1 for details). Stimulation of the respiratory burst of phagocytes results in an increase production of superoxide anion ($O_2^-$) (reviewed by Klebanoff, 1992). There is some controversy as to whether $O_2^-$ by itself is directly toxic to ingested microbes or to host tissues. As Clark *et al.* (1987b) and Cox *et al.* (1987) suggested that $O_2^-$ does not have the necessary reactivity to be directly toxic to cells. However,

dismutation of $O_2^-$ results in rapid formation of hydrogen peroxide ($H_2O_2$) which is a powerful bactericidal agent and may be directly toxic to surrounding host tissues. It is scavenged by catalase, which breaks it down to oxygen and water, and by the glutathione cycle in which the degradation of $H_2O_2$ is coupled to the increased activity of the hexose monophosphte shunt. The toxicity of $H_2O_2$ can be increased several fold by a number of mechanisms including reaction with myeloperoxidase (MPO) (from azurophilic granules) and halide to form hypohalous acids (such as hypochlorous acid) and subsequently hydroxyl radicals. Hypochlorous acid (HOCl) reacts readily with free amines such as free amino acids, extracellular and intracellular proteins and macromolecular structures on bacteria yielding the chloramines. In the presence of MPO, $H_2O_2$ can act on amino acids to generate aldehyde thus damaging bacterial surfaces. Phagocytic cells may also form nitric oxide by the oxidation of L-arginine to L-citrulline by the action of nitric oxide-synthase (Marletta *et al.*, 1988). This cytosolic process may contribute, at least in mononuclear phagocytes, to tumour cell injury, fungistasis and antiparasitic activity (Green *et al.*, 1990). The nitric oxide (NO) system has the potential for interaction with the superoxide pathway by forming peroxynitrite ion ($ONOO^-$), which is itself a potent oxidant and decomposes when protonated to form a strong oxidant with hydroxyl radicals (McCall *et al.*, 1989). The NO system is particularly important in protection against mycobacteria, parasites and pathogenic fungi.

In addition the data demonstrated that neutrophils in the peripheral blood of SCD patients have a limited capacity to respond to priming with cytokines and this may contribute to the susceptibility of these patients to infection.

One consequence of sub-optimal oxygen radical production after priming would be the failure to kill micro-organisms that require high concentrations of $H_2O_2$ for their destruction. Such organisms are likely to be those that have developed protective mechanisms against the phagocyte respiratory burst, such as encapsulation, catalase production, or oxidase inhibition (Perry *et al.*, 1994). Thus the neutrophil defect could potentially exacerbate the infective risks with *Streptococcus pneumoniae* and *Haemophilus influenzae* that accompany abnormalities of opsonisation and the hyposplenic state in sickle cell syndromes (Pearson 1977).

Surprisingly, there was little evidence of recent or sustained *in vivo* priming of neutrophils as markedly elevated responses to agonist were not observed in either the NADPH oxidase or $PLA_2$ assays when cells were stimulated in the absence of *in vitro* cytokines. This might have been expected if cells had been recently primed *in vivo*, as demonstrated by previous studies in our laboratory (Jaswon *et al.*, 1990; Khwaja *et al.*, 1992) but there are two possible explanations why evidence of recent priming was not observed. Firstly, activation of neutrophils by interaction with damaged

endothelium may only be transient. For example, the primed state of the neutrophil NADPH oxidase can be short-lived when induced by platelet activating factor (1-2 hours) (Kitchen *et al.*, 1996), or by cytokines such as IL-8 (30 minutes) and TNFα (1 hour), whereas the response to GM-CSF can last for several hours (Roberts *et al.*, 1993a). It is possible that there had been *in vivo* priming with such a transient priming agent and the majority of cells were tested during a post-priming refractory period. Secondly, inflammatory events in SCD may produce a range of activated states in the neutrophil. Fully activated phagocytes are lost from the circulation, as evidenced by a reduction in levels of circulating neutrophils after *in vivo* infusions of cytokines (Devereux *et al.*, 1987) due to margination or migration into the tissues (Devereux *et al.*, 1989). In this study, neutrophils fully primed *in vivo* may similarly have left the circulating pool, leaving the less activated cells to be collected from the peripheral blood for *in vitro* assays. The discovery of defective priming in sickle cell disease is one of a very few examples. Another example of defective priming is the neutrophils from patients with human immunodeficiency virus (HIV), which have shown an impaired oxidative burst after cytokine priming (Elbim *et al.*, 1994).

In conclusion, these data show definitive evidence of neutrophil activation with raised phospholipase $A_2$ activity in the steady state of SCD which may contribute to the triggering of vaso-occlusive crises. This observation may provide a rationale for beneficial therapeutic intervention with 'anti-inflammatory drugs'.

Therefore, the following chapter investigates the possible mechanism(s) involved in defective priming of neutrophils from patients with SCD and explores the possibility of correction of the defect when neutrophils are reconstituted in normal plasma from healthy individuals.

# CHAPTER 4

# A STUDY OF THE MECHANISMS INVOLVED IN DEFECTIVE PRIMING OF NEUTROPHILS IN SICKLE CELL DISEASE

## 4.1 INTRODUCTION

As described in Chapter 3, the priming defect of neutrophil $H_2O_2$ production was not due to an inherent defect of NADPH oxidase as neutrophils from patients with sickle cell disease (SCD) responded to phorbol myristate acatate (PMA) (one of the most potent activators of NADPH oxidase) with the same magnitude as their same day control. In addition, there was also a defect of cytokine-mediated priming of phospholipase $A_2$ ($PLA_2$). These observations indicate a global priming defect of neutrophils in patients with SCD and prompted us to determine the possible underlying mechanisms involved.

In the study carried out in the previous Chapter (3), the majority of patients with SCD were of Afro-Caribbean origin whilst the "same-day" controls were generally Caucasian. Several studies have shown lower mean neutrophil counts and monocyte counts in black males than white males (Freedman *et al.*, 1997; Reed and Diehl, 1991). Neutropenia among blacks has also been observed in both women (Bain *et al.*, 1984) and children (Bao *et al.*, 1993). However, the data in Tables 3.3 and 3.4 comparing white cell counts between the patients and their respective Caucasian controls, showed no difference between the two populations. In addition, the analysis of $H_2O_2$ production by the DCF assay was independent of the absolute neutrophil count, as it was based on the mean cell fluorescence and percentage of positive cells that were counted, therefore any neutropenia due to racial origin should not have influenced the results obtained. However, there may be subtle differences in the neutrophil function in people of different racial origins. Therefore it was important to investigate the priming of $H_2O_2$ production in neutrophils from a group of non-SCD, healthy Afro-Caribbean individuals in comparison with a Caucasian group.

Major irreversible organ failure is one of the characteristics associated with SCD that causes loss of splenic function, chronic lung disease (Powars *et al.*, 1988), leg ulcers (Koshy *et al.*, 1989), retinopathy with loss of sight (Talbot *et al.*, 1983) and end stage renal failure (Allon, 1988). The spleen is the first organ to show functional impairment (hypertrophy) which is apparent as early as 6 months (Pearson *et al.*, 1979) and characterised by splenomegaly. The spleen is impaired in its ability to remove damaged red blood cells (RBC), pitted (vacuoles containing cellular debris) erythrocytes and Howell-Jolly bodies (DNA remnant) from red blood cells (Casper *et al.*, 1976). The spleen has a dual function: it is a lymphoid organ capable of producing antibody but phagocytes in the spleen are capable of phagocytosing and culling scenescent erythrocytes. In patients with SCD the splenic function as a bacterial filter especially *Streptococcus pneumoniae* and *Haemophilus influenzae* is impaired. Previous studies have shown that patients with SCD have a defect in opsonisation of

bacteria which could be due to abnormalities of alternative complement pathway although some studies suggest increased activity (Wilson *et al.*, 1976; Mold *et al.*, 1995) some stated defective function of the alternative complement pathway (Johnston *et al.*, 1973) yet others found no evidence for altered complement and immunoglobulin levels in patients with SCD (Mohamed *et al.*, 1992). Asplenic patients have a life-long susceptibility to infection, although the liver plays an important role in removing well opsonised bacteria but the spleen has a crucial role in removal of bacteria which are not opsonised. In addition the spleen also produces coagulation factor VIII and tuftsin, a tetrapeptide (Thr-Lys-Pro-Arg) derived from the heavy chain of IgG molecule (residues 289-292). Tuftsin stimulates oxidative activity of PMN. It has been reported that the spleen performs a vital role in the fight against malignant tumors and bacterial infection (Liu *et al.*, 1995) by stimulation of oxidative activity of macrophages (Singh *et al.*, 1992). Splenectomy which is sometimes carried out incidentally, because of trauma or in patients with Hodgkin's disease, also leads to an immundeficiency state in which patients are susceptible to bacterial infection. It was important to determine whether the defective priming of neutrophils in patients with SCD was related to splenic dysfunction, and so a group of non-SCD splenectomised patients were studied.

SCD is also associated with anaemia, the degree of which varies between individuals. Indeed, it has been shown that high total haemoglobin levels in SCD are a risk factor for painful crisis (Platt *et al.*, 1991) and acute chest syndrome (Castro *et al.*, 1994). Several factors such as increased rigidity of the cell membrane and instability of HbS which causes the binding of large amount of immunoglobin G (IgG) to the membrane cause their recognition and destruction by macrophages. This contributes to the diminished life span of sickle erythrocytes from 120 days to 15-20 days. It has been shown that complement could mediate intravascular haemolysis of SCD (Test and Woolworth, 1994). Loegering, (1996) has demonstrated that phagocytosis of IgG-coated erythrocytes could depress neutrophil functions such as the respiratory burst, phagocytosis and bacterial killing possibly due to the reaction of haemoglobin iron with reactive oxygen metabolites which cause an oxidant damage to the phagocyte.

It was important to study the effect of anaemia on the priming responses of neutrophils and to compare this with other forms of haemolytic anaemias such as $\beta$-thalassaemia intermedia. This is a genetic disorder characterised by reduced rate of production of $\beta$ globin chains, hence imbalanced $\alpha/\beta$ globin chains which is manifested by ineffective erythropoiesis and an increase rate of intravascular haemolysis. Therefore, a group of patients with anaemia were selected whose anaemia was not due to blood loss following surgery. This is because the neutrophils may have

already been activated due to cytokine release, infection or trauma due to recent surgery.

New concepts of sickle cell disease suggests involvement of a constant low grade inflammation during symptom free steady state which is significantly increased during painful crises which may be caused by abnormal adhesion of sickled red blood cells to endothelium due to expression of various adhesion molecules (Natarajan *et al.*, 1996). The raised resting levels of arachidonate release from the neutrophils of patients with SCD which was described in the previous Chapter (3) could support this hypothesis. To test whether the observation of defective priming in SCD merely reflected a state of generalised ongoing inflammation, patients with acute seropositive erosive rheumatoid arthritis were studied who met the revised criteria of the American College of Rheumatology (Arnett *et al.*, 1988). RA is a common systemic, autoimmune, inflammatory disorder which is mainly affects the joints which are heavily infiltrated with neutrophils. There is controversial evidence on both priming of neutrophil function in RA (Eggleton *et al.*, 1995; Miesel *et al.*, 1996; Kowanko *et al.*, 1996) and the effect of drugs such as methotrexate, sulphasalazine and aurothiomalate on polymorphonuclear function in RA (Laurindo *et al.*, 1995; Storgaard *et al.*, 1996) therefore the peripheral blood neutrophils from a group of patients was tested for their priming responses.

To determine the effect of plasma on neutrophils from patients with SCD, experiments were set up to test whether defective priming of neutrophils was reversible when cells were reconstituted in plasma from healthy controls. Finally it is possible that some factor (s) in the plasma of patients with SCD was responsible for inhibiting cytokine responses, and that if removed and replaced with plasma from a healthy control the defect could be reversed. Therefore experiments were performed to determine whether this was the case.

## 4.2 MATERIALS AND METHODS

Materials

See Chapter 3, Section 3.2 for stock materials and cytokines being used.

### 4.2.1 Patients and controls studied

The following patients from University College London and the North Middlesex Hospital were studied:

*i) SCD patients*

(a) 4 patients with sickle cell anaemia (HbSS) in steady state between vaso-occlusive crisis (age 24-34, 2 males ) were tested. All of these patients were asymptomatic at the time of study and none were receiving repeated blood transfusion, opiate analgesics or hydroxyurea; (b) 4 hospitalised HbSS patients during vaso-occlusive crisis treated by prophylactic antibiotics and analgesia (age 24-53 years, 2 males) .

*ii) non-SCD patients*

(a) 3 splenectomised patients in long term remission from Hodgkin's disease (age 35-50 years, 2 males), (b) 3 patients with β-thalassaemia intermedia (age 25-38 years, 1 male) treated by prophylactic antibiotics, folic acid and none were receiving blood transfusion, (c) 6 patients with rheumatoid arthritis, (d) 6 patients with iron deficiency anaemia (details for c and d are given in Table 4.1).

*iii) healthy controls*

(a) 6 healthy individuals of Afro-Caribbean origin (age 25-36 years, 2 males), (b) 24 healthy laboratory personnel (age 24-62 years, 10 males, tested on 32 occasions) one of whom was tested each time that blood from the above categories was tested (same day controls). In all cases peripheral blood was obtained with the informed consent of the donor.

Methods

### 4.2.2 $H_2O_2$ Production Assay

Intracellular $H_2O_2$ production in whole blood was measured using the DCFH-DA assay, as detailed in Chapter 3, Section 3.2.

## Table 4.1: Patient details

### a) Rheumatoid Arthritis

| *Patients* | *Sex* | *Age* | *Hb (g/L)* | *ESR (mm/hr)* | *Medication* |
|---|---|---|---|---|---|
| 1 | F | 41 | 121 | 45 | Methotrexate |
| 2 | F | 62 | 147 | 2 | Intramuscular Gold |
| 3 | F | 54 | 141 | 5 | Hydroxychloroquine |
| 4 | F | 42 | 129 | 38 | Meloxicam |
| 5 | F | 22 | 87 | 112 | Salazopyrine |
| 6 | F | 70 | 148 | 12 | Salazopyrine |
| **Mean±SE** | - | 48 ± 7 | 129 ± 9 | 36 ± 17 | - |
| Normal range | - | - | 115 - 155 | <40yrs=1-7<br>>40yrs=1-20 | - |

### b) Iron-deficiency anaemia

| *Patients* | *Sex* | *Age* | *Hb (g/L)* | *Medication* | *Cause of anaemia* |
|---|---|---|---|---|---|
| 1 | F | 60 | 73 | - | - |
| 2 | F | 35 | 71 | Oral Iron | Pregnant (20 weeks) |
| 3 | F | 32 | 87 | - | Gastric Ulcer |
| 4 | F | 27 | 57 | - | Menorrhaegia |
| 5 | F | 31 | 73 | - | Menorrhaegia |
| 6 | F | 48 | 93 | Iron | Dietary |
| **Mean±SE** | - | 39 ± 5 | 76 ± 5 | - | - |
| Normal range | - | - | 115 - 155 | - | - |

F(Female), ESR(Erythrocyte Sedimentation Rate), Hb(Haemoglobin)

### 4.2.3 Cross-over study of blood cells and plasma

*4.2.3.1) plasma from patients with SCD during crisis*

Peripheral blood (10 ml) from SCD patients during painful crises or healthy controls was taken into 10 IU/ml of preservative-free heparin and plasma was isolated from the blood cells by centifugation at 180 x g for 7 minutes at room temperature. Plasma was carefully collected into a fresh tube and further centrifuged at 400 x g for 7 minutes to remove the platelets and finally diluted two fold in PBS supplemented with 5 mM glucose (PBSG). The cell pellet was washed twice by resuspension in PBSG (without calcium or magnesium). The washed cell pellet from the patient was resuspended to the original volume with either control plasma or autologus plasma. Similarly, the cell pellet from the control was resuspended in patients plasma or their own plasma.

*4.2.3.2) plasma from patients with SCD in steady state*

The above procedure (Section 4.2.3.1) was carried out using 10 ml heparinised blood from SCD patients in steady state and their same day controls. Separated plasma was centrifuged at 400 x g for 7 minutes. Original volume of blood was restored by resuspension of the cell pellet in either control plasma or autologus plasma. $H_2O_2$ production in neutrophils was measured from each sample with the DCFH-DA assay as described in Chapter 3, Section 3.2.

### 4.2.4 Statistical analysis

Unless otherwise stated the data are the mean ± 1SE of the number of experiments given in the text. Statistical analysis of the data was performed by Wilcoxon's matched-pairs signed-ranks test (Siegel, 1956).

## 4.3 RESULTS

### 4.3.1 Resting level of $H_2O_2$ prodction

Resting levels of neutrophils $H_2O_2$ from all 5 different groups studied showed no significant differences compared to their same day control as shown by the mean cell fluorescence (MCF) values of the ungated population (Table 4.2).

### 4.3.2 NADPH oxidase activity in non-SCD patients and healthy control groups

*i) Asplenic patients*

Adult patients with SCD have poor or absent splenic function. The effect of incubation of whole blood with either 1 μM FMLP or 20 μM PMA were the same magnitude as their same day controls as shown in Table 4.3. To investigate whether asplenic patients had a defect in priming (as shown in Chapter 3), neutrophil $H_2O_2$ production and cytokine priming was measured in 3 patients in long-term remission from Hodgkin's disease who had been splenectomised many years previously. No defect in cytokine-mediated priming was observed compared to non-splenectomised controls tested on the same day (Figure 4.1).

*ii) Afro-Caribbean controls*

All but two of the SCD patients studied were of African or Afro-Caribbean descent, whereas the same day controls were non-Afro-Caribbeans. To examine whether any racial factor may have contributed to the results of the priming experiments, blood from six healthy non-SCD Afro-Caribbean was studied in parallel with samples from healthy non-Afro-Caribbeans. Neutrophil NADPH oxidase response to either FMLP or PMA showed no significant difference from their respective same day control (Table 4.4). There was also no significant differences between the values for these two groups in their priming response to either GM-CSF or TNFα as shown in Figure 4.2.

**Table 4.2: Total MCF values**

| *Subjects* | *Total MCF* | *n* |
|---|---|---|
| **Asplenia** | 35 ± 6 | 3 |
| Control | 44 ± 9 | |
| **Afro-Caribbean** | 56 ± 7 | 6 |
| Control | 54 ± 6 | |
| **RA‡** | 37 ± 3 | 6 |
| Control | 40 ± 3 | |
| **Anaemia** | 36 ± 2 | 6 |
| Control | 38 ± 2 | |
| **β-Thalassaemia** | 37 ± 1 | 3 |
| Control | 45 ± 1 | |

Data shown are the total mean cell fluorescence values of the ungated neutrophil population in different groups studied. Data are the mean ± 1SE of the number of experiments (n). ‡RA = Rheumatoid Arthritis, $P>0.05$ in all cases.

**Table 4.3: NADPH oxidase activity in unprimed neutrophils from asplenic patients**

| | ***Total $H_2O_2$ Production*** [(% positive) x (MCF)] | | |
|---|---|---|---|
| **Subjects** | **PBS** | **FMLP** | **PMA** |
| **Asplenia** | 2685 ± 1854 | 3695 ± 141 | 89452 ± 4726 |
| Control | 2818 ± 1745 | 2690 ± 189 | 90362 ± 4328 |
| **(n)** | (3) | (3) | (3) |

Data shown are the total cellular $H_2O_2$ production in arbitrary fluorescence units and are the mean ± 1 SE of the number of experiments shown in parentheses. $P>0.05$ versus same day control.

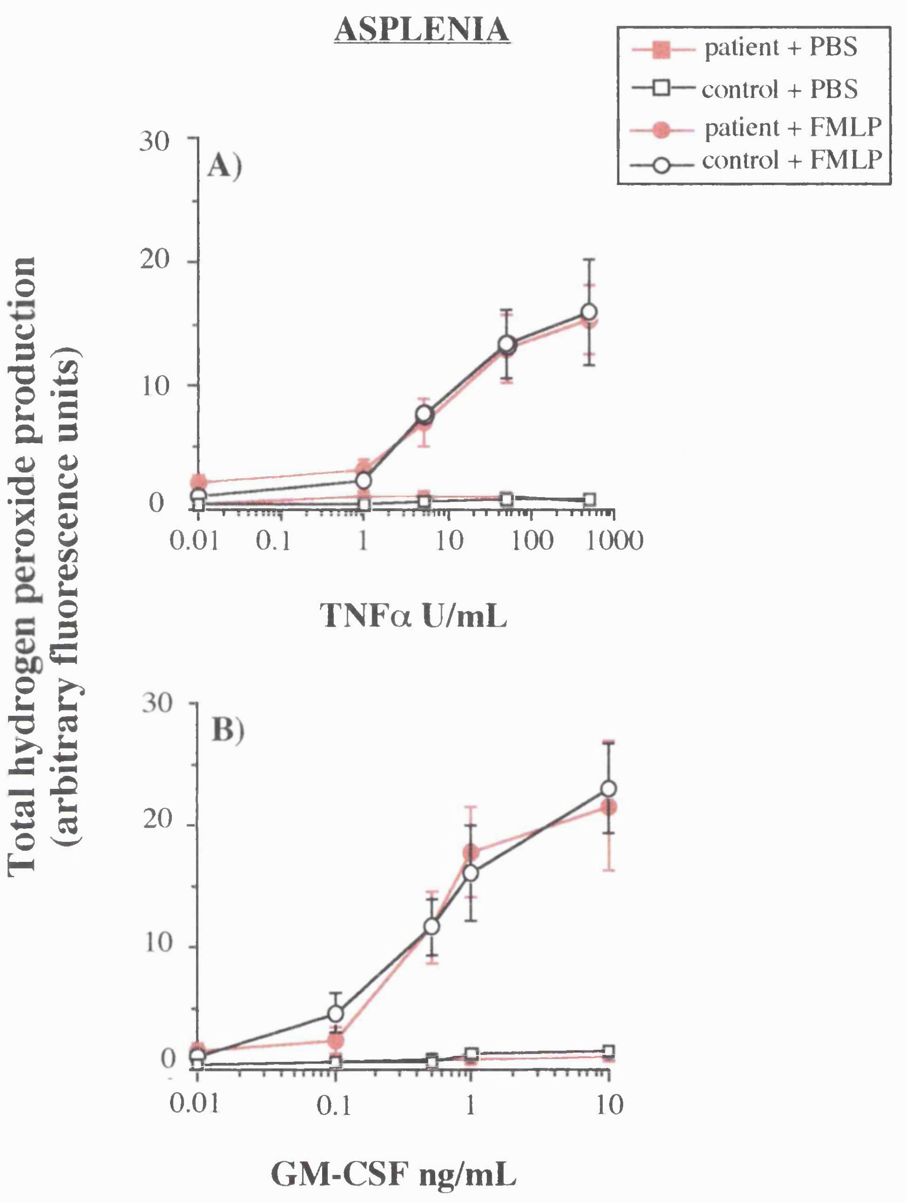



**Figure 4.1: Total $H_2O_2$ production in splenectomised patients.** Cytokine mediated priming of neutrophil NADPH oxidase activity in splenectomised patients compared with their same day controls.. Total $H_2O_2$ production was calculated as the product of the percent of positive cells and their standardised MCF. The data shown are the mean ± 1 SE of 3 experiments. No significant difference was observed between the two groups.

**Table 4.4: NADPH oxidase activity in unprimed neutrophils from healthy Afro-Caribbean controls**

| | *Total $H_2O_2$ Production* [(% positive) x (MCF)] | | |
|---|---|---|---|
| **Subjects** | **PBS** | **FMLP** | **PMA** |
| **Afro-Caribbean** | 1303 ± 178 | 3803 ± 562 | 76169 ± 2698 |
| Control | 1114 ± 119 | 2262 ± 200 | 80939 ± 1939 |
| **(n)** | (6) | (6) | (6) |

Data shown are the total cellular $H_2O_2$ production in arbitrary fluorescence units and are the mean ± 1 SE of the number of experiments shown in parentheses. *P*>0.05 versus same day control.

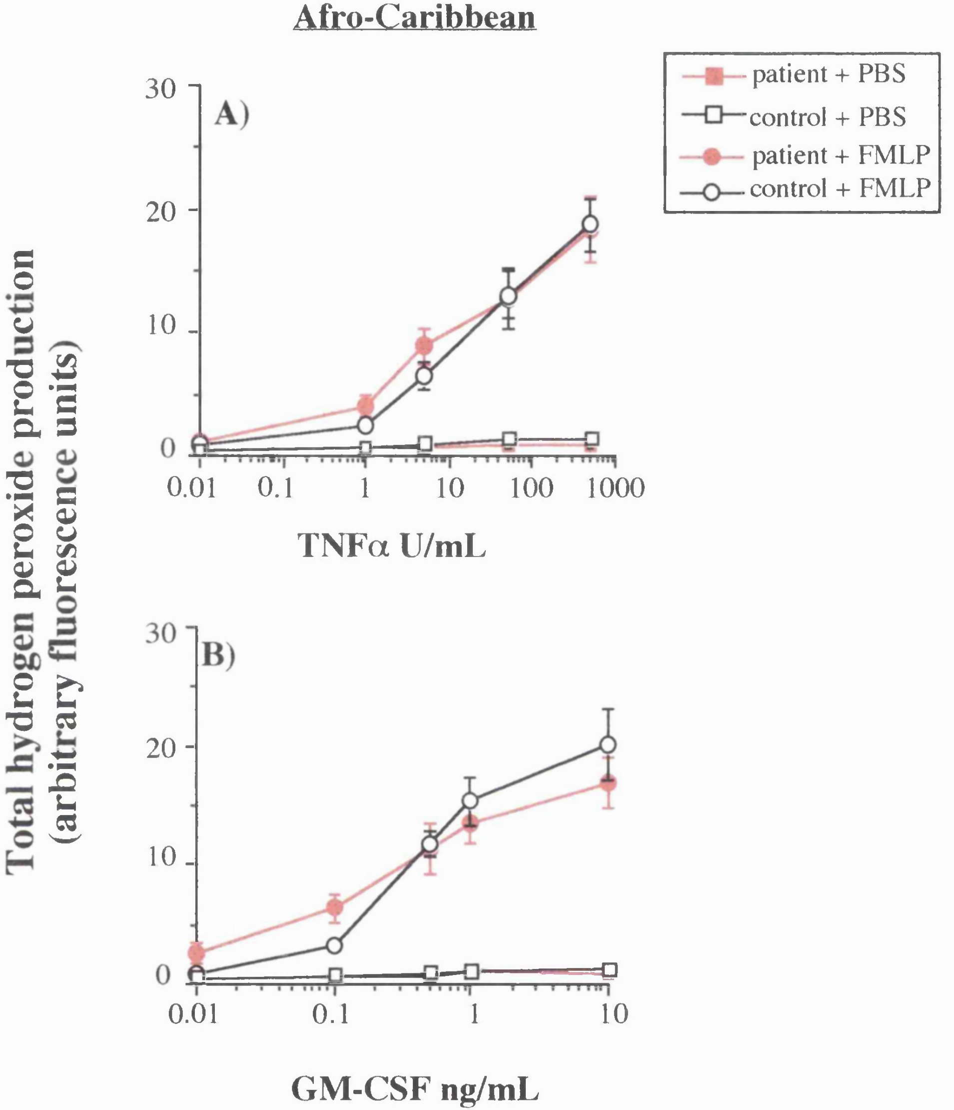



**Figure 4.2: Total $H_2O_2$ production in Afro-Caribbean individuals.** Cytokine mediated priming of neutrophil NADPH oxidase activity in a group of Afro-Caribbean individuals compared with non-Afro-Caribbean controls tested on the same day. Total $H_2O_2$ production was calculated as the product of the percent of positive cells and their standardised MCF. The data shown are the mean ± 1 SE of 6 experiments. No significant difference was observed between the two groups.

*iii) Patients with arthritis*

6 patients with seropositive rheumatoid arthritis, classified according to the revised criteria of the American Rheumatism Association (Arnett *et al.*, 1988), were studied to determine whether the defect in cytokine-mediated priming could be observed in patients with ongoing inflammation. They were receiving a variety of medications (see Table 4.1a for patient details). The unprimed neutrophil responses to either chemotactic peptide FMLP or PMA was not significantly different from controls as shown in Table 4.5.
There was also no significant difference in neutrophil $H_2O_2$ production after priming with either TNF$\alpha$ (Figure 4.3 A) or GM-CSF (Figure 4.3 B), between the patient and control groups. The total $H_2O_2$ production for patients with arthritis expressed as percentage of the value for the same day controls and the data are shown in Table 4.6.

*iv) Patients with iron-deficiency anaemia*

To investigate whether the priming defect was associated with the anaemia of sickle cell disease, 6 females with iron deficiency anaemia were studied. Iron deficient patients were selected who were not infected, or recovering from surgery, or suffering from any form of malignancy, as this might affect their neutrophil responses. The mean haemoglobin values for these patients was 76 ± 5 g/L (mean ± 1SE) compared with 85 ± 6 g/L (n=10) for patients with steady-state SCD (Chapter 3, Table 3.3), and 84 ± 7 g/L (n=6) for SCD patients in crisis (Chapter 3, Table 3.4). There was no significant difference in either FMLP or PMA stimulated neutrophil $H_2O_2$ production between patients with iron deficiency anaemia and their sex-matched controls as shown in Table 4.7. There was no significant differences in neutrophil responses with prior priming by TNF$\alpha$ or GM-CSF as shown in Figure 4.4 (in all analyses, $P > 0.05$, n=6). The total $H_2O_2$ production for anaemic patients expressed as percentage of the value for same day controls and the data are shown in Table 4.8.

*v) Patients with β-Thalassaemia intermedia*

To investigate whether the priming defect associated with neutrophils from patients with SCD might be due to the haemolytic condition of the disease, 3 patients with β-Thalassaemia intermedia (β-Thal), another condition associated with chronic haemolysis, were selected who were not receiving blood transfusion or iron-chelation therapy but receiving folic acid, and who had haemoglobin values between 80 and 88 g/L associated with significant haemolysis. As shown in Figure 4.5 less $H_2O_2$ was

**Table 4.5: NADPH oxidase activity in unprimed neutrophils from patients with rheumatoid arthritis**

| Subjects | *Total $H_2O_2$ Production* [(% positive) x (MCF)] PBS | FMLP | PMA |
|---|---|---|---|
| **RA‡** | 1810 ± 292 | 3942 ± 2006 | 77544 ± 1528 |
| Control | 1980 ± 283 | 3454 ± 896 | 80066 ± 1778 |
| **(n)** | (6) | (6) | (6) |

Data shown are the total cellular $H_2O_2$ production in arbitrary fluorescence units and are the mean ± 1 SE of the number of experiments shown in parentheses. $P>0.05$ versus same day control. (‡RA = Rheumatoid Arthritis).

## Rheumatoid Arthritis

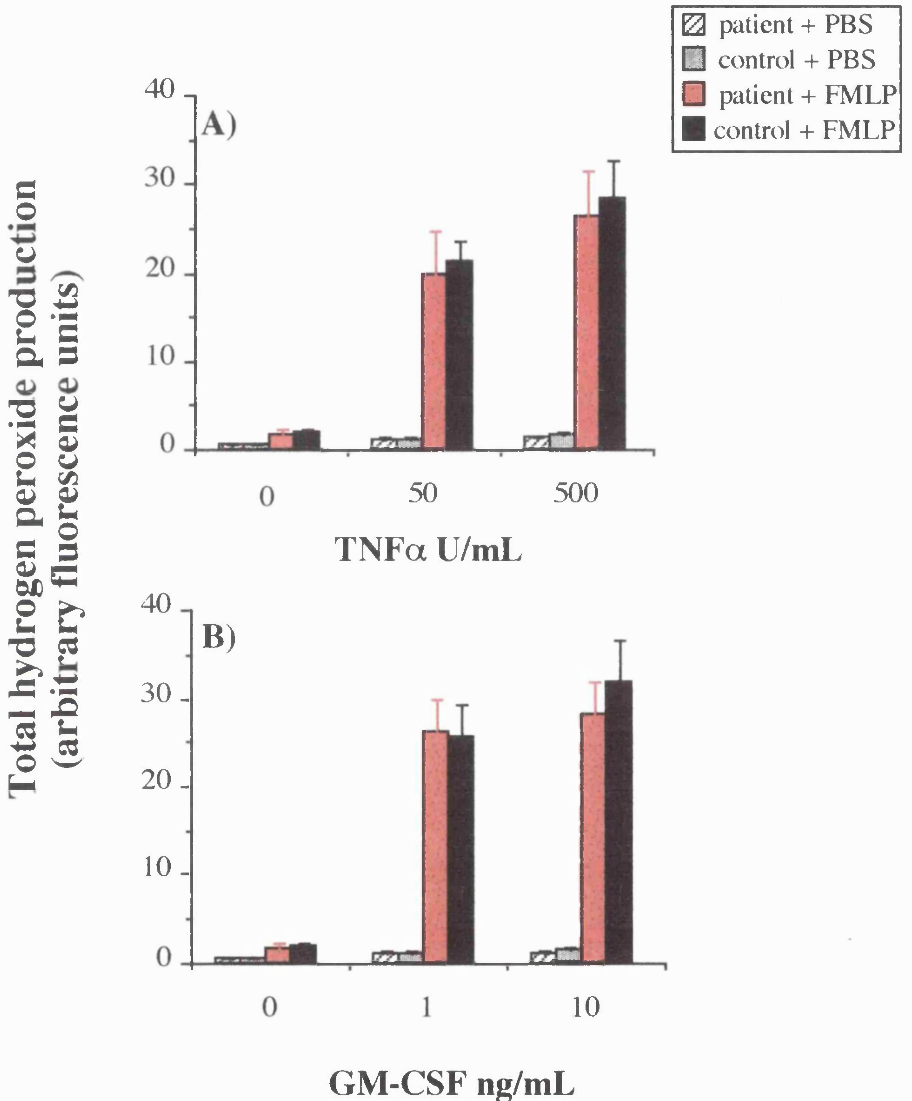



**Figure 4.3: Total $H_2O_2$ production in patients with RA.** Cytokine mediated priming of neutrophil NADPH oxidase activity in patients with rheumatoid arthritis compared with their same day controls. Total $H_2O_2$ production was calculated as the product of the percent of positive cells and their standardised MCF. The data shown are the mean ± 1 SE of 6 experiments. No significant differences was observed between the two groups.

**Table 4.6: Total $H_2O_2$ production for the patients with rheumatoid arthritis expressed as a percentage of values for the same day controls**

| *$H_2O_2$ production* | | | |
|---|---|---|---|
| *TNFα* (U/ml) | *% of control* | *GM-CSF* (ng/ml) | *% of control* |
| **0** | 98 ± 18 | **0** | 98 ± 18 |
| **50** | 98 ± 23 | **1** | 107 ± 14 |
| **500** | 100 ± 20 | **10** | 95 ± 5 |

Data shown are the mean ± 1SE of 6 experiments. The effect of TNFα and GM-CSF priming of FMLP stimulated $H_2O_2$ production for patients was expressed as a percentage of same day controls. $P>0.05$ in all cases

**Table 4.7: NADPH oxidase activity in unprimed neutrophils from patients with iron-deficiency anaemia**

| Subjects | *Total $H_2O_2$ Production* [(% positive) x (MCF)] PBS | FMLP | PMA |
|---|---|---|---|
| **Anaemia** | 1708 ± 185 | 3318 ± 804 | 77579 ± 1673 |
| Control | 1563 ± 118 | 1930 ± 334 | 81885 ± 1719 |
| **(n)** | (6) | (6) | (6) |

Data shown are the total cellular $H_2O_2$ production in arbitrary fluorescence units and are the mean ± 1 SE of the number of experiments shown in parentheses. $P>0.05$ versus same day control.

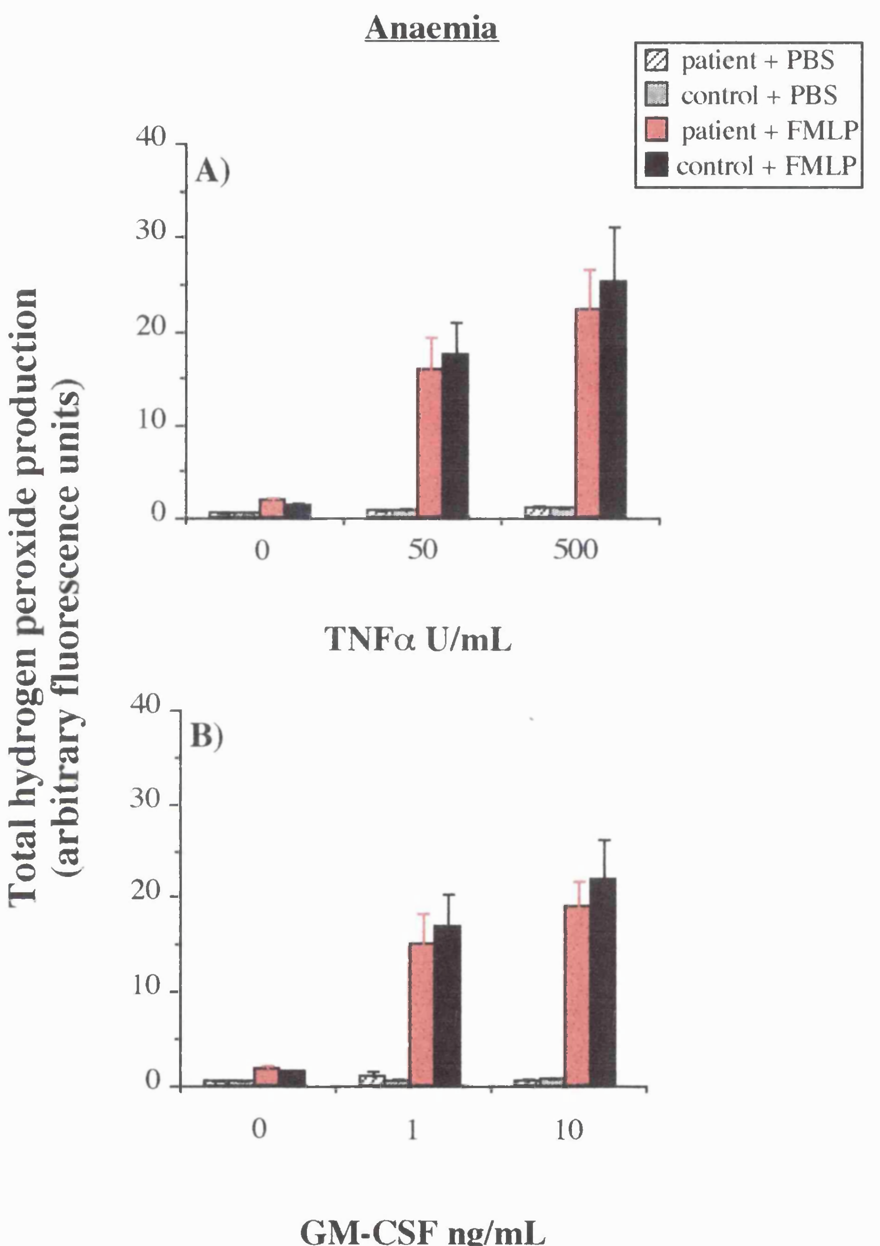



**Figure 4.4: Total $H_2O_2$ production in patients with anaemia.** Cytokine mediated priming of neutrophil NADPH oxidase activity in patients with iron-deficiency anaemia compared with their same day controls. Total $H_2O_2$ production was calculated as the product of the percent of positive cells and their standardised MCF. The data shown are the mean ± 1 SE of 6 experiments. No significant difference was observed between the two groups.

**Table 4.8: Total $H_2O_2$ production for the patients with iron-deficiency anaemia expressed as a percentage of values for the same day controls**

| *$H_2O_2$ production* | | | |
|---|---|---|---|
| *TNFα* (U/ml) | *% of control* | *GM-CSF* (ng/ml) | *% of control* |
| **0** | 138 ± 17 | **0** | 138 ± 17 |
| **50** | 91 ± 9 | **1** | 100 ± 30 |
| **500** | 94 ± 13 | **10** | 95 ± 14 |

Data shown are the mean ± 1SE of 6 experiments. The effect of TNFα and GM-CSF priming of FMLP stimulated $H_2O_2$ production for patients was expressed as a percentage of same day controls. $P>0.05$ in all cases

## β-Thalassaemia intermedia

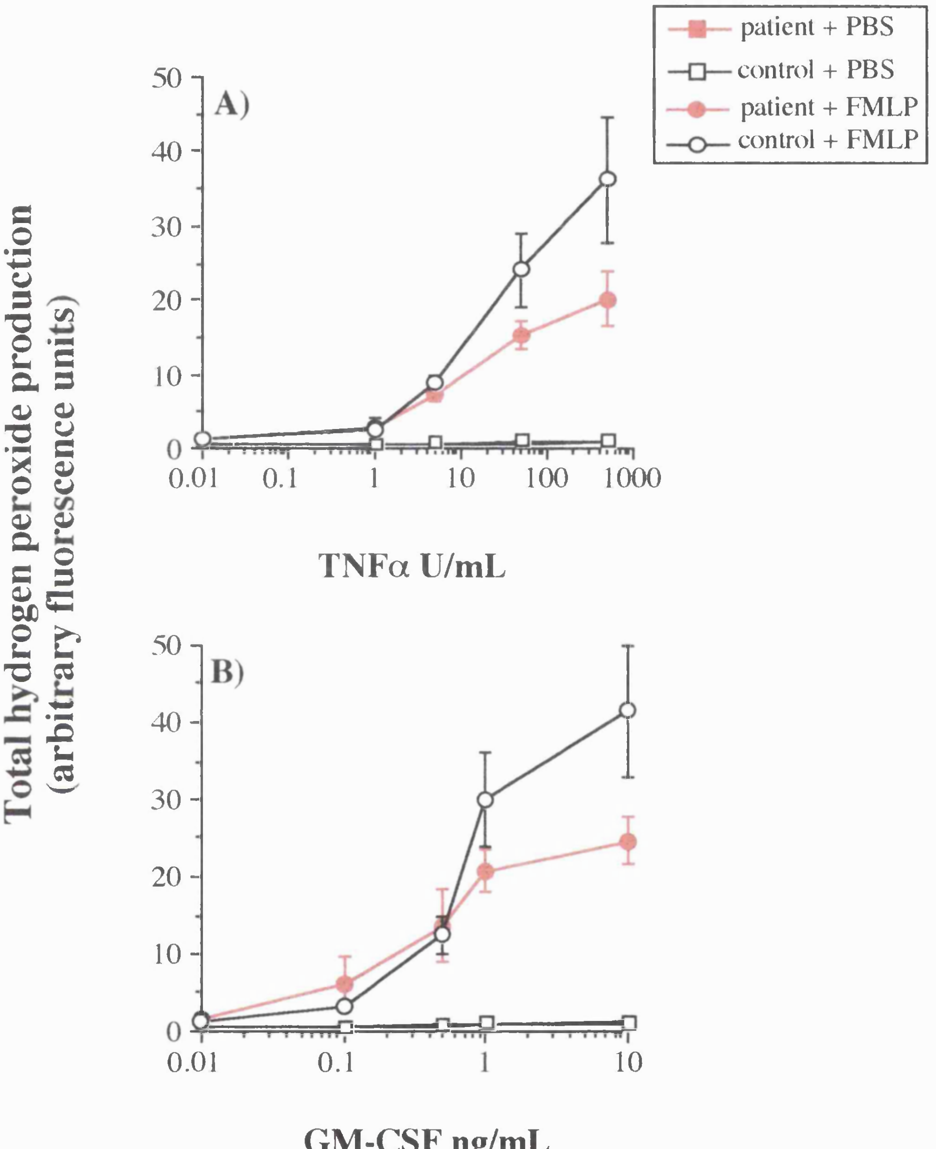



**Figure 4.5: Total $H_2O_2$ production in patients with β-thalassaemia intermedia.** Cytokine mediated priming of neutrophil NADPH oxidase activity in a group of patients with β-thalassaemia intermedia compared with controls tested on the same day. Total $H_2O_2$ production was calculated as the product of the percent of positive cells and their standardised MCF. The data shown are the mean ± 1 SE of 3 experiments. No significant difference was observed between the two groups.

produced in neutrophils from patients with β-Thal when primed with cytokines, than control cells tested on the same day.

The effect of FMLP or PMA on NADPH oxidase activity in unprimed neutrophils were investigated. As shown in Table 4.9 the PMA responses of neutrophils from patients with β-thalassaemia were on average only 89% of their same-day controls. This small difference was statistically significant ($P<0.01$, n=3).

The total $H_2O_2$ production for patients with β-Thal expressed as percentage of the value for same day controls shows at maximal concentration of cytokines $H_2O_2$ production was approximately 60% of control as shown in Table 4.10. However, there was no statistically significant difference in FMLP-stimulated neutrophil $H_2O_2$ production between patients with β-Thal and their same day controls either with prior priming by TNFα or GM-CSF (in all analyses, $P>0.05$, n=3).

### 4.3.4 Effect of plasma on priming

*i) SCD patients during painful crisis*

In order to determine whether the priming defect derived from the neutrophils or plasma, further experiments were performed. Neutrophils from sickle cell patients were washed free of plasma as described in the Materials and Method (Section 4.2), and the effect of adding back plasma from healthy controls or the patients was tested. If the defect lay entirely with the plasma then the cellular defect might be expected to be reduced when cells were put into plasma from healthy controls. In these experiments blood cells were reconstituted in plasma diluted 1:1 in PBSG (5 mM). If the inhibitor was solely in the plasma therefore the defect in the patients cells should be corrected by the addition of normal plasma.

Firstly, neutrophils from healthy individuals were incubated with DCFH-DA for 30 minutes at 37°C prior to priming with cytokines for further 30 minutes and cells were stimulated with FMLP. Initial control experiments showed that the function of neutrophils in reconstituted blood was not different from untreated blood (Figure 4.6), indicating that the washing procedure was not deleterious to neutrophil function.

Secondly blood from patients during crises were tested. The function of the washed cells was not restored by re-addition of 50% normal plasma (see Figure 4.7). In addition, there was a small reduction in function when neutrophils from healthy individuals were reconstituted with plasma from patients during crises.

The maintenance of defective priming despite of reconstitution with normal plasma in crises might be due to the large amounts of analgesia that these patients were receiving, therefore replicate experiments were performed in patients with steady state

**Table 4.9: NADPH oxidase activity in unprimed neutrophils from patients with β-Thalassaemia intermedia**

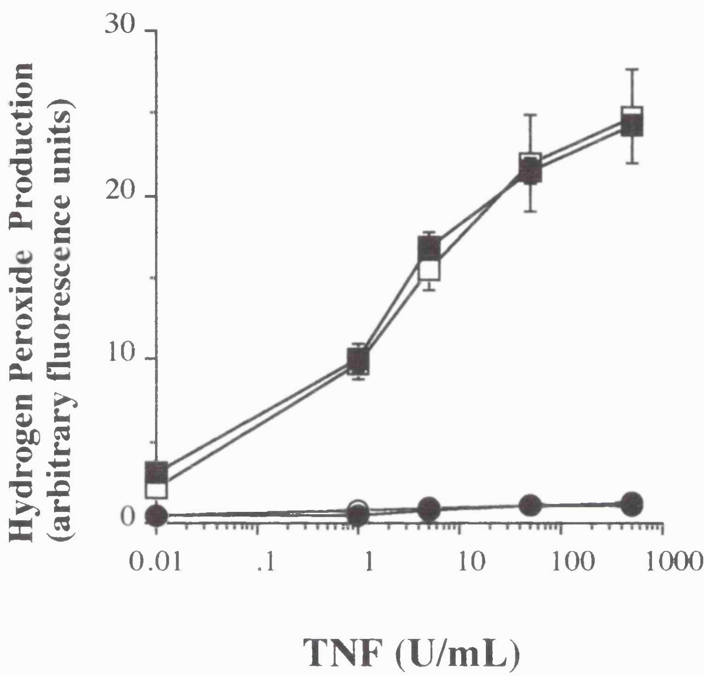
| Subjects | *Total $H_2O_2$ Production* [(% positive) x (MCF)] PBS | FMLP | PMA |
|---|---|---|---|
| **β-Thalassaemia** | 1584 ± 2 | 2466 ± 313 | *75820 ± 904 |
| Control | 1677 ± 5 | 1988 ± 52 | 85113 ± 626 |
| **(n)** | (3) | (3) | (3) |

Data shown are the total cellular $H_2O_2$ production in arbitrary fluorescence units and are the mean ± 1 SE of the number of experiments shown in parentheses. **P*<0.01 versus same day control.

**Table 4.10: Total $H_2O_2$ production for the patients with β-Thalassaemia intermedia expressed as percentage of values for the same day control**

| *$H_2O_2$ production* | | | |
|---|---|---|---|
| *TNFα* (U/ml) | *% of control* | *GM-CSF* (ng/ml) | *% of control* |
| **0** | 108 ± 20 | **0** | 118 ± 25 |
| **1** | 108 ± 3 | **0.1** | 165 ± 79 |
| **5** | 82 ± 9 | **0.5** | 105 ± 18 |
| **50** | 67 ± 9 | **1** | 72 ± 9 |
| **500** | 59 ± 7 | **10** | 62 ± 6 |

Data shown are the mean ± 1 SE of 3 experiments. The effect of TNFα and GM-CSF priming of FMLP stimulated $H_2O_2$ production for patients was expressed as a percentage of same day controls. $P>0.05$ in all cases



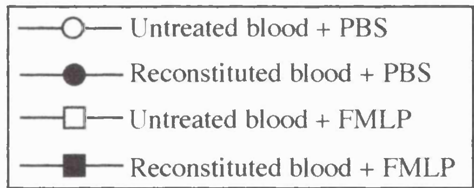




**Figure 4.6: Effect of washing process by centrifugation on $H_2O_2$ production in neutrophils from healty individuals.** The data shown are the mean ± range of 2 experiments, where blood cells were either used as untreated or washed free of plasma and then reconstituted in autologous plasma. $p>0.05$

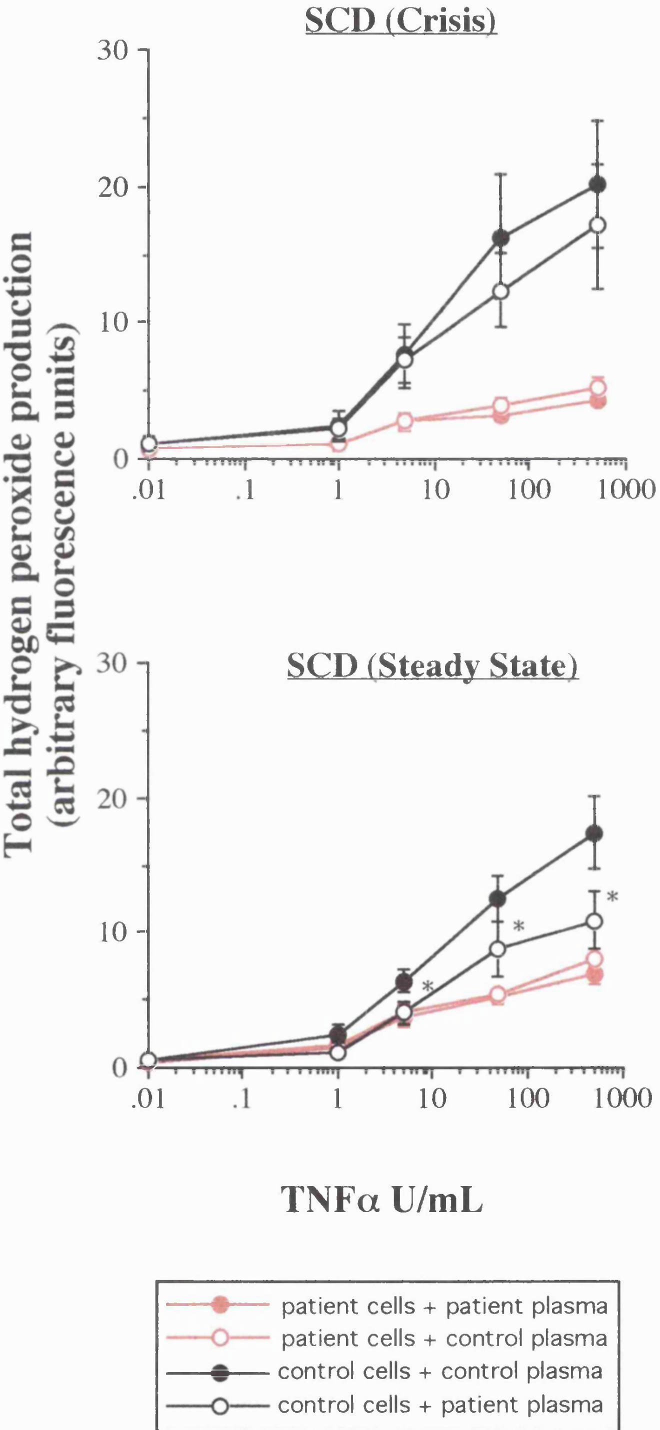



**Figure 4.7: Effect of plasma on $H_2O_2$ production in neutrophils from SCD patients.** Total $H_2O_2$ production in primed neutrophils stimulated with FMLP in patients with SCD was calculated as the product of the percent of positive cells and their standardised MCF. The data shown are the mean ± 1 SE of 4 experiments. *$P$<0.02

of SCD. This might also account for the decrease in function of neutrophils from healthy individuals with the diluted plasma.

*ii) SCD patients in steady state*

The total mean cell fluorescence (MCF) of the ungated population is shown in Table 4.11, which indicates no significant difference between the groups ($P>0.05$ in all cases). As shown in Figure 4.7 addition of undiluted patient plasma to the cells from the same day control caused a significant reduction in $H_2O_2$ production when samples were primed with TNFα. The total $H_2O_2$ production expressed as a percentage of same day controls was 68% ± 7% and 62% ± 5% ($P<0.02$, n=4) for samples primed with 50 and 500 U/ml TNF, respectively. Addition of normal plasma to the cells from SCD patients enhanced the $H_2O_2$ production to 108% ± 8% and 121% ± 11% ($P>0.05$, n=4) of the same day control when samples were primed with 50 and 500 U/ml TNF, respectively (Table 4.12). Although the data were not statistically significant, it indicated that plasma from patients with SCD contains factors that inhibit priming of FMLP stimulated $H_2O_2$ production in neutrophils from healthy controls and washing the cells from SCD patients free of autologus plasma caused slight improvement on the priming ability of the neutrophils.

**Table 4.11: Total MCF of neutrophils from SCD patients in steady state or during painful crisis**

| Patient | *Total MCF* | | | |
|---|---|---|---|---|
| | CC | CP | PP | PC |
| Steady State | 64 ± 2 | 64 ± 4 | 69 ± 5 | 72 ± 5 |
| Crisis | 62 ± 7 | 63 ± 7 | 66 ± 8 | 68 ± 7 |

Data shown are the mean ± 1SE of 4 experiments with $P>0.05$ in all cases.

CC = control cells + control plasma PP = patient cells + patient plasma

CP = control cells + patient plasma PC = patient cells + control plasma

**Table 4.12: Total $H_2O_2$ production in primed neutrophils stimulated by FMLP from SCD patients in steady state or during crisis**

| *$H_2O_2$ Production* | | | | |
|---|---|---|---|---|
| TNFα (U/ml) | Steady-State | | Crisis | |
| | PC‡ (%) | CP‡ (%) | PC‡ (%) | CP‡ (%) |
| 0 | 94 ± 12 | 160 ± 50 | 103 ± 7 | 94 ± 6 |
| 1 | 120 ± 5 | 52 ± 10* | 78 ± 5 | 133 ± 65 |
| 5 | 117 ± 14 | 62 ± 5* | 107 ± 18 | 102 ± 15 |
| 50 | 108 ± 8 | 68 ± 7* | 124 ± 23 | 81 ± 8 |
| 500 | 121 ± 11 | 62 ± 5* | 118 ± 10 | 85 ± 10 |

Data shown are the mean ± 1SE of 4 experiments using undiluted plasma from SCD patients in steady state and 4 experiments using diluted plasma from SCD patients in crisis. $H_2O_2$ production is expressed as a percentage of control (ie; PC as a percentage of CC and CP as a percentage of PP). *$P<0.02$
‡CP = control cells + patient plasma ‡PC = patient cells + control plasma
‡CC = control cells + control plasma ‡PP = patient cells + patient plasma

## 4.4 DISCUSSION

In an attempt to further understand the mechanism of altered neutrophil function in sickle cell disease (SCD) the data presented in this chapter test whether the various complications associated with SCD could account for the priming defect of neutrophils.

There is evidence for low neutrophil counts among blacks in comparison to whites (Freedman *et al.*, 1997) and because SCD patients in this study were of Afro-Caribbean background, I decided to investigate whether defective priming of neutrophils could be explained in terms of racial differences. A group of healthy Afro-Caribbean individuals were tested and the data obtained excluded the racial differences as a mechanism as no defects in neutrophil $H_2O_2$ production were seen in healthy Afro-Caribbean subjects.

Another feature common to patients with SCD is hyposplenism. As this organ is inactive in SCD patients, the neutrophil $H_2O_2$ production was tested in a group of non-SCD patients who received splenectomies previously. The data showed that there was no defects in neutrophils $H_2O_2$ production in asplenic patients.

The effects observed in SCD were also not reproduced in a group of patients with active rheumatoid arthritis. This study appears to be the first to test neutrophil function in whole blood from patients with arthritis, whereas previous studies using purified cells or diluted whole blood have produced contradictory results, some showing evidence of activated (Laurindo *et al.*, 1995; Eggleton *et al.*, 1995; Miesel *et al.*, 1996) or defective (Al-Balla *et al.*, 1990; Mur *et al.*, 1997) neutrophils in the circulation with reduced responses after cytokine-mediated priming (Eggleton *et al.*, 1995; Al-Balla *et al.*, 1990). However, Kowanko (1996), found no differences between patients with RA and healthy individuals with respect to their TNF$\alpha$ priming of neutophils respiratory burst. It has been shown that high levels of platelet activating factor (PAF) may also be a contributing factor to the inflammatory state of SCD (Oh *et al.*, 1997). These findings with rheumatoid arthritis patients emphasize that the defective priming seen in SCD is likely to be specifically associated with events in the circulation rather than being a general feature of inflammatory disorders.

It was further investigated whether the priming defect could be attributed to the marked anaemia of the patients with SCD, who had haemoglobin levels of 80-90 g/L. However, this study showed no evidence for reduced $H_2O_2$ production in a series of patients with iron-deficiency anaemia whose mean haemoglobin was 76 $\pm$ 5 g/L (n=6). Finally, one other factor associated with SCD is the premature destruction of sickled red blood cells thus giving rise to increased plasma haemoglobin levels. There is no previous evidence to suggest a correlation between the haemolytic events of SCD

and defective priming of neutrophils, therefore priming of $H_2O_2$ production in neutrophils was measured in patients with β-thalassaemia intermedia, a group of diseases resulting from decreased beta-globin mRNA expression and characterised by ineffective erythropoiesis and excessive haemolysis. The data from patients with β-thalassaemia showed reduced neutrophil $H_2O_2$ production when cells were primed by either GM-CSF or TNFα to 62% and 59% respectively. This suggets that the intravascular haemolysis may be a common feature in each condition accounting for defective priming of their neutrophils with cytokines. However, these data were not statistically significant and this needs to be verified by a larger study population. One other feature of β-thalassaemia is an elevated level of bilirubin which has also been shown in the plasma of patients with SCD which reduces the antioxidant capacity of plasma (Dailly *et al.*, 1998) which may possibily increase membrane damage, and hence increase adherence. If haemolysis is one of the contributing factors in defective priming of neutrophils in SCD, the replacement of sickle cell plasma with plasma from healthy individuals should ameliorate this defect. This hypothesis was tested in patients with SCD either in steady state or during crisis. Those in steady state of disease showed 20% increase in $H_2O_2$ production when neutrophils were primed with TNF (500 U/ml) and 2) those during crisis showed the same pattern. As this abnormality was only partially reversed by the addition of normal plasma, it suggests that the priming defect was of cellular origin which was partially corrected by normal plasma.

The effect of plasma was further studied when normal cells were suspended in plasma from SCD patients in steady state which showed reduction of $H_2O_2$ production to 38% of the control and with diluted plasma from patients in crises the reduction was 15% of the same day control. These data support the possibility of soluble inhibitory factor(s) in plasma from SCD patients that partially reduced $H_2O_2$ production in neutrophils. The effect of plasma factors on priming of neutrophils in SCD patients has not been investigated previously, and the nature of plasma inhibitors were not identified.

However, plasma factors such as C-reactive protein (CRP) which is mainly synthsised in the liver has been reported to be moderately increased in SCD patients during asymptomatic steady state and significantly increased during painful crisis (Akinola *et al.*, 1992; Stuart *et al.*, 1994), production of these proteins could be triggered by inflammatory cytokines such as, IL-6, IL-1, TGFβ-1 and TNFα (Croizat *et al.*, 1994). There is also evidence to show an increased thrombotic risk in these patients which could be due to increased thrombin and fibrin formation (Leslie *et al.*, 1975; Francis, 1989) with reduced levels of heparin cofactor II (Porter *et al.*, 1993),

antithrombin III, protein C and protein S (Caccioloa *et al.*, 1989; Karayalcin *et al.*, 1989; Francis, 1988).

$H_2O_2$ production in unprimed neutrophils stimulated by FMLP or PMA showed no significant differencefrom contols indicating that the defect observed in priming was not due to defect of NADPH oxidase itself. Taken together these findings imply both cellular and plasma defects associated with SCD.

# CHAPTER 5

# MITOGEN-ACTIVATED PROTEIN KINASE CASCADES UTILISED BY TNF$\alpha$ AND GM-CSF DURING PRIMING OF NADPH OXIDASE IN HUMAN NEUTROPHILS

## 5.1 INTRODUCTION

Neutrophils play a crucial role in host protection against microbial infection. Neutrophils are activated in response to a variety of stimuli, including chemoattractants, cytokines and immune complexes (Metcalf, 1991; Marucha *et al.*, 1990; Ohta *et al.*, 1991). After stimulation by the chemotactic peptide, FMLP, neutrophils can generate toxic reactive oxygen intermediates such as superoxide catalysed by NADPH oxidase (see Chapter 1 for details). In addition, activation of neutrophils gives rise to a range of functional responses including, adherence, migration, degranulation, calcium flux and actin polymerisation (Sandborg and Smolen, 1988). These responses can be initiated by binding of FMLP to a seven-transmembrane-spanning receptor and further downstream signalling mediated by the heterotrimeric G-protein, $G_{i2}$ (Boulay, *et al.*, 1990; Bommakanti *et al.*, 1992; Murphy, 1994). Signal transduction pathways activated by FMLP lead to activation of phospholipases including PLC-$\beta$ (which increases inositol phosphate and calcium ions) (Camps *et al.*, 1992), PLD (Cockroft *et al.*, 1994). FMLP also activates kinases such as PI3-kinase (Traynor-Kaplan and Harris, 1988; Stephens *et al.*, 1991; Coffer *et al.*, 1998), and MEK which leads to activation of MAP kinase (Grinstein and Furuya, 1992; Torres *et al.*, 1993; Worthen *et al.*, 1994). FMLP also stimulates serine/threonine or tyrosine phosphorylation (Tauber *et al.*, 1990). The response to FMLP can be potentiated (primed) through the interaction of the cell with variety of priming agents such as TNF$\alpha$ and GM-CSF (Chapter 1, Figure 1.4). This priming of neutrophils by cytokines for enhanced functional activity has been recognised for many years although the mechanism(s) involved in the cytokine-mediated priming response of mature neutrophils are not fully elucidated.

The NADPH oxidase is relatively quiescent in neutrophils from healthy individuals, and requires a 'priming' step in order to generate reactive oxygen metabolites. Studies have shown that priming can be induced by a variety of cytokines and inflammatory mediators including, GM-CSF and TNF$\alpha$ which do not directly activate the NADPH oxidase when neutrophils are in suspension, but enable the cell to give enhanced responses when stimulated by agonist (for review see Gasson, 1991). Priming has also been observed at the level of phospholipase $A_2$ and D activation (Sullivan *et al.*, 1987; DiPersio *et al.*, 1988; Roberts *et al.*, 1996; Bourgoin *et al.*, 1990).

Protein phosphorylation is fundamentally important as a molecular switch for turning cellular processes on or off and the intracellular transmission of signals initiated by stimuli at the cell surface is mediated by activation of protein kinases which are combined into a complex network. Most protein phosphorylation occurs on serine

and threonine residues but tyrosine phosphorylation also plays an important role in cellular functions. The biochemical events which lead to activation of neutrophil effector functions involve phosphorylation and dephosphorylation of many proteins (Bokoch, 1995). Other reports support the hypothesis that phosphorylation events mediate the activation of neutrophils. The activity of protein kinases were inferred by studies using agents such as genestein and erbstatin which inhibit tyrosine kinases (Kusunoki *et al.*, 1992; Naccache *et al.*, 1990). It has been shown that the protein kinase cascades from cytokine receptors involve activation of mitogen-activated protein kinase (MAPK) pathways which are highly conserved. In mammalian cells multiple MAPK pathways have been identified, and they are essential for many biological processes. Three members of the MAPK family are the ERKs (including $p42^{ERK2}$), p38 MAPK and JNK (see Chapter 1). These pathways are involved in regulation of growth, differentiation and inflammatory responses. Gomez-Camronero *et al.* (1993) have demonstrated that GM-CSF can activate $p42^{ERK2}$ in human neutrophils and found that this activity is most likely due to tyrosine phosphorylation of MAPK triggered by the binding of GM-CSF to its receptor. Yuo *et al.* (1993) have shown that TNFα increased tyrosine phosphorylation of a 42 kDa protein. In contrast, others suggested that TNFα fails to activate ERK in neutrophils when in suspension (Waterman and Sha'afi, 1995) whereas TNFα increases tyrosine phosphorylation of a 42 kDa protein in adherent neutrophils (Rafiee *et al.*, 1995).

Previous studies have found that PI3-kinase, tyrosine kinases and PKC are components of signal transduction pathways leading to activation of the respiratory burst (Vlahos *et al.*, 1995; Coffer *et al.*, 1998; Kodama *et al.*, 1999; Gomez-Cambronero *et al.*, 1989; Wilson *et al.*, 1986; Niwa *et al.*, 1996; Rane *et al.*, 1997). It was demonstrated that introduction of active form of PI3-kinase into monoblastic phagocyte line (GM-1) caused activation of a serine/threonine protein kinase downstream of PI3-kinase, PKB, and also phosphorylation of $p47^{phox}$ (Didichenko *et al.*, 1996).

As discussed in Chapter 1 (general introduction) priming of the respiratory burst may occur at several different sites either in the agonist signal transduction pathway or in the components of the NADPH oxidase (Figure 1.4). Previous studies have shown that although the number of FMLP receptors is increased after cytokine priming this does not account for the primed response (Roberts *et al.*, 1990) and therefore changes to signalling pathways downstream from the receptor may be involved in priming. The roles of MAPKs in cellular functions of the neutrophils are not fully understood. Therefore in this study the involvement of $p42^{ERK2}$ and p38 MAPK pathways in the activation of NADPH oxidase in unprimed and primed human neutrophils was investigated.

## 5.2 MATERIALS AND METHODS

Materials

- PD 098059- A stock solution was prepared in DMSO at concentration of 30 mM and was stored at -20°C. Serial dilutions were prepared in DMSO at concentrations indicated in the text on the day of experiment. (CalBioChem-Novabiochem Corporation, La Jolla, CA).
- SB 203580- A stock solution was prepared in DMSO at concentration of 30 mM and was stored at -20°C. Serial dilutions were prepared on the day of experiment. (Alexis Corporation Ltd., Nottingham, UK)
- 40% (wt/vol) Acrylamide solution (Gibco BRL, Paisley, UK)
- 2% (wt/vol) Bis/Acrylamide solution (Gibco BRL)
- Acrylamide/N'N'-bis-methylene (bis)-acrylamide ratio of 37.5:1 (30% monomer:2.67% cross-linking monomer concentration) (Boehringer, Mannheim, Germany).
- Polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore Ltd., UK)
- Erk-2 antibody (C-14) (Santa Cruz Biotechnology Inc., Santa Cruz, CA)
- p38 antibody (C-20) (Santa Cruz Biotechnology Inc.)
- Phospho-specific p38 antibody (New England Biolabs, Inc, Beverly, MA)
- Goat-anti-rabbit antisera (Dako Ltd., High Wycombe, Bucks, UK)
- Rabbit-anti-goat antisera (Dako Ltd.)
- Pefabloc® SC [4-(2-aminoethyl)-benzenesulfonyl fluoride, hydrochloride (ABSF), a serine protease inhibitor]. A stock solution was prepared at 100 mM in water and stored at -20°C.
- [$\gamma$-$^{32}$P]ATP (specific activity of 3000 Ci/mmol) (Amersham Pharmacia Biotech, UK)
- Protein G-Sepharose (Sigma, Poole, Dorset)

- **Buffer A:-** 0.5% Triton-X-100, 1 mM EDTA, 50 mM Tris/HCl pH 8.0, 100 mM NaCl

- **Kinase buffer:-** 25 mM Tris/HCl pH 8.0, 10 mM $MgCl_2$, 2 mM $MnCl_2$,

- **p38 kinase Assay Kit:-** (Upstate biotechnology, Lake Placid, NY 12946) containing:-
  1) Assay dilution buffer (ADB):-20 mM MOPS pH 7.2, 25 mM β- glycerol phosphate, 5mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol.

2) Magnesium/ATP cocktail: 500 mM cold ATP and 75 mM magnesium chloride in ADB.
3) GST-MAPKAP kinase-2 (residues 46-400 of recombinant human MAPKAP Kinase-2 with an N-terminal glutathione S-transferase (GST) *tag* and a C-terminal *myc* epitope).

- **Separating gel preparation (15%):-** 1.25 ml distilled water, 3.73 ml 1 M Tris-HCl pH 8.8, 3.75 ml acrylamide (40%), 0.38 ml bisacrylamide (2%) (acrylamide/bis 15%:0.075%, ratio 200:1), 0.1 ml 10% SDS, 0.05 ml 10% (wt/vol), ammonium persulphate, 0.005 ml TEMED

Methods

### 5.2.1 Neutrophil purification

Neutrophils were separated by Ficoll-Hypaque centrifugation, dextran sedimentation and hypotonic lysis of erythrocytes as described in Chapter 2 Section 2.1.

### 5.2.2 Measurement of superoxide anion production

The production of superoxide anions by neutrophils was measured as the superoxide dismutase-inhibitable reduction of ferricytochrome *c* (as described in detail in Chapter 2, Methods section). Neutrophil samples in duplicate at a concentration of $1 \times 10^6$ cells/ml were incubated at 37°C with either PD 098059 or SB 203580 (at various doses and for various incubation times as indicated in the text). The optimal preincubation time used thereafter was 10 minutes for PD 098059 and 30 minutes for SB 203580, prior to stimulation or priming with cytokines as described in Chapter 2.

### 5.2.3 Measurement of MAP kinase phosphorylation

*i) $p42^{ERK2}$*

The $p42^{ERK2}$ analysis was performed by electrophoresis on a 15% (acrylamide/bis 15%:0.075%) polyacrylamide gel (20 cm x 20 cm x 0.3 cm spacers) using a large gel apparatus as instructed by the manufacturer (Protean II, BioRad, UK). Following stimulation with agonist as indicated in the result section, neutrophil samples were prepared as described in Chapter 2 and proteins equivalent to 1-2 x $10^5$ cells were

loaded into each lane and electrophoresis carried out at a constant 120 V for 15 hours. The proteins were transferred onto a PVDF membrane using a semi-dry transfer apparatus (Milliblot, Millipore, USA) at constant current of 0.45 Amps for 45 minutes. The non-specific binding sites were blocked by 2 hours incubation of the membrane in TBST/5% non-fat dried milk. Then the blot was probed with polyclonal Erk-2 antibody diluted 1:1000 in TBST/5% non-fat dried milk for 1 hour. The blot was then washed three times in TBST and incubated with a 1:2000 dilution (in TBST/5% non-fat dried milk) of peroxidase-conjugated goat anti-rabbit antibody for 1 hour at room temperature. The blot was washed in TBST and once in TBS. Detection of the protein bands were carried out by using an enhanced chemiluminescence method according to the manufacturers instructions (Amersham International), prior to analyses by autoradiography.

*ii) p38 MAPK*

Samples were analysed by electrophoresis on a 15% (Acrylamide/Bis 30%:0.8%) polyacrylamide gel using the minigel apparatus (Mini-Protean II, BioRad, CA, USA) as described in Chapter 2. The resolved proteins were transferred to a nitrocellulose membrane at a constant current of 0.45 Amps for 45 minutes. The non-specific binding sites were blocked in TBST/5% non-fat dried milk for 2 hours at room temperature. The blots were incubated overnight at 4°C with a 1:1000 dilution of either primary rabbit-anti-human p38 antibody or goat-anti-human p38 (phospho-specific) antibody which detects p38 MAP kinase only when phosphorylated on tyrosine 182 and threonine 180. Then the membranes were washed with TBST (three washes for 10 minutes each) and incubated with 1:2000 dilution of the second antibody, peroxidase conjugated goat anti-rabbit or rabbit anti-goat antibody, for one hour at 4°C followed by two washes with TBST and one wash with TBS. Then the blots were developed with the enhanced chemiluminescence detection system.

### 5.2.4 p38 MAP kinase assay

Neutrophils at $2x10^7$ cells/ml were incubated with SB 203580 (various doses as indicated in the text) or diluent (DMSO) for 30 minutes at 37°C followed by stimulation with either PBS (control) or 1μM FMLP. For priming experiments, samples were incubated with either diluent control (0.01% FCS), GM-CSF (10 ng/ml) or TNFα (500 U/ml) for 10 minutes at 37°C prior to stimulation. The reactions were terminated by adding ice cold lysis buffer [containing Pefabloc (2 mM final concentration) in addition to other protease and phosphatase inhibitors listed in

Chapter 2] for 15-30 minutes. All the subsequent steps were performed at 4°C. The lysates were then cleared by centrifugation at 12000 x g for 10 minutes and the supernatant was transferred to a fresh microfuge tube containing 2 μg/ml (final concentration) of p38 MAPK polyclonal antiserum for 30 minutes on a rotating wheel, followed by addition of 50% protein G-Sepharose (in PBS) for a further 30 minutes. After incubation, complexes were centrifuged at 12000 x g for 5 minutes and the supernatant was removed. The immune complexes containing p38 MAP kinase were then washed twice with buffer A and once with kinase buffer. Then the phosphorylation of MAPKAPK-2, the downstream substrate of p38 MAPK, was used to measure p38 MAPK activity. The immunoprecipitates were incubated with 200 ng GST-MAPKAP kinase-2, 2 μl magnesium/ATP cocktail, 10 μl ADB and 5 μCi of [$\gamma$-$^{32}$P]ATP, for 15 minutes at 30°C with occasional mixing. The reaction was then stopped by the addition of 4 x Laemmli's sample buffer to the samples which were boiled for 10 minutes at 95°C. Samples were separated by 12.5% SDS/PAGE and transferred to a nitrocellulose membrane at a constant current of 0.8 Amps for 50 minutes. Then MAPKAP kinase-2 phosphorylation was detected by autoradiography.

### 5.2.5 Statistical analysis of data

The statistical significance of the differences between sample populations was determined by the Student's paired-*t* test, with significance accepted at $P<0.05$.

## 5.3 RESULTS

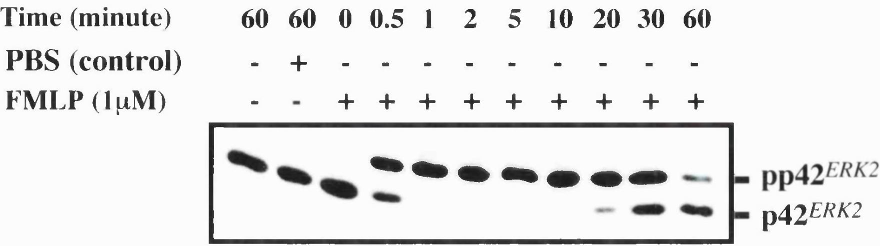
### 5.3.1 Phosphorylation and activation of $p42^{ERK2}$ and p38 MAPK in human neutrophils

*i) Effect of agonists*

Purified neutrophils were stimulated with either FMLP (1 μM) or PMA (500 ng/ml) for various time intervals as indicated in the Figure 5.1. Whole cell lysates were prepared and activation of $p42^{ERK2}$ was measured using a gel retardation assay. Migration of phosphorylated $p42^{ERK2}$ is slower than the unphosphorylated form, thus enabling the two bands to be separated. Others have confirmed that the appearance of the slow migrating upper band, is characteristic for MAPK activation which is associated with its enhanced phosphorylation (Leevers & Marshall, 1992; Waterman *et al.*, 1995). Stimulation of the cells with FMLP was observed within 30 seconds and responses were maximal within 1 minute and decreased within 20 minutes (Figure 5.1 A). In contrast, the shift induced by PMA was detectable within 1 minute after stimulation, was maximal within 2 minutes, and was sustained until at least 60 minutes (Figure 5.1 B).

Neutrophil samples stimulated with agonists (as explained above) were separated on 12.5% SDS/PAGE in order to detect activation of p38 MAPK in these cells by western blotting and gel retardation assay (Figure 5.2). It was noted that the phosphorylated form of p38 MAPK does not migrate more slowly than the unphosphorylated form (i.e. phosphorylated p38 MAPK did not show an electrophoretic mobility shift). Therefore a different approach was used. Neutrophil samples were loaded on the 12.5% gel in duplicate and transferred to a PVDF membrane. Then the membrane was cut into two identical halves in order to probe one blot with an anti-p38 antibody and the second blot with a phospho-specific p38 antibody (Tyr-182 and Thr-180). FMLP (1 μM) stimulated p38 MAPK phosphorylation within 30 seconds and this peaked at 2 minutes. In contrast, there was no detectable phosphorylation of p38 MAPK when neutrophils were stimulated by 500 ng/ml PMA, although total p38 MAPK was detected in samples stimulated for up to 60 minutes (Figure 5.2). These data suggests that FMLP signals via both $p42^{ERK2}$ and p38 MAPK whereas the PMA response is mainly via $p42^{ERK2}$.

A)




B)

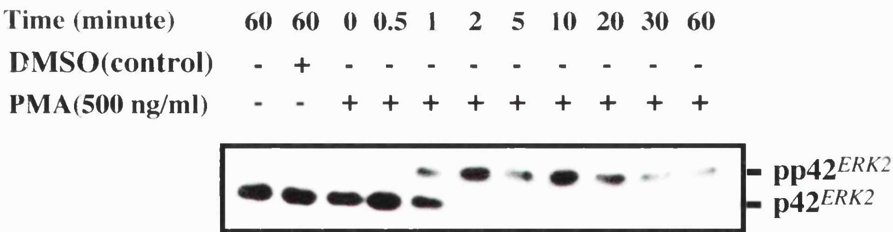



**Figure 5.1: Western analysis of p42*ERK2* activation in neutrophils.** Cells were incubated with (A) FMLP (1μM) (B) PMA (500 ng/ml) for the time points indicated at 37°C. Phosphorylation of p42*ERK2* was detected by SDS/PAGE and western blotting as described in the Materials and Method section. The autoradiographs shown are from a single experiment that was carried out A) three times and B) twice, with similar results.

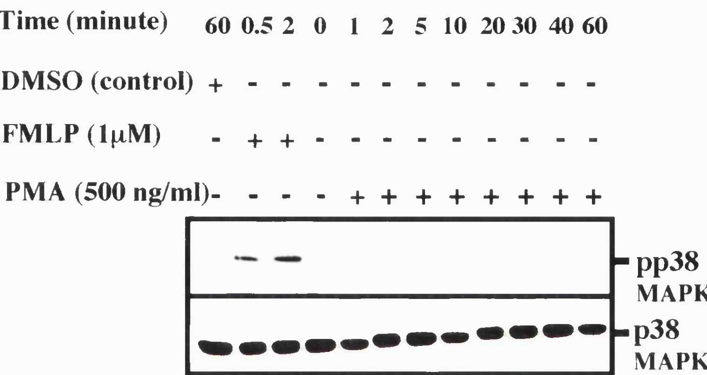



**Figure 5.2: Activation of p38 MAPK in FMLP and PMA-stimulated neutrophils.** Cells were stimulated with either 1μM FMLP (positive control) or 500 ng/ml PMA (DMSO diluent control) for the time points indicated. After lysis, phosphorylation of p38 MAPK was detected by SDS/PAGE and western blotting as described in the Materials and Method section. The autoradiographs are from a single experiment that was carried out twice with similar results.

*ii) Effect of cytokines*

Stimulation of neutrophil $p42^{ERK2}$ with the cytokines, TNFα (500 U/ml) and GM-CSF (10 ng/ml) provided a different kinetic profile from the agonists used in section (i). Addition of TNFα to the cells resulted in a transient phosphorylation of $p42^{ERK2}$, which was observed at 5 minutes, with optimal activation at 10 minutes after stimulation and activity decreased to baseline by 20 minutes (Figure 5.3 A), whereas stimulation with GM-CSF was of longer duration. As shown in Figure 5.3 B $p42^{ERK2}$ was phosphorylated by GM-CSF within 5 minutes after stimulation and phosphorylation decreased gradually at 30 minutes and completely disappeared at 60 minutes. These data demonstrate that activation of $p42^{ERK2}$ by these cytokines is distinct from that of the agonists that were used.

Activation of p38 MAPK with TNFα was detectable within 5 minutes of stimulation and disappeared within 20 minutes (Figure 5.4 A), whereas, GM-CSF (10 ng/ml) phosphorylated p38 MAPK within 1 minute after stimulation and this was sustained for 15 minutes and it was not detectable at 30 minutes. (Figure 5.4 B)

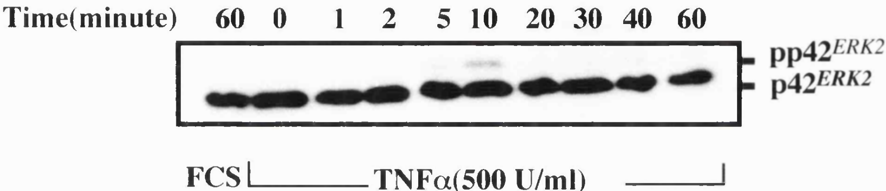
## 5.3.2 Establishment of optimal conditions for $p42^{ERK2}$ and p38 MAPK inhibitors

In order to investigate the involvement of MAP kinases in cytokine-mediated priming of the respiratory burst the effect of two inhibitors of the MAP kinase family was studied.

### *5.3.2.1 Inhibition of $p42^{ERK2}$ activation by PD 098059 in neutrophils*

PD 098059 (Figure 5.5), is a reportedly specific (non-competitive) inhibitor of MEK1 (Alessi *et al.*, 1995). It acts by binding to the inactivated form of MEK, therefore preventing its phosphorylation by c-Raf or MEK kinase. The specificity of the inhibitory effect of PD 098059 *in vitro* and *in vivo* is indicated by its failure to inhibit 18 other protein serine/threonine kinases (Table 5.1) (Alessi *et al.*, 1995). In order to test the efficacy of PD 098059 in inhibiting $p42^{ERK2}$ activation of neutrophils, the following experiments were performed. Neutrophil $p42^{ERK2}$ responses stimulated by GM-CSF and PMA were chosen, as these agonists gave the most potent and sustained activation on which to test PD 098059. Purified neutrophils at 1 x $10^6$ cells/ml were incubated with PD 098059 for 10 minutes prior to stimulation by either GM-CSF (10 ng/ml) or PMA (500 ng/ml) for 10 and 5 minutes, respectively. The reaction was stopped by addition of ice cold lysis buffer for 15-30 minutes.

A)




B)

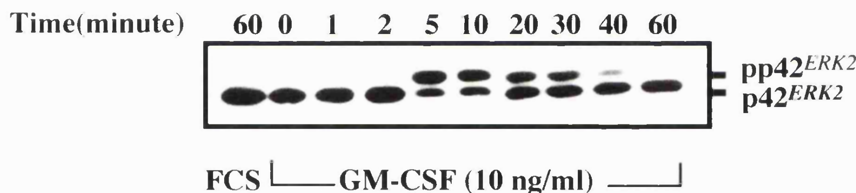
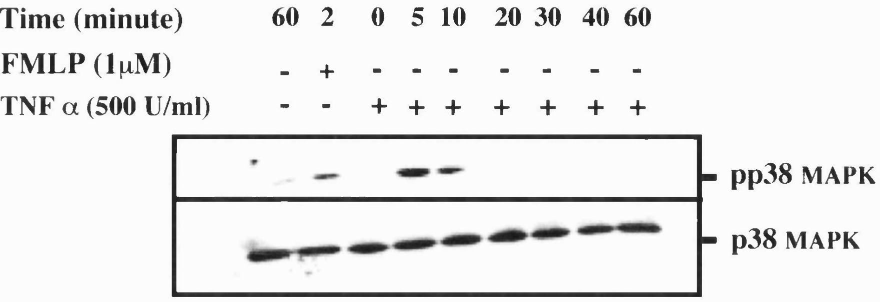



**Figure 5.3: Kinetics of p42$^{ERK2}$ activation in neutrophils measured by gel retardation assay.** Cells were stimulated with cytokine diluent (0.01% FCS), TNFα (500 U/ml) or GM-CSF (10 ng/ml) for the time points indicated. After lysis, phosphorylation of p42$^{ERK2}$ was detected by SDS/PAGE and western blotting as described in the Materials and Method section. The autoradiographs are from a single experiment that was carried out A) twice and B) three times, with similar results.

A)




B)

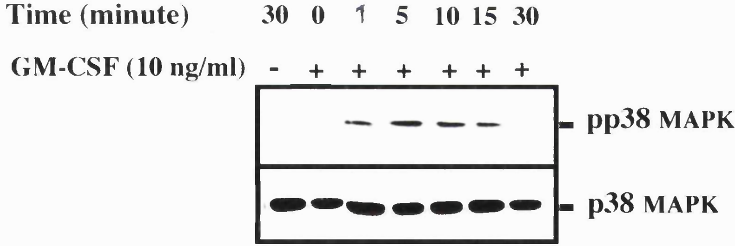



**Figure 5.4: Ability of cytokines to activate p38 MAP kinase in neutrophils.** Neutrophils were stimulated with either TNFα (500 U/ml), GM-CSF (10 ng/ml) or FMLP (1μM) for the time points indicated before cell lysis. Samples were resolved on SDS/PAGE as described in the Materials and Method section. The autoradiographs are from a single experiment that was performed twice.

**Table 5.1 : Effect of PD 098059 (50 μM) on the activities of purified protein kinases *in vitro.***

| *Protein Kinases* | *Activity (% of control)* |
|---|---|
| MAP kinase kinase -1 }* | 102 ± 1 |
| MAP kinase kinase -2 | 98 ± 3 |
| p42 MAPK | 101 ± 1 |
| MAPKAP kinase-1 | 98 ± 1 |
| RK kinase | 88 ± 9 |
| MKK4 | 87 ± 5 |
| JNK | 99 ± 5 |
| RK | 97 ± 6 |
| MAPKAP kinase-2 | 109 ± 7 |
| Protein kinase A | 94 ± 3 |
| Protein kinase Cα | 100 ± 1 |
| AMP-activated protein kinase | 97 ± 3 |
| Cyclin A/ cdk2 | 95 ± 1 |
| Phosphorylase kinase | 99 ± 0 |
| Glycogen synthase kinase-3α | 87 ± 1 |
| Glycogen synthase kinase-3β | 93 ± 1 |
| p70 S6 kinase | 94 ± 2 |
| Myosin light chain kinase | 88 ± 2 |

* MAPKK-1 and MAPKK-2 were activated by c-Raf *in vitro* and it has been demonstrated that PD 098059 does not inhibit the phosphorylated forms of MAP kinase kinase

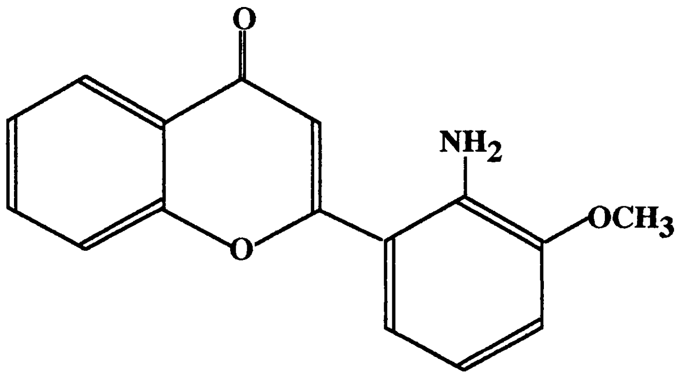



**Figure 5.5: Structure of PD 098059**
[2-)2-amino-3-methoxyphenyl)-oxanaphthalen-4-one]
(Adapted from Alessi *et al.*, 1995)

After centrifugation clear lysates were collected into a fresh tube followed by addition of 4 x Laemmli sample buffer. Then samples were boiled for 10 minutes at 95°C and $p42^{ERK2}$ phosphorylation was measured by western blotting as described in the Materials and Method section 5.2. Figures 5.6 A and B show the dose response of PD 098059 when neutrophils stimulated with either GM-CSF (10 ng/ml) for 10 minutes or PMA (500 ng/ml) for 5 minutes. When cells were stimulated with GM-CSF the inhibitory effect of PD 098059 on $p42^{ERK2}$ was observable at 1 μM with maximum inhibition at 10 μM. Whereas, 5 μM PD 098059 showed partial inhibition of PMA stimulated $p42^{ERK2}$ with maximal inhibition at 20 μM.

Figure 5.6 C shows that preincubation of neutrophils with PD 098059 (40 μM) for different time points at 37°C inhibited $p42^{ERK2}$ phosphorylation within 10 minutes and the effect of PD 098059 was sustained until at least 60 minutes. This was important for priming experiments where incubation of cells with PD 098059 for 10 minutes was followed by 30 minutes stimulation by cytokines.

When the optimal condition for the use of PD 098059 was established then neutrophil samples were treated with PD 098059 (40 μM) in order to inhibit $p42^{ERK2}$ activity prior to stimulation of neutrophils with either 1μM FMLP or 500 ng/ml PMA (Figures 5.7 A and B). The inhibitory effect of PD 098059 was also observed when neutrophils were stimulated with either 500 U/ml TNFα (Figure 5.8 A) or 10 ng/ml GM-CSF (Figure 5.8 B). Thus it seems that all these stimulants require MEK1 for activation of $p42^{ERK2}$ in human neutrophils.

*5.3.2.2 Inhibition of p38 MAP kinase activation by SB 203580 in neutrophils*

SB 203580 (Figure 5.9), is reported to be a specific inhibitor of cellular p38 MAPK *in vitro* and *in vivo* as it inhibits both p38 MAPK and its isoform p38β, but has no inhibitory action on SAPK3 and SAPK4 (Cuenda *et al.*, 1997; Goedert *et al.*, 1997). It exerts its inhibitory effect by binding to the ATP binding site of p38 MAPK. As shown in Table 5.2 it did not inhibit 12 other protein kinases tested *in vitro* (Cuenda *et al.*, 1995).

In order to investigate the effect of SB 203580 on p38 MAPK activity, it was required to establish the optimal time point for detection of the activation of p38 MAPK; 1) Purified human neutrophils were stimulated with FMLP (1 μM) for various time points. The cells were then lysed and p38 MAPK was immunoprecipitated with specific antiserum. It has been shown that MAPKAPK-2 is a substrate for phosphorylation by p38 MAPK (Freshney, *et al.*, 1994), therefore in order to detect the enzymatic acivity of p38 MAPK, a glutathione S-transferase (GST)-MAPKAPK-2 was used as a substrate as described in the Method section 5.2. The assay showed that

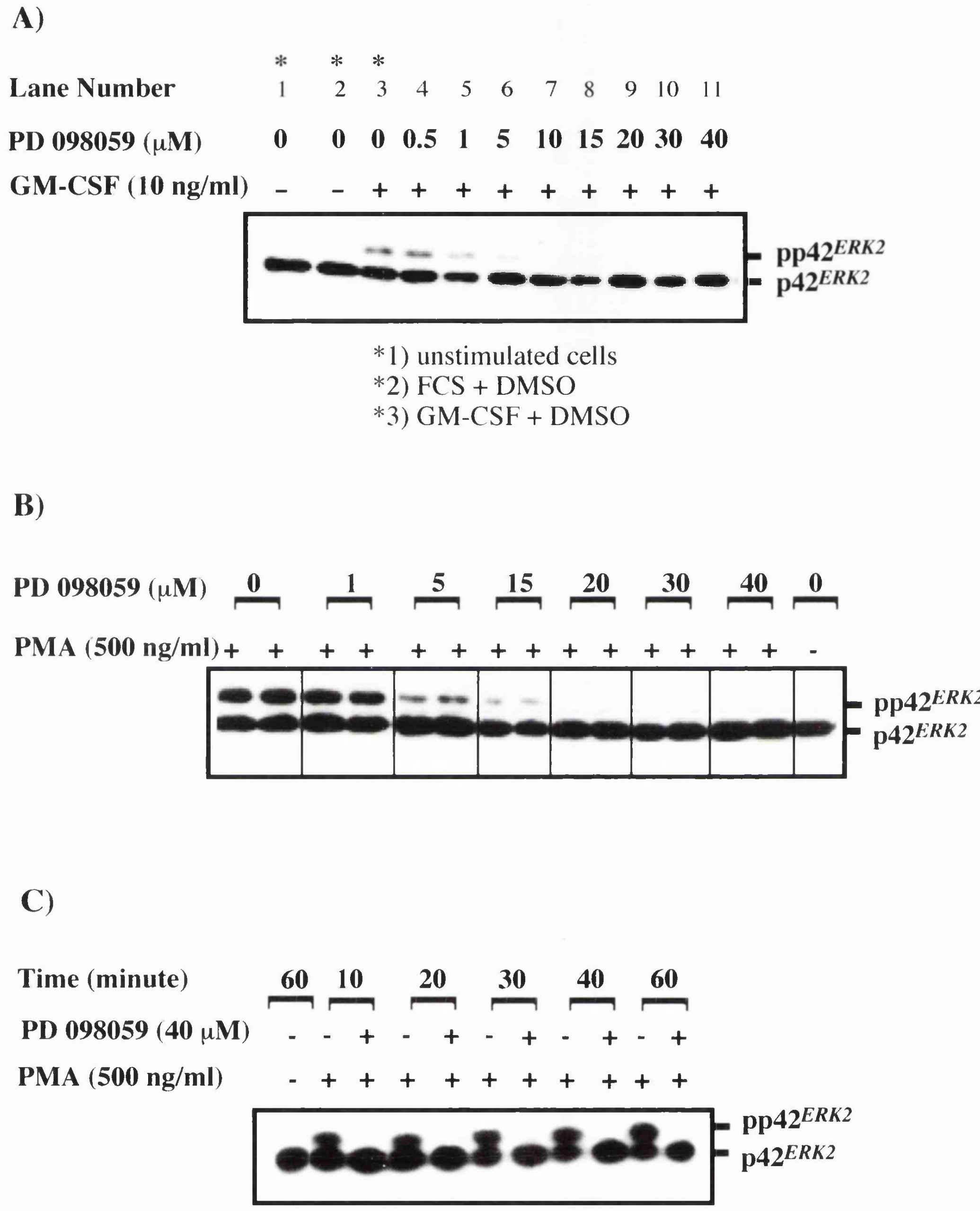
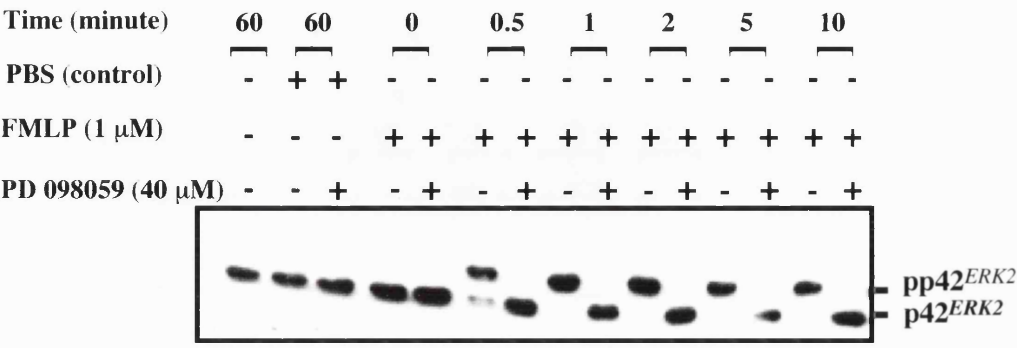



**Figure 5.6: Dose response and time-course of PD 098059 measured by gel retardation assay.** Neutrophil samples were preincubated with various doses of PD 098059 for 10 minutes followed by stimulation with A) cytokine diluent (0.01% FCS) or GM-CSF (10 ng/ml) for 10 minutes, B) diluent (0.01% DMSO) or PMA (500 ng/ml) for 5 minutes. C) Neutrophil samples were preincubated with 40 μM PD 098059 for different time points prior to stimulation with PMA (500 ng/ml) for 5 minutes. After lysis, phosphorylation of $p42^{ERK2}$ was detected by SDS/PAGE and western blotting as described in the Method section. The autoradiographs shown are as follows: (A) is from a single experiment that was performed twice, (B) is from a single experiment performed in duplicate and (C) is from a single experiment.




B)

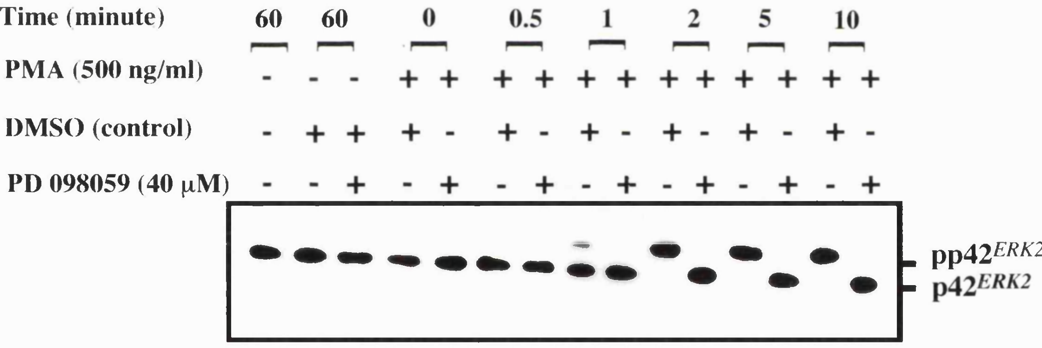
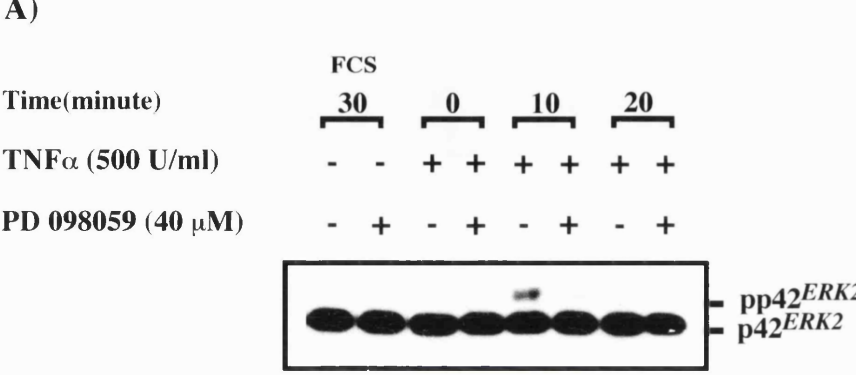



**Figure 5.7: Effect of PD 098059 on phosphorylation of $p42^{ERK2}$ stimulated by agonists.** Neutrophil samples were stimulated with either A) FMLP (1 μM) or B) PMA (500 ng/ml) for the time points indicated. After lysis, phosphorylation of $p42^{ERK2}$ was detected by SDS/PAGE and western blotting as described in the Method section. The autoradiographs shown are from a single experiment that was performed twice.



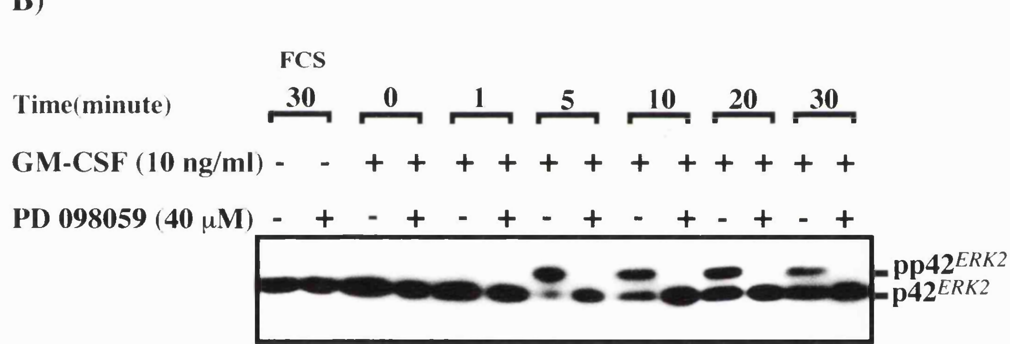




**Figure 5.8: Effect of PD 098059 on $p42^{ERK2}$ phosphorylation by cytokines in neutrophils.** Purified neutrophils were incubated with either DMSO or 40 μM PD 098059 for 30 minutes and then stimulated with either A) TNFα (500 U/ml), B) GM-CSF (10 ng/ml) or 0.01% FCS (diluent control) at time points indicated. Phosphorylation of $p42^{ERK2}$ was measured by gel retardation assay as described in the Method section. The autoradiographs shown are from a single experiment that was performed twice.

**Table 5.2: Effect of SB 203580 (100 μM) on the activities of protein kinases and phosphatases**

| *Protein Kinase* | *Activity (% of control)* |
|---|---|
| RK/p38 | 35 ± 1 (1 μM SB 203580) |
| RK/p38 | 5 ± 1 (10 μM SB 203580) |
| MAPKAP kinase-2 | 92 ± 3 |
| JNK/SAP kinase | 86 ± 4 |
| p42 MAP kinase | 97 ± 3 |
| MAP kinase kinase | 93 ± 4 |
| c-Raf | 87 ± 2 |
| p90 S6 kinase | 103 ± 2 |
| p70 S6 kinase | 97 ± 4 |
| protein kinase A | 99 ± 2 |
| phosphorylase kinase | 97 ± 1 |
| cyclin A/cdk2 | 91 ± 4 |
| cyclin E/cdk2 | 112 ± 6 |
| casein kinase-2 | 99 ± 2 |
| protein phosphatase-1 | 97 ± 4 |
| protein phosphatase-2A | 106 ± 6 |

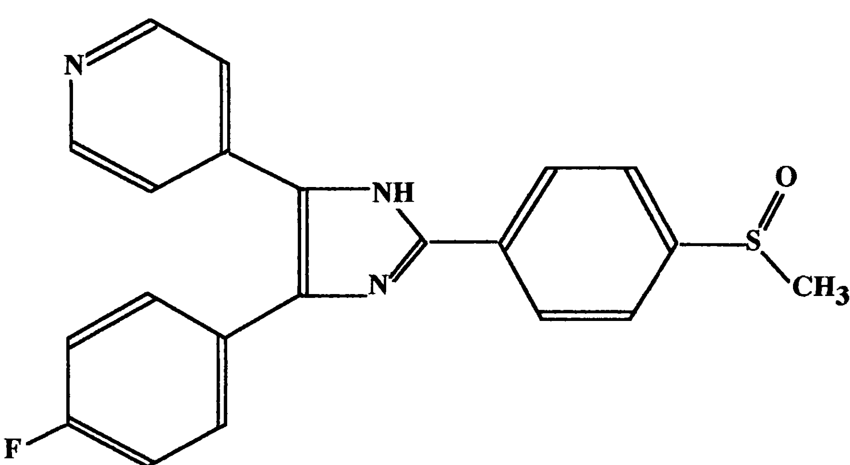



**Figure 5.9: Structure of SB 203580**
4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole
(Cuenda *et al.*, 1995)

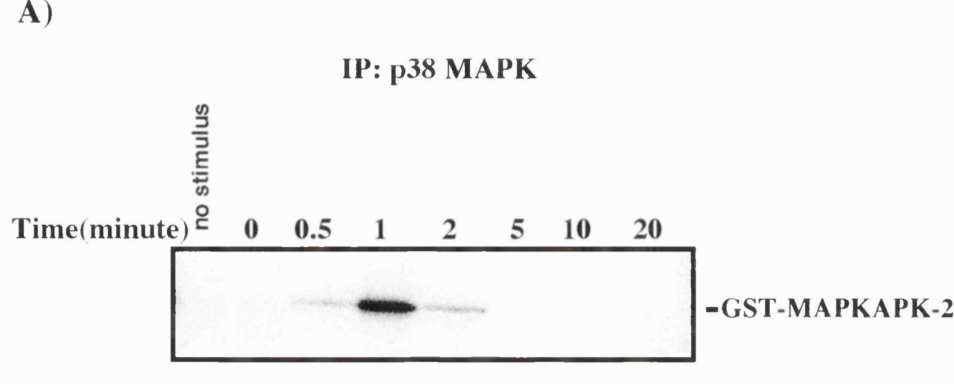
FMLP stimulated a time-dependent increase in phosphorylation of MAPKAPK-2 which was detectable within 30 seconds, reached maximal levels around 1 minute and persisted for up to 2 minutes (Figure 5.10). 2) Neutrophil samples were treated with various doses of SB 203580 prior to stimulation with FMLP (1μM) for 1 minute. Cells were then lysed and immunoprecipitated as described in (1). Upon treatment with SB 203580, there was a progressive decrease in p38 MAPK activity with increasing concentration of SB 203580 as shown in Figures 5.11 A and B. Both figures show an inhibitory effect of SB 203580 at 1 μM which was more pronounced in Figure 5.11 B, indicating the differential sensitivity of neutrophils from different individuals to SB 203580. The findings were confirmed by examining total cellular p38 MAPK, and Figures 5.10 a, 5.11 a and b indicate that the differences observed for the induced protein phosphorylation of the cellular MAPKAPK-2 did not result from differences in loading or from cellular protein digestion. In both Figures 5.11 A and B, 30 and 40 μM SB 203580 are shown to inhibit p38 MAPK activity. In Figure 11 B there was increased background activity in the right hand side of the autoradiograph, making the data for 20 μM SB 203580 difficult to interpret.

In a control experiment neutrophil samples were treated with PD 098059 or SB 203580 prior to stimulation with 500 ng/ml PMA. As shown in Figure 5.12, SB 203580 had no inhibitory effect on $p42^{ERK2}$ phosphorylation whereas, PD 098059 completely inhibited phosphorylated $p42^{ERK2}$ and this demonstrates the specificity of SB 203580 for p38 MAPK rather than $p42^{ERK2}$.

### 5.3.3 Role of $p42^{ERK2}$ and p38 MAPK in activation of the respiratory burst in neutrophils

*i) unprimed response*

To study the role of $p42^{ERK2}$ and p38 MAPK in mediating the stimulation of the respiratory burst by FMLP (1 μM) or PMA (500 ng/ml), the effect of various concentrations of PD 098059 and SB 203580 (which inhibit $p42^{ERK2}$ and p38 MAPK, respectively) were analysed in a cytochrome *c* reduction assay of superoxide production (described in the Materials and Method section 5.2). Figure 5.13 shows the effect of these inhibitors on the basal levels of superoxide production and on the FMLP-induced respiratory burst. There was no change in the basal levels of superoxide production with either inhibitor. Figure 5.13 shows that the level of superoxide production of samples treated with PD 098059 when expressed as a percentage of the diluent control was 85% ± 10% and 86% ± 14% with 30 μM and 40 μM PD 098059, respectively (Figure 5.13 A), but this level of inhibition was not



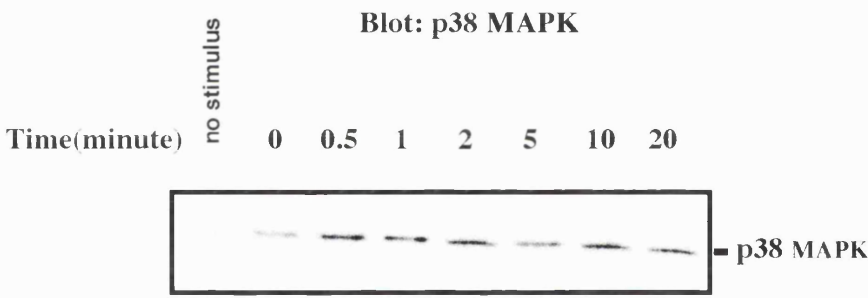
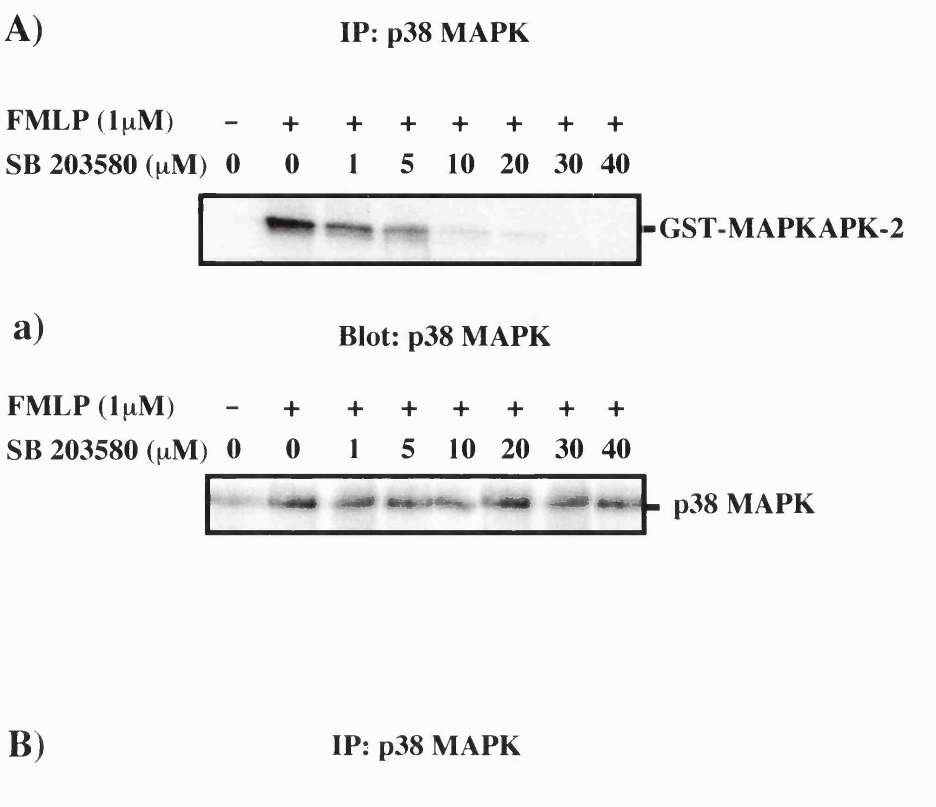



**Figure 5.10: Activation of p38 MAPK in FMLP-stimulated neutrophils.** PMN were stimulated with 1μM FMLP for various time points. p38 MAPK was immunoprecipitated and then subjected to a kinase assay using glutathione *S*-transferase-tagged (GST)-MAPKAPK-2 as a sunstrate in the presence of [$\gamma$-$^{32}$P]ATP. Samples were then subjected to SDS-PAGE and transferred to PVDF membrane as described in the Methods and Materials section 5.2. A) The region corresponding to the MAPKAPK-2 is illustrated. a) The membrane was probed using p38-specific antibody in order to check that equal amounts of p38 MAPK were present in each lane. Results shown are representative of one experiment.



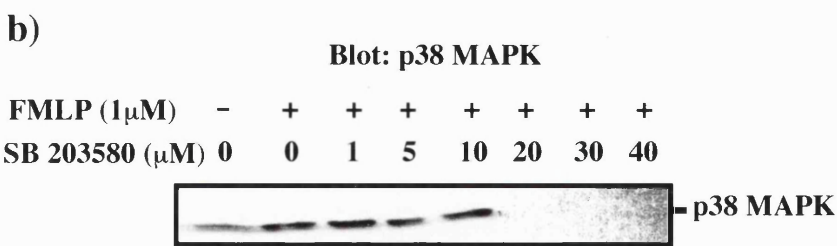




**Figure 5.11: Dose response of SB 203580.** Neutrophils ($2x10^7$ cells/ml) were preincubated with either SB 203580 (at various doses) or DMSO (diluent control) prior to stimulation with 1 μM FMLP for 1 minute. Then cells were lysed and p38 MAPK was immunoprecipitated and subjected to a kinase assay using GST-MAPKAPK-2 as a substrate in the presence of [γ-$^{32}$P]ATP. Samples were then subjected to 12.5% SDS/PAGE and transferred to PVDF membrane as described in the Method section. A & B are two identical experiments using PMN from different individuals. (a) and (b) PVDF membranes from the above experiments were probed using p38-specific antibody in order to check that equal amonuts of p38 MAPK were present in each lane and the autoradiographs are shown in (a) and (b).

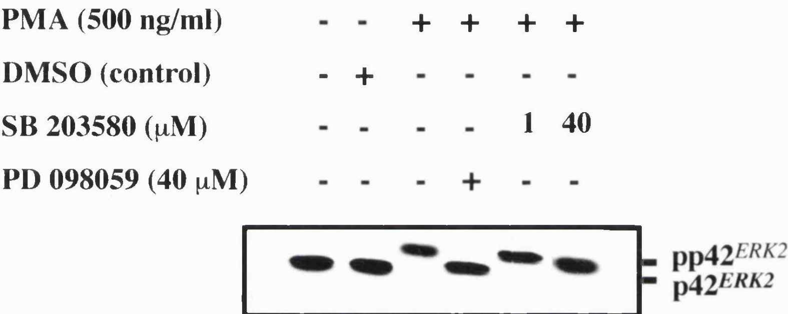
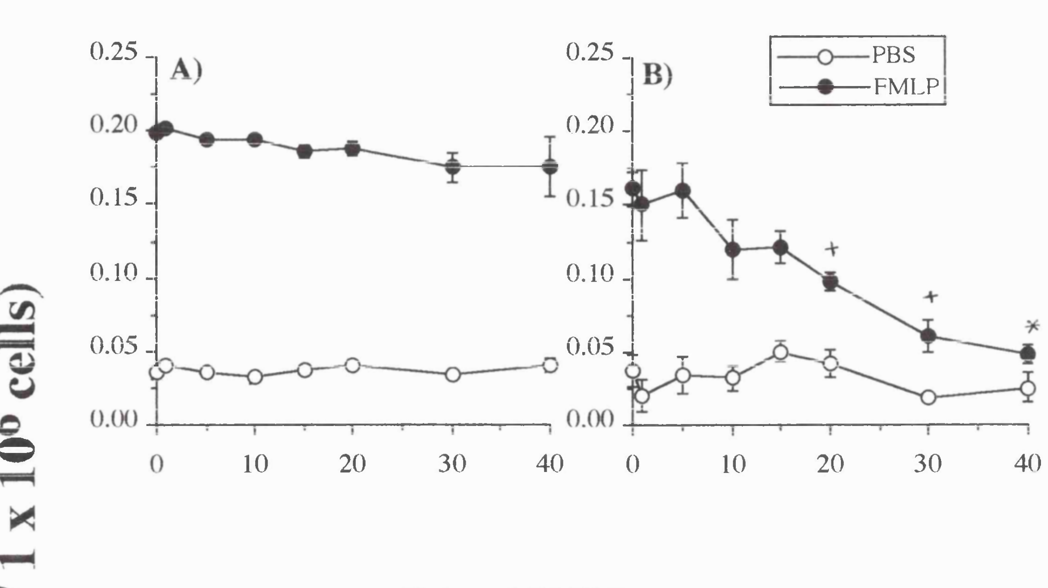
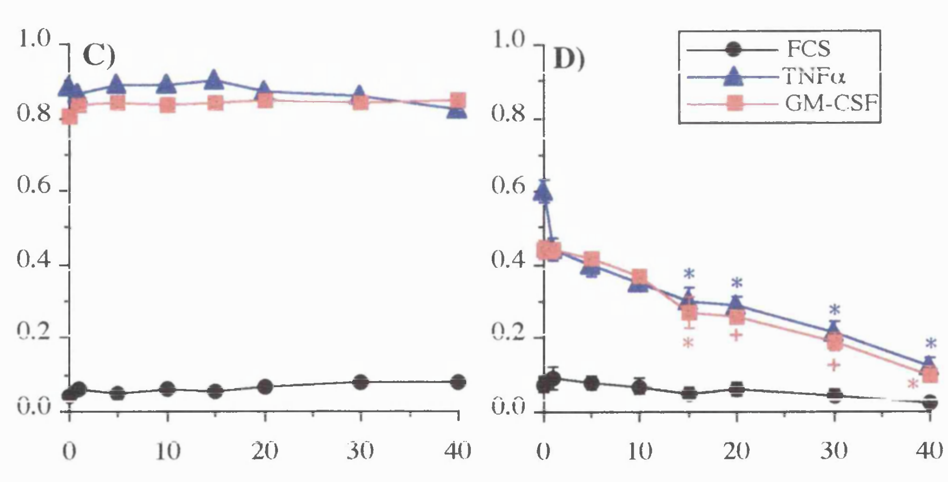



**Figure 5.12: Effect of SB 203580 on phosphorylation of p42$^{ERK2}$ stimulated by PMA in neutrophils.** Purified human neutrophils were treated with either PD 098059, SB 203580 or diluent control (DMSO) prior to stimulation with PMA (500 ng/ml) for 5 minutes at 37°C. Cell lysates were resolved on 12.5% SDS/PAGE and the blot was probed with p42$^{ERK2}$ antibody. Autoradiograph shown is from a single experiment.

## Unprimed FMLP response




## Primed FMLP response




## PMA response

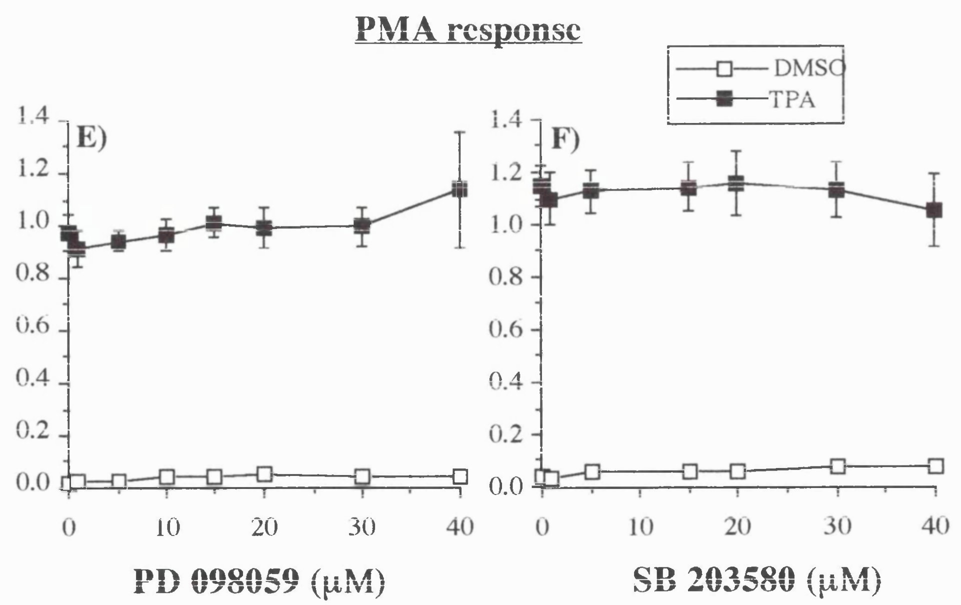


**Figure 5.13: Effect of MEK and p38 MAPK inhibitors on superoxide production in neutrophils stimulated with FMLP**. The cells were preincubated with different concentrations of either PD 098059 or SB 203580 before stimulation with 1 μM FMLP as described in the Method section. The differences in cytokine-primed superoxide release in samples that were not exposed to the inhibitors shown in (C) and (D) is due to differential response of neutrophils from different individuals used in this study. The data shown are the mean ± 1 SE of (A) 4 experiments, (B) and (C) 3 experiments, (D) 3-7 experiments, (E) 3 experiments and (F) 6-8 experiment. The absolute values for superoxide production in the absence of any inhibitor (DMSO) shown in A) is 1.6 ± 0.2 and B) is 1.8 ± 0.5 nmoles/$10^6$ cells/6 minutes. Significant differences are shown as: $^+P<0.05$, $^*P<0.01$

statistically significantly different from the DMSO diluent control. In contrast, preincubation with SB 203580 caused a dose-dependent inhibition of the FMLP-stimulated respiratory burst. Statistically significant inhibition was observed at 20 μM SB 203580. As shown in Figure 5.13 B, SB 203580 at 30 μM (35% ± 11% of DMSO control) and at 40 μM (18% ± 7% of DMSO control) showed a greater inhibitory effect than PD 098059 (Table 5.3).

*ii) primed response*

The unprimed neutrophils released a modest amount of superoxide when stimulated with FMLP (2.0 ± 0.5 nmoles/$10^6$ cells/6 minutes) (as shown in Figure 5.13 A and B). However, primed neutrophils exhibit a markedly enhanced release of superoxide production when stimulated by FMLP (41 ± 1 and 37 ± 2 nmoles/$10^6$ cells/6 minutes when primed with either TNFα or GM-CSF, respectively) (as shown in Figure 5.13 C and D). The effect of the MAP kinase inhibitors on the FMLP-induced respiratory burst in neutrophils that were pretreated with cytokines was analysed. As the extent of priming was different in the two sets of experiments with PD 098059 and SB 098059, the data were standardised by expressing the values for samples treated with inhibitors as a percentage of the value for samples treated with the diluent alone. As shown in Figure 5.13 C treatment of neutrophils with PD 098059 prior to priming with either TNFα (95% ± 3% of no PD 098059 control) or GM-CSF (104% ± 1% of no PD 098059 control) had no effect on the FMLP-stimulated respiratory burst (Table 5.4), even at the highest concentrations of PD 098059 which were shown to inhibit total $p42^{ERK2}$ activity (Figure 5.2 PD dose response).
Treatment of neutrophils with SB 203580 inhibited the respiratory burst of neutrophils primed with either TNFα or GM-CSF (Figure 5.13 D). As shown in Table 5.5 inhibition of superoxide production expressed as a percentage of control (i.e. no inhibitor) was 19% ± 6% and 20% ± 6%, respectively, for samples primed with TNFα (500 U/ml) or GM-CSF (10 ng/ml) in the presence of 40 μM SB 203580.

*iii) PMA response*

As shown in Figure 5.13 E and F, preincubation of neutrophils with either DMSO diluent, PD 098059 or SB 203580 in a concentration range of 0-40 μM for 30 minutes at 37°C, did not inhibit the respiratory burst activity induced by PMA. These data indicate that the inhibitory effect of SB 203580 on FMLP superoxide responses was not due to any non-specific toxic effects of the inhibitors on neutrophil respiratory burst activity.

**Table 5.3: Effect of MEK and p38 MAPK inhibitors on FMLP-stimulated respiratory burst in unprimed neutrophils**

| *PD 098059* (*μM*) | $O_2^{\cdot -}$ *Production* (% of control) | *SB 203580* (*μM*) | $O_2^{\cdot -}$ *Production* (% of control) |
|---|---|---|---|
| 0 | 100 ± 0 | 0 | 100 ± 0 |
| 1 | 101 ± 5 | 1 | 96 ± 20 |
| 5 | 96 ± 4 | 5 | 102 ± 2 |
| 10 | 96 ± 4 | 10 | 71 ± 18 |
| 15 | 92 ± 7 | 15 | 57 ± 17 |
| 20 | 91 ± 16 | 20 | 46 ± 1$^{+}$ |
| 30 | 85 ± 10 | 30 | 35 ± 11$^{+}$ |
| 40 | 86 ± 14 | 40 | 18 ± 7* |

Superoxide production in neutrophils (1 x $10^6$ cells/ml) stimulated with 1 μM FMLP following preincubation at 37°C with various concentrations of PD 098059 (10 minutes) or SB 203580 (30 minutes). Basal release of superoxide anions in unstimulated samples was subtracted from the FMLP-stimulated values, and the data expressed as the percentage of no inhibitor (i.e. DMSO control). The data shown are the mean ± 1 SE of three experiments with PD 098059 and four experiments with SB 203580. The significance of difference between samples are shown as $^{+}P < 0.05$, $^{*}P < 0.01$.

**Table 5.4: Effect of MEK inhibitor, PD 098059, on cytokine mediated priming of the respiratory burst stimulated with FMLP in neutrophils**

| *PD 098059 (μM)* | *Superoxide Production* (% of control) | | |
|---|---|---|---|
| | *FCS* | *TNFα* | *GM-CSF* |
| 0 | 100 ± 0 | 100 ± 0 | 100 ± 0 |
| 1 | 105 ± 6 | 98 ± 1 | 101 ± 1 |
| 5 | 99 ± 2 | 99 ± 1 | 102 ± 1 |
| 10 | 100 ± 2 | 97 ± 3 | 102 ± 1 |
| 15 | 94 ± 9 | 100 ± 1 | 101 ± 2 |
| 20 | 91 ± 11 | 98 ± 2 | 103 ± 1 |
| 30 | 81 ± 15 | 97 ± 2 | 103 ± 1 |
| 40 | 71 ± 18 | 95 ± 3 | 104 ± 1 |

The ability of PD 098059 to inhibit cytokine, [TNFα (500 U/ml), GM-CSF (10 ng/ml)] or diluent (0.01% FCS)-primed FMLP-stimulated superoxide production in neutrophils was measured at 37°C. Basal release of $O_2^-$ in unstimulated samples was subtracted from the FMLP-stimulated values, and the data expressed as the percentage of no PD 098059 (i.e. DMSO control). The data shown are the mean ± 1 SE of three individual experiments. There was no significant difference between samples with or without inhibitor (i.e. $P > 0.05$ in all experiments).

**Table 5.5: Effect of p38 MAPK inhibitor, SB 203580, on cytokine mediated priming of the respiratory burst stimulated with FMLP in neutrophils**

| *SB 203580 (μM)* | *Superoxide Production* (% of control) *FCS* | *TNFα* | *GM-CSF* |
|---|---|---|---|
| 0 | 100 ± 0 | 100 ± 0 | 100 ± 0 |
| 1 | 106 ± 4 | 79 ± 4 | 97 ± 2 |
| 5 | 99 ± 7 | 71 ± 2 | 94 ± 6 |
| 10 | 84 ± 17 | 63 ± 4 | 84 ± 7 |
| 15 | 71 ± 14 | 57 ± 9* | 66 ± 6* |
| 20 | 71 ± 16 | 50 ± 5* | 60 ± 8+ |
| 30 | 38 ± 2 | 33 ± 4* | 44 ± 7+ |
| 40 | 34 ± 6 | 19 ± 6* | 20 ± 6* |

Effect of various doses of SB 203580 on superoxide production in neutrophils (1 x $10^6$ cells/ml) stimulated with 1 μM FMLP following preincubation with 500 U/ml TNFα, 10 ng/ml GM-CSF or 0.01% FCS at 37°C. Basal release of $O_2^-$ in unstimulated samples was subtracted from the FMLP-stimulated values, and the data expressed as the percentage of no SB 203580 (i.e. DMSO control). The data shown are the mean ± 1 SE of 4-8 experiments. Significant differences between samples incubated with or without SB 203580 are shown as: $^+P< 0.05$, $^*P< 0.01$.

### 5.3.4 Effect of a combination of both PD 098059 and SB 203580 on respiratory burst activity in neutrophils

Neutrophil samples were treated with a combination of both PD 098059 and SB 203580 prior to priming with cytokines followed by stimulation with FMLP. In order to examine the effect of blocking both $p42^{ERK2}$ and p38 MAPK signalling pathways simultaneously on superoxide production. Figure 5.14 shows that the combination of PD 098059 and SB 203580 was inhibitory to the FMLP but not the PMA response. However, it should be noted that the extent of priming by TNFα and GM-CSF was greater in these experiments than in the priming experiments shown in Figure 5.13 D, making a comparison of the absolute data difficult to perform. Therefore the data were standardised by re-expressing them as a percentage of no inhibitor (control). Table 5.6 shows that both inhibitors blocked superoxide production to slightly greater degree than incubation with either inhibitor alone. For example Table 5.5 show that 20 μM SB 203580 inhibited TNFα or GM-CSF primed superoxide responses to 50% ± 5% and 60% ± 8% of the DMSO control, respectively whereas, PD 098059 at 20 μM had no inhibitory effect. The combination of inhibitors at 20 μM inhibited TNFα or GM-CSF primed responses to 34% ± 7% and 40% ± 5% of the DMSO control, respectively, which was statistically significantly greater than either inhibitor used alone.

Experiments were performed in parallel to demonstrate that the inhibitory effect of both PD 098059 and SB 203580 used in combination was not due to toxicity. Neutrophils were preincubated with both PD 098059 and SB 203580 prior to stimulation with PMA (500 ng/ml). As shown in Figure 5.14 B the combination of inhibitors did not block superoxide production.

### 5.3.5 Comparison of the kinetics of the FMLP-stimulated respiratory burst with the kinetics of $p42^{ERK2}$ and p38 MAPK activation by FMLP

The kinetics of superoxide production in unprimed neutrophils stimulated with FMLP was measured in a dual beam spectrophotometer by continuous measurement of the rate of cytochrome *c* reduction (Figure 5.15 A and B). The traces in Figure 5.15 show rapid stimulation of $O_2^-$ production following the addition of 1 μM FMLP (response detected within 10 seconds) with maximal linear production of superoxide being maintained for at least one minute, followed by down regulation to resting levels at about 2 minutes. These kinetics match those of p38 MAPK activation by the same agonist (Figure 5.10 A), whereas they do not match the sustained activation of

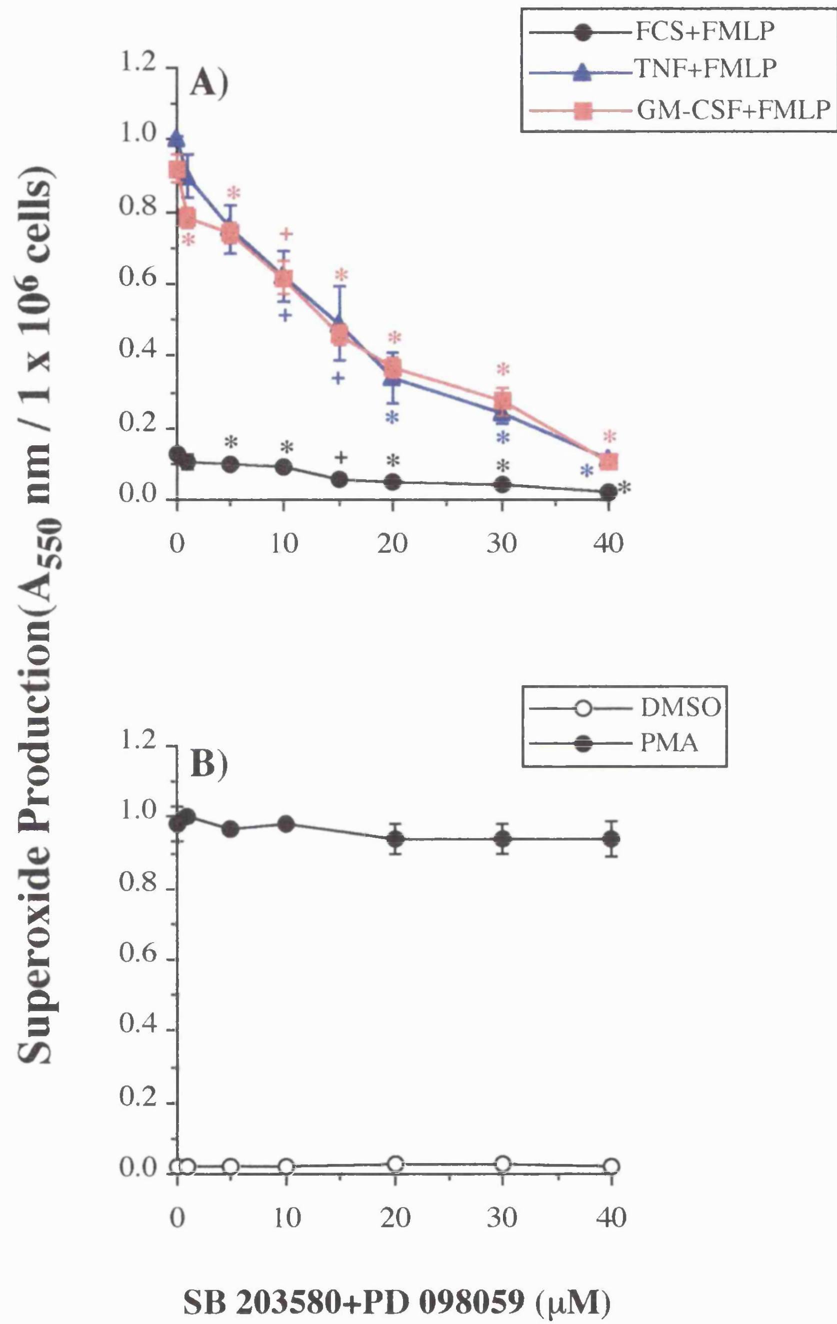



**Figure 5.14: Effect of PD 098059 + SB 203580 on cytokine-mediated primed superoxide production in neutrophils stimulated with FMLP.** Purified neutrophils were preincubated with different concentrations of both PD 098059 + SB 203580 prior to A) incubation with either TNFα (500 U/ml), GM-CSF (10 ng/ml), or 0.01% FCS followed by stimulation with 1μM FMLP at 37°C as described in the Method section. B) In control experiment neutrophils were stimulated with PMA (500 ng/ml) to show that the inhibitory effect in (A) is not due to toxicity of the inhibitors. The data shown are the mean ± 1 SE of 3 experiments. The significant differences between samples with or without the inhibitors are shown as: $^{+}P<0.05$, $^{*}P<0.01$

**Table 5.6: Effect of a combination of both MEK and p38 MAPK inhibitors on cytokine mediated priming of the respiratory burst stimulated with FMLP in neutrophils**

| *PD 098059 + SB 203580 (μM)* | *Superoxide Production* (% of control) | | |
|---|---|---|---|
| | *FCS* | *TNFα* | *GM-CSF* |
| 0 | 100 ± 0 | 100 ± 0 | 100 ± 0 |
| 1 | 88 ± 19 | 89 ± 5 | 85 ± 1* |
| 5 | 78 ± 2* | 75 ± 7 | 80 ± 1* |
| 10 | 71 ± 2* | 62 ± 7+ | 67 ± 7+ |
| 15 | 49 ± 6+ | 49 ± 10+ | 50 ± 4* |
| 20 | 40 ± 3* | 34 ± 7* | 40 ± 5* |
| 30 | 32 ± 3* | 24 ± 4* | 30 ± 5* |
| 40 | 19 ± 2* | 11 ± 1* | 12 ± 2* |

Effect of both PD 098059SB and SB 203580 on superoxide production in neutrophils (1 x $10^6$ cells/ml) stimulated with 1 μM FMLP following preincubation with either 0.01% FCS, 500 U/ml TNFα or 10 ng/ml GM-CSF at 37°C. Basal release of $O_2^-$ in unstimulated samples was subtracted from the FMLP-stimulated values, and the data expressed as the percentage of no inhibitor (i.e. DMSO control). The data shown are the mean ± 1 SE of 3 experiments. Significant differences between samples incubated with or without the inhibitors are shown as: $^+P < 0.05$, $^*P < 0.01$.

p42$^{ERK2}$ by FMLP shown in Figure 5.1 A. These data support the finding that stimulation of the respiratory burst by FMLP is via p38 MAPK.

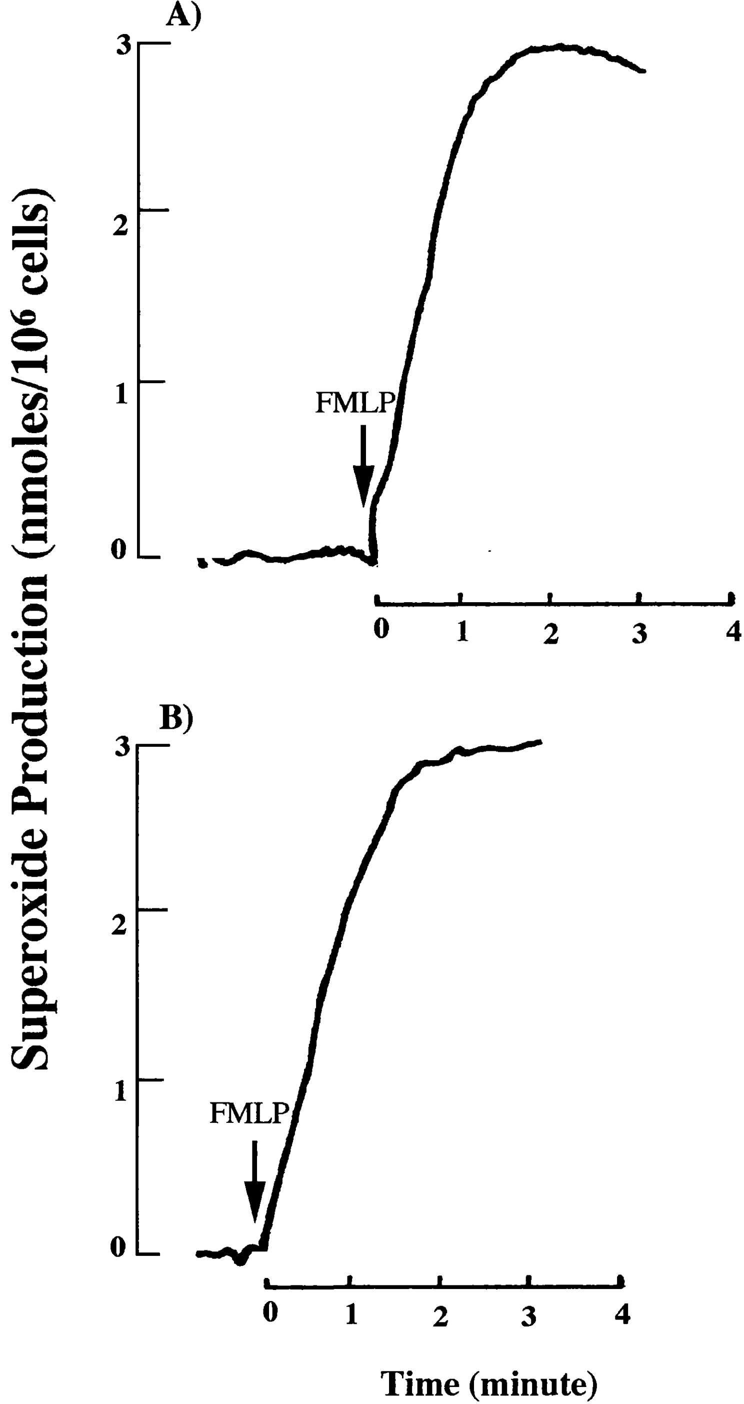



**Figure 5.15: Kinetics of superoxide production in neutrophils stimulated with FMLP.** Superoxide production was measured immediately after the addition of stimulus by the superoxide dismutase-inhibitable reduction of ferri-cytochrome *c* in a dual-beam spectrophotometer (model SP8-200, Pye-Unicam, Cambridge, England). The cuvettes contained purified neutrophils (3 x $10^6$ cells/ml) were pre-warmed for 5 minutes at 37°C prior to addition of FMLP (1μM). Two traces of continuous measurement of cytochrome *c* reduction are shown in (A) and (B) are from identical experiments using neutrophils from two different individuals.

## 5.4 DISCUSSION

The molecular mechanisms involved in the priming of the respiratory burst by GM-CSF and TNFα are not well understood. This study investigates the activation of the MAPK signal transduction pathways in cytokine-mediated priming of the respiratory burst in human neutrophils. At the time of doing this study others have been published similar data supporting our findings.

In this study analysis of the kinetics of $p42^{ERK2}$ showed that activation by either FMLP or PMA was extremely rapid as measured by gel retardation assay. Phosphorylation of $p42^{ERK2}$ was observed within 30 seconds of stimulation with FMLP and decreased to basal levels within 20 minutes. Whereas, the effect of PMA on phosphorylation of $p42^{ERK2}$ was detected within 1 minute which was sustained until at least 60 minutes. FMLP also activated p38 MAPK, but it was noted that unlike $p42^{ERK2}$, the phosphorylated form of p38 MAPK did not migrate slowly than the unphosphorylated form therefore, both total and phosphorylated p38 MAPK were detected by western blotting and also by p38 MAP kinase assay where immunoprecipitates of p38 MAPK were incubated with MAPKAPK-2 (substrate) in the presence of [$\gamma$-$^{32}$P]ATP. Both assays showed a rapid and transient activation of p38 MAPK with FMLP. These findings are in agreement with other reports indicating that FMLP stimulates both $p42^{ERK2}$ and p38 MAPK in neutrophils (Worthen *et al.*, 1994; Avdi *et al.*, 1996). In contrast, PMA did not show stimulation of p38 MAPK in my experiments when analysed by western blotting. However, El Benna *et al.*, (1996b) found that PMA stimulates p38 MAPK as determined by immunoblotting with anti-phosphotyrosine antibody. These data indicate that the time course of phosphorylation of $p42^{ERK2}$ by FMLP and PMA was rapid and sustained, whereas, phosphorylation of p38 MAPK by FMLP was maximal within 1 minute and undetectable within 5 minutes.

The development of the two specific inhibitors of $p42^{ERK2}$ and p38 MAP kinase, PD 098059 and SB 203580, has allowed the investigation of the roles of MAPK pathways in priming of the respiratory burst. PD 098059 inhibits MEK activation by either Raf kinase or MEKK1, thus preventing ERK activation (Alessi *et al.*, 1995). SB 203580 is one of a group of related compounds that is specific for inhibition of p38 MAPK (Cuenda *et al.*, 1995). SB 203580 has been shown to be selective against p38 and p38β MAPK but to have no inhibitory effect on SAPK3/SAPK4, $p42/p44^{MAPK}$ and JNK (Cuenda *et al.*, 1995; Goedert *et al.*, 1997). This is confirmed by the finding that different doses of SB 203580 did not inhibit phosphorylation of $p42^{ERK2}$ by PMA, whereas the effect of PD 098059 was inhibitory (Figure 5.12). Incubation of neutrophils with PD 098059 for various time points showed an

inhibitory effect within 10 minutes which was sustained for at least 60 minutes. This was important for the priming experiments where longer incubation periods with cytokines were required. The inhibitory effect of PD 098059 was observed at 1 μM and was complete at 10 μM when cells were stimulated by GM-CSF, whereas the PMA response was inhibited with 5 μM PD 098059 and completed with 20 μM indicating that the PMA response required 5 times more PD 098059 for inhibition of $p42^{ERK2}$ as compared to GM-CSF.

These two inhibitors, PD 098059 and SB 203580, were utilised in order to determine whether inhibition of either $p42^{ERK2}$ or p38 MAPK, respectively, could influence neutrophil functions. Our data has shown that $p42^{ERK2}$ is phosphorylated by FMLP and this was inhibited to below detectable levels by PD 098059. However, FMLP-stimulated respiratory burst activity was only minimally inhibited by PD 098059 to a level which was not significantly different from the control (86% ± 14% of the control $P>0.05$, n=3). This is supported by the report that ERK activation could be dissociated from FMLP-stimulated respiratory burst activity in HL-60 cells (Yu *et al.*, 1995). In contrast to our data, Avdi *et al.* (1996) reported inhibition of FMLP-stimulated release of superoxide anion from cytochalasin B-treated neutrophils when cells were preincubated with 10 μM PD 098059 for one hour at 37°C. However, cytochalasin-B, an alkaloid from fungi which interferes with the assembly of actin filaments, has profound and non-physiological effects on neutrophils. In comparison SB 203580 showed an inhibitory effect on the unprimed FMLP-stimulated respiratory burst (18% ± 7% of the control with 40 μM SB 203580, $P<0.01$, n=4) but not basal activity. The rapid stimulation of p38 MAPK activation by FMLP shown in the kinase assay using MAPKAPK-2 as the downstream substrate, was similar to the time course of superoxide generation elicited by FMLP suggesting that p38 MAPK activation is necessary for stimulation of the respiratory burst. This conclusion is supported by a report that p38 MAPK inhibition blocked FMLP-stimulated superoxide release in a concentration-dependent manner (Nick *et al.*, 1997). The concomitant activation of p38 MAPK and superoxide anion generation suggests that p38 MAPK activation may be necessary for stimulation of respiratory burst activity. Additionally p38 MAPK phosphorylates $p47^{phox}$, a component of NADPH oxidase (El Benna *et al.*, 1996b). However, the report that p38 MAPK inhibition blocked FMLP-stimulated superoxide production in a concentration dependent manner supported this hypothesis (Nick *et al.*, 1997). Our data indicate the involvement of p38 MAPK but not $p42^{ERK2}$ in the FMLP-stimulated respiratory burst. This is in accord with the finding of Yu *et al.* (1995) demonstrating that an increased intracellular concentration of cAMP can inhibit the FMLP-stimulated respiratory burst, while not affecting the FMLP-induced activation of ERK1 and ERK2 in either HL-60 cells differentiated with DMSO or

mature neutrophils. Therefore, under these conditions ERK activation could be dissociated from FMLP-stimulated superoxide production in neutrophils and HL-60 cells. Other studies have shown inhibition of ERK activity by PD 098059 was associated with significant inhibition of the FMLP-stimulated respiratory burst in neutrophils (Avdi *et al.*, 1996; Rane *et al.*, 1997). In contrast, a report by Zu *et al.* (1998) has showed that blocking of neutrophil p44/p42 MAP kinase with PD 098059 had no inhibitory effect on the FMLP-induced respiratory burst.

PMA, an agonist that bypasses receptors on the cell surface, elicits maximal superoxide release in neutrophils. PMA phosphorylates $p42^{ERK2}$ and this was blocked by PD 098059. However, neither PD 098059 nor SB 203580 inhibited superoxide production stimulated by PMA suggesting a $p42^{ERK2}$/p38 MAPK-independent activation of the respiratory burst by this agonist. This finding is in contrast with a study by El Benna *et al.* (1996b) showing phosphorylation of p38 MAPK by immunoprecipitation of $^{32}$P-labelled p38 MAPK from neutrophils that had been loaded with $^{32}P_i$ before stimulation by PMA. The phosphorylation of p38 MAPK by PMA has also been confirmed by immunoblotting with the anti-phosphotyrosine antibody (Nick *et al.*, 1996; El Benna *et al.*, 1996b). It has been shown that p38, like ERK, can be activated both by a PKC-independent pathway and by a pathway dependent on a G protein-coupled receptor such as, FMLP (El Benna *et al.*, 1996b). In another study it has been shown that inhibition of PKC had no effect on Ras or Raf activation by FMLP in human neutrophils, although PMA is capable of activating Raf (Worthen *et al.*, 1994). The lack of inhibition of the PMA-stimulated respiratory burst by either inhibitors showed that these inhibitors were not toxic to neutrophils.

Activation of the respiratory burst during the process of phagocytosis is crucial for the production of antimicrobial reactive oxygen metabolites. Activation of this effector function in mature neutrophils requires a priming step by cytokines such as TNFα and GM-CSF. This study shows that stimulation of neutrophils with TNFα and GM-CSF results in activation of both $p42^{ERK2}$ and p38 MAPK when analysed by SDS/PAGE and western blotting. TNFα activates $p42^{ERK2}$ weakly within 10 minutes whereas, stimulation with GM-CSF was of much longer duration. This is confirmed by Coffer *et al.* (1998) who found that both TNFα and GM-CSF activate $p42^{ERK2}$ and that TNFα activates ERK2 transiently and weakly. Activation of p38 MAPK by TNFα was also transient which is in agreement with Waterman and Sha'afi (1995) finding that TNFα caused phosphorylation of a 40 kDa protein, possibly p38 MAPK. Although there was differential activation of the two MAPK pathways by TNFα and GM-CSF both these factors prime the respiratory burst to an equal extent when the optimal doses of these cytokines where used. This confirms the data shown in Chapter 3, Figure 3.8 using the whole blood assay. This is in accord with previous data that

although the magnitude of priming is the same, the kinetics of priming by TNFα and GM-CSF are different (Khwaja *et al.*, 1992; Roberts *et al.*, 1993a). PD 098059 inhibited the activation of $p42^{ERK2}$ when neutrophils were stimulated by either TNFα or GM-CSF. The use of various concentrations of PD 098059 in order to inhibit $p42^{ERK2}$ activation did not affect the respiratory burst of neutrophils when cells were primed with either GM-CSF or TNFα. This is in contrast with findings of El Benna *et al.*, (1996b) suggesting that MAPK can phosphorylate $p47^{phox}$ *in vitro* and hence, the activation of the respiratory burst. Therefore, our data indicate that the $p42^{ERK2}$ signalling pathway does not have an important role in activation of cytokine-mediated priming of respiratory burst. In contrast to the effect of PD 098059, the addition of SB 203580 inhibited FMLP-stimulated superoxide production with the same dose response of inhibition for both TNFα or GM-CSF. McLeish *et al.*, (1998), reported that PD 098059 and SB 203580 had no inhibitory effect on FMLP-stimulated superoxide, while these two inhibitors did significantly attenuate the TNFα and GM-CSF priming of superoxide generation stimulated by FMLP. These data indicate that the site of inhibition of SB 203580 is more likely to be at the level of the oxidase itself, as it inhibits both the unprimed and primed responses.

It is possible that another MAP kinase family member may be involved. A third proline-directed kinase present in human neutrophils is known as JNK. With regard to activation of JNK in human neutrophils controversial results are reported. It has been reported that FMLP does not activate JNK in normal human neutrophils (Nick *et al.*, 1997; Suzuki *et al.*, 1999), whereas a recent report shows that FMLP activates JNK in mature HL-60 cells differentiated by DMSO (Rane *et al.*, 1997).

Collectively these data indicate that in both unprimed and primed neutrophils, FMLP activates the respiratory burst via a p38 MAPK but not a $p42^{ERK2}$-dependent pathway.

# CHAPTER 6

# INVESTIGATION OF THE IMPORTANCE OF THE MITOGEN-ACTIVATED PROTEIN KINASE PATHWAYS IN THE PRIMING OF PHOSPHOLIPASE $A_2$ IN HUMAN NEUTROPHILS

## 6.1 INTRODUCTION

The phospholipase $A_2$s ($PLA_2$) are a diverse family of enzymes which catalyse the hydrolysis of *sn*-2 fatty acyl ester bond of glycerophospholipids producing free fatty acid and lysophospholipid. $PLA_2$ has a pivotal role in regulating phospholipid metabolism and liberation of arachidonic acid (AA) from cellular phospholipids. Many different cell types including neutrophils liberate AA in response to stimulation. AA is a rate-limiting precursor for the biosynthesis of biologically active lipids such as prostaglandins, leukotrienes, thromboxane $A_2$ and platelet-activating factor (PAF) which play an important role in host-defence and inflammatory reactions (Roshak *et al.*, 1994; Bauldry *et al.*, 1996; Tithof *et al.*, 1998).

In resting cells, most of the $PLA_2$ is localised in the cytoplasm and the release of AA from membrane phospholipids is rather low (basal activity). AA can act as signal transduction molecule, as it activates enzymes such as protein kinase C (McPhail *et al.*, 1984; Nishizuka, 1992), MAP kinase (Rao *et al.*, 1994), neutral sphingomylinase (Jayadev *et al.*, 1994) and also increases intracellular calcium levels (Smith *et al.*, 1987; van der Zee *et al.*, 1995) and gene expression (Rizzo *et al.*, 1995). In addition, AA has an influential effect on both the activation and priming of the respiratory burst when stimulated by FMLP. It was previously shown that inhibition of $PLA_2$ activity by mepacrine, an inhibitor of $sPLA_2$, was associated with inhibition of the FMLP stimulated respiratory burst in neutrophils whether these were primed with cytochalasin B or GM-CSF (Roberts *et al.*, 1996).

Multiple forms of mammalian $PLA_2$ have been identified in phagocytic cells (Dennis, 1997) and depending on their mode of action are divided into; 1) low molecular weight, secretory $PLA_2$ ($sPLA_2$) the structure of which is stablised by disulphide bonds and require calcium for catalysis (Barbour & Dennis, 1993). $sPLA_2$ can exist as an extracellular form in inflammatory exudates and in a cell-associated form, 2) cytosolic $PLA_2$ ($cPLA_2$) which requires submicromolar concentrations of calcium for translocation rather than for catalysis and has a preference for arachidonic acid (AA) (Kramer *et al.*, 1991; Clark *et al.*, 1990) and 3) calcium-independent $PLA_2$ ($iPLA_2$) (Balsinde and Dennis, 1997) (see Chapter 1 for details). It has been shown that according to the cell type different isoforms of $PLA_2$ are expressed and some cells are capable of expressing multiple isoforms, such as macrophages that express $sPLA_2$, $cPLA_2$ and $iPLA_2$ (Balsinde and Dennis, 1996).

Receptor mediated stimulation of many different cell types activates $PLA_2$. In human granulocytes, receptor mediated stimulation of $PLA_2$ causes little release of AA (Bauldry *et al.*, 1988), but incubation with certain cytokines including TNF$\alpha$ (Seeds *et al.*, 1998) and GM-CSF (DiPersio *et al.*, 1988; Durstin *et al.*, 1994; Roberts *et al.*,

1996) enhances $PLA_2$ activity in a process known as "priming". The mechanism of cytokine-priming of different isoforms of $PLA_2$ has not been fully elucidated, but it is known that it is not dependent on new protein synthesis. However, it has been shown that primed neutrophils release increased amounts of AA by activation of membrane receptors (Volpi *et al.*, 1985; Bauldry *et al.*, 1991a; McDonald *et al.*, 1993). At the present it is not known which isoforms of $PLA_2$ are activated during priming, but there is evidence for activation of both $cPLA_2$ and $sPLA_2$ by TNFα in neutrophils (Seeds *et al.*, 1998). Other $PLA_2$ enzymes might be involved in AA release such as $iPLA_2$ (Larsson *et al.*, 1998).

Different regulatory mechanisms are involved in the activation of $cPLA_2$. One is an increase in intracellular calcium levels which is required for translocation of $cPLA_2$ from the cytosol to the cell membranes (Clark *et al.*, 1991; Schievella *et al.*, 1995), and this is mediated by the N-terminal calcium-dependent phospholipid binding domain (CaLB) (Channon and Leslie, 1990; Nalefski *et al.*, 1994). However many cytokines that prime $PLA_2$ including TNFα, do not cause an increase in cytosolic calcium (Bauldry *et al.*, 1991) so a rise in calcium may not be involved the priming process. Another mechanism of activating $PLA_2$ is by the phosphorylation of serine residues 437, 454, 505 and 727 (de Carvalho *et al.*, 1996). Serine 505 is important in activation of $cPLA_2$ *in vivo* since overexpression of mutant $cPLA_2$ (replacement of serine 505 with alanine ) in Chinese hamster ovary cells fails to enhance agonist-induced arachidonic acid release compared to the expression of wild type enzyme (Lin *et al.*, 1993). Furthermore, serine 505 in the $cPLA_2$ sequence is a major phosphorylation site which resides within the MAPK consensus sequence (Pro-Leu-$Ser^{505}$-Pro) and therefore enhancement of the MAPK activity by priming agents such as GM-CSF may be important for the priming of $cPLA_2$ activity. Another proline-directed kinase that phosphorylates $cPLA_2$ is p38 MAPK (Kramer *et al.*, 1996; Börsch-Haubold *et al.*, 1998), however the p38 MAPK inhibitor, SB 203580, was shown to partially inhibit the phosphorylation of both serine 505 and serine 727, suggesting that other kinases may be involved (de Carvalho *et al.*, 1996). Nemenoff *et al.* (1993) reported that purified $cPLA_2$ can be phosphorylated by either purified PKC or MAPK.

In the previous chapter (Chapter 5), it was shown that the priming effect of TNFα and GM-CSF on the respiratory burst of neutrophils was mediated via p38 MAPK rather than the $p42^{ERK2}$ pathway, based on the selective activities of the two inhibitors, PD 098059 and SB 203580. Therefore, these inhibitors were used to investigate whether inhibition of cytokine-primed $PLA_2$ activity leads to a similar pattern of inhibition to priming of the respiratory burst. Therefore neutrophils were primed with either TNFα or GM-CSF and stimulated with FMLP in the presence or

absence of the MAPK inhibitors. If the same pattern of inhibition was observed, this would be a further evidence that $PLA_2$ and the NADPH oxidase in neutrophils were primed via similar mechanisms.

## 6.2 MATERIALS AND METHODS

Methods

### 6.2.1 Assay of $PLA_2$ activity

Both the incorporation and release of $[^3H]$-AA from neutrophils were analysed as described in detail in Chapter 2. Radiolabelled neutrophil samples (0.5ml) in duplicate at $1 \times 10^6$ cells/ml were incubated with the 5-lipoxygenase inhibitor, MK886 (200 nM final concentration) for 5 minutes at 37°C (this blocked the downstream metabolism of AA and maximised the recovery of $[^3$-H]-AA). This was followed by addition of either DMSO (diluent control), PD 098059 for 10 minutes or SB 203580 for 30 minutes (at various doses as indicated in the text). Then cells were primed with either cytokine diluent (0.01% FCS), TNFα (500 U/ml) or GM-CSF (10 ng/ml) for 20 minutes, followed by stimulation with either calcium ionophore, A23187 (1 μM), or FMLP (1μM) in the presence of BSA (1mg/ml) for 15 minutes. The reaction was terminated by placing the samples on ice for 10 minutes. The samples were then centrifuged at 12000 x g for 4 minutes and aliquots of the supernatants were assayed for radioactivity by liquid scintillation spectroscopy.

### 6.2.2 Analysis of $p42^{ERK2}$ and p38 MAPK activation

Activated $p42^{ERK2}$ was measured by gel retardation assay and western blotting whereas p38 MAPK activity was measured by blotting with either rabbit-anti-human p38 antibody or goat-anti-human p38 (phospho-specific) antibody which detects p38 MAP kinase only when phosphorylated on tyrosine 182 and threonine 180 as described in Chapter 5, section 5.2.

### 6.2.3 Statistical analysis of data

Where appropriate, the significance of the differences between samples were analysed by paired Student's *t*-test and were considered significant when $P<0.05$.

## 6.3 RESULTS

### 6.3.1 Effect of MEK1 and p38 MAPK inhibitors on $PLA_2$ activity in human neutrophils

To investigate the role of $p42^{ERK2}$ and p38 MAPK in mediating the activation of $PLA_2$ in unprimed and primed neutrophils stimulated with either calcium-ionophore or FMLP, selective inhibitors of these two respective MAPK pathways, PD 098059 and SB 203580 were used. It was previously demonstrated in Chapter 5 that PD 098059 and SB 203580 were effective inhibitors of $p42^{ERK2}$ and p38 MAPK, respectively, in the neutrophil system.

*i) FMLP response*

In order to test whether activation of $p42^{ERK2}$ was required for $PLA_2$ activity in human neutrophils, an inhibitor of MEK1, PD 098059 (as described in Chapter 5) was used to block $p42^{ERK2}$ activity and look at the effect on AA release. Purified neutrophils were incubated with either PD 098059, SB 203580 (at various concentrations) or DMSO diluent control for 10 minutes or 30 minutes respectively, and AA release after priming with cytokines and activation with FMLP (1μM) was measured (see Chapter 2 for details). Firstly it was noted that there was very little $PLA_2$ activity in unprimed neutrophils when these were stimulated with FMLP. The basal AA release was 2250 ± 190 cpm/1 x $10^6$ cells and the unprimed AA release stimulated by FMLP was 2405 ± 225 cpm/1 x $10^6$ cells (mean ± 1SE, n=8). The mean TNFα-primed response was 3180 ± 77 (mean ± 1SE, n=4) and the mean primed GM-CSF response was 4644 ± 1004 (mean ± 1SE, n=4, absolute data). As shown in Figure 6.1 A and 6.2 A, preincubation of neutrophils with PD 098059 in a concentration range of 1-40 μM inhibited AA release in a dose-dependent manner. Maximal inhibition was achieved at 15 μM and 10 μM PD 098059 when cells were primed with either TNFα or GM-CSF, respectively (Table 6.1). These doses correlated with the doses required to inhibit $p42^{ERK2}$ activity as shown by gel retardation assay in Chapter 5, Figure 5.6, as maximum inhibition was observed with 10 μM PD 098059 when cells were stimulated with 10 ng/ml GM-CSF. In contrast SB 203880, a specific inhibitor of p38 MAPK, did not block AA release (Table 6.2) when cells were primed with either TNFα (Figure 6.1 B) or GM-CSF (Figure 6.2 B), when it was used at a concentration of 1-30 μM. In the previous chapter it was shown that neutrophil p38 MAPK activity was blocked by 1 μM SB 203580.

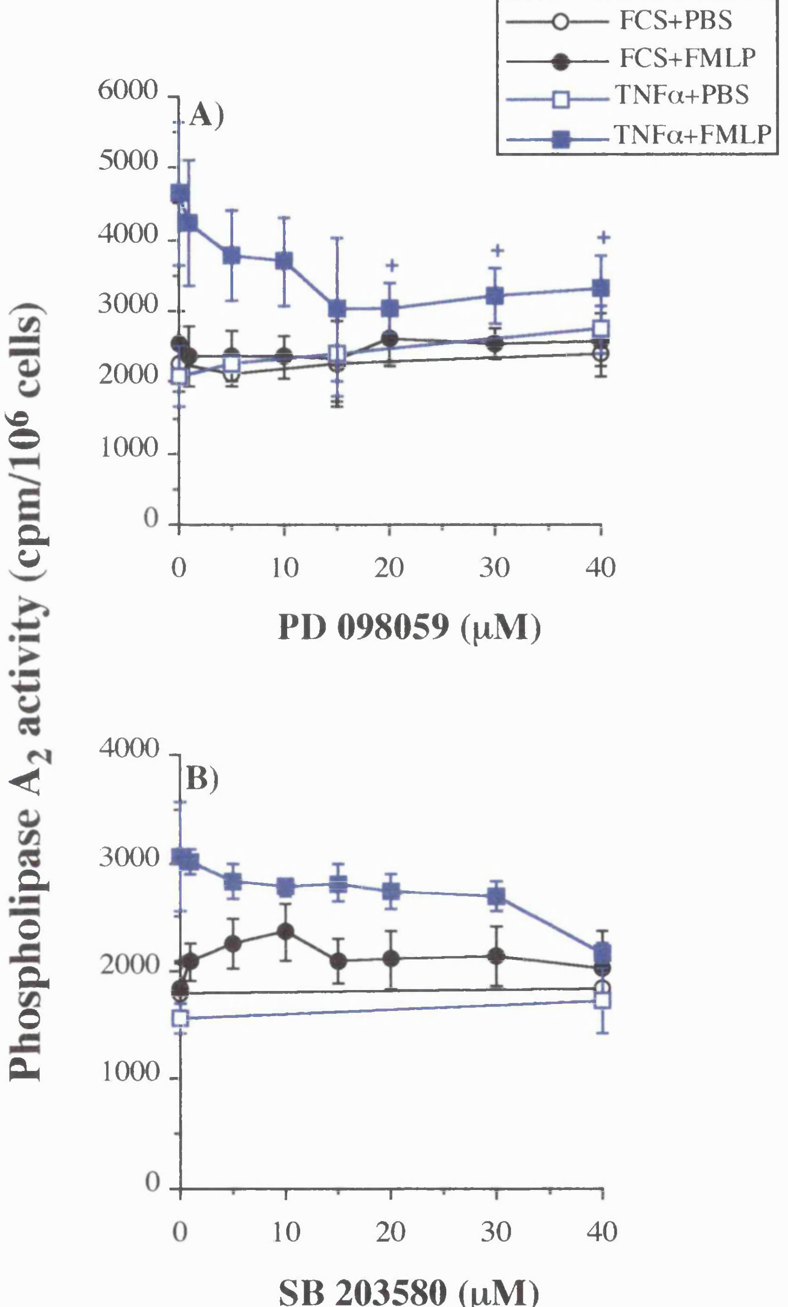



**Figure 6.1: Effect of MEK1 and p38 MAPK inhibitors on TNFα priming of AA release in neutrophils stimulated with FMLP.** Purified neutrophils were preincubated with different concentrations of either PD 098059 or SB 203580 before priming with TNFα (500 U/ml) followed by activation with 1 μM FMLP as described in Method section. The data shown are the mean ± 1 SE of (A) 4 experiments and (B) 3 experiments performed in duplicates. Note the difference in y-axis between A & B. When the absolute data for samples incubated with or without the inhibitors were compared by paired t-test there was significant differences which is shown as: $^{+}P$<0.05.

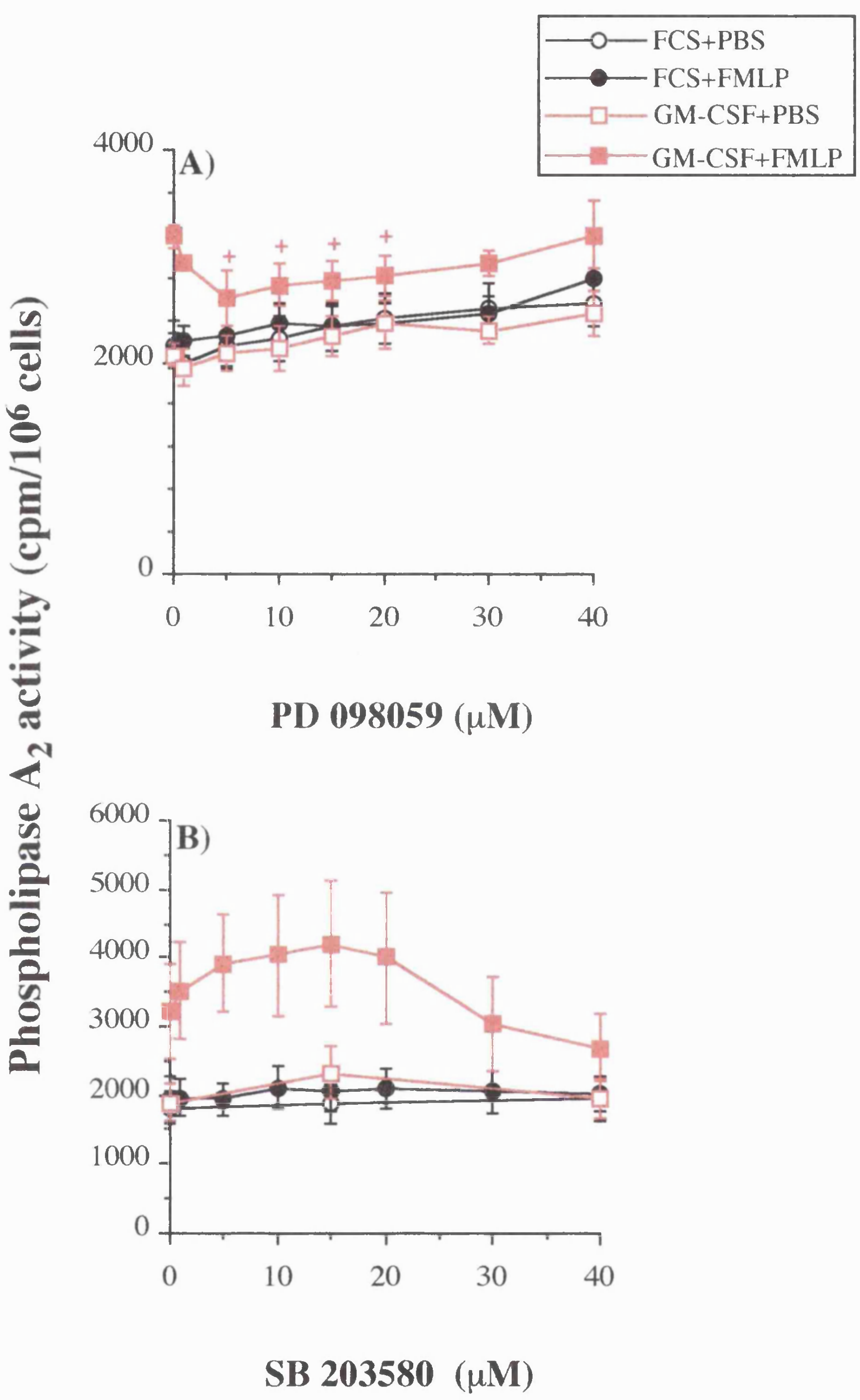



**Figure 6.2: Effect of MEK1 and p38 MAPK inhibitors on GM-CSF priming of AA release in neutrophils stimulated with FMLP.** Purified neutrophils were preincubated with different concentrations of either PD 098059 or SB 203580 before priming with GM-CSF (10 ng/ml) followed by with 1 μM FMLP as described in the Method section. The data shown are the mean ± 1 SE of (A) 4 experiments and (B) 3 experiments, performed in duplicates. Note that the difference in y-axis between A & B. There was a significant difference between samples incubated with or without PD 098059 when the absolute data were analysed (i.e. $^{+}P<0.05$).

**Table 6.1: Effect of the MEK inhibitor, PD 098059, on cytokine-mediated AA release in neutrophils stimulated by FMLP**

| *PD 098059 (μM)* | *AA release* (% of control) | | |
|---|---|---|---|
| | *FCS* | *TNFα* | *GM-CSF* |
| 0 | 100 ± 0 | 100 ± 0 | 100 ± 0 |
| 1 | 117 ± 56 | 80 ± 3* | 88 ± 10 |
| 5 | 119 ± 11 | 61 ± 3* | 49 ± 25 |
| 10 | 92 ± 21 | 52 ± 8+ | 50 ± 3* |
| 15 | 90 ± 16 | 53 ± 24 | 46 ± 8* |
| 20 | 94 ± 18 | 28 ± 6* | 40 ± 8+ |
| 30 | 226 ± 53 | 30 ± 4* | 57 ± 4* |
| 40 | 204 ± 48 | 26 ± 1* | 50 ± 20 |

Neutrophils were preincubated with PD 098059 for 10 minutes at 37°C before priming with either TNFα (500 U/ml), GM-CSF (10ng/ml) or diluent (0.01% FCS) for 20 minutes, followed by stimulation with FMLP (1 μM) for 15 minutes. Basal release of arachidonate in the unstimulated samples was subtracted from the FMLP-stimulated values, and the data were expressed as a percentage of the value for no inhibitor (i.e. DMSO). The data shown are the mean ± 1SE of 4 experiments with TNFα, 3 experiments with GM-CSF and 4 experiments with the FCS diluent control, performed with duplicate samples. The significant differences between the samples in the presence or absence of the PD 098059 is shown as: $^{+}P<0.05$, $^{*}P<0.01$.

**Table 6.2: Effect of the p38 MAPK inhibitor, SB 203580, on cytokine-mediated priming of $PLA_2$ in neutrophils stimulated with FMLP**

| *SB 203580 (μM)* | *AA release* (% of control) | | |
|---|---|---|---|
| | ***FCS*** | ***TNFα*** | ***GM-CSF*** |
| 0 | 100 ± 0 | 100 ± 0 | 100 ± 0 |
| 1 | 118 ± 41 | 136 ± 36 | 125 ± 11 |
| 5 | 88 ± 56 | 113 ± 27 | 159 ± 24 |
| 10 | 140 ± 24 | 107 ± 26 | 145 ± 18 |
| 15 | 147 ± 59 | 107 ± 23 | 149 ± 19 |
| 20 | 153 ± 5 | 109 ± 33 | 130 ± 19 |
| 30 | 88 ± 12 | 91 ± 20 | 78 ± 30 |
| 40 | 84 ± 6 | 34 ± 18 | 61 ± 12 |

Neutrophils were preincubated with SB 203580 for 30 minutes at 37°C before priming with TNFα (500 U/ml), GM-CSF (10 ng/ml) or diluent (0.01% FCS), followed by stimulation with FMLP (1μM) for 15 minutes. Basal release of arachidonate in unstimulated samples was subtracted from the FMLP -stimulated values, and the data were expressed as a percentage of the relevant no inhibitor control (i.e. DMSO). The data shown are the mean ± 1SE of 3 experiments with TNFα, 3 experiments with GM-CSF and 3-4 experiments with FCS diluent control, performed with duplicate samples. No significant difference between the samples incubated with or without SB 203580 was observed, (i.e. $P > 0.05$ in all experiments).

However, the highest concentration of SB 203580 that was used partially inhibited AA release, although this may well be due to non-specific toxicity.

*ii) A23187-response*

In an attempt to clarify the effects of PD 098059 and SB 203580 on neutrophil $PLA_2$ activity a more potent agonist than FMLP was used. Calcium-ionophore, A23187, is one of the most potent agonists of $PLA_2$ in neutrophils. As shown in Figures 6.3 and 6.4, the magnitude of AA release in response to A23187 was much greater than the response to FMLP. The mean ± SE for the basal AA release was 2012 ± 176 cpm/1 x $10^6$ cells and was 4484 ± 828 cpm/1 x $10^6$ cells when cells were stimulated with A23187. The mean TNFα-primed response was 7212 ± 1520 cpm/1 x $10^6$ cells (mean ± 1SE, n=4) as shown in Figure 6.3. The GM-CSF-primed response was 11145 ± 1772 cpm/1 x $10^6$ cells as shown in Figure 6.4. The absolute data were standardised by expressing them as a percentage of the control value with no inhibitor (i.e. DMSO) and these data are shown in Tables 6.3 and 6.4.

In contrast, SB 203580 had a stimulatory effect on TNFα primed AA release stimulated with A23187, although this effect was not statistically significant (158% ± 23% of DMSO control, $P>0.05$, n=4). Note, that the primed activity from SB203580-treated cells was increased. As shown in Figure 6.4 A, when neutrophil samples were primed with GM-CSF (10 ng/ml) 40 μM PD 098059 inhibited AA release (39% ± 7% of DMSO control, $P<0.01$, n=3), but SB 203580 only minimally inhibited the GM-CSF primed response at 40 μM (95% ± 9% of DMSO control, $P>0.05$, n=4) (Table 6.4).

### 6.3.2 Activation of MAP kinase pathways by arachidonic acid and calcium ionophore

*i) $p42^{ERK2}$*

To investigate the effect of calcium-ionophore, A23187, and arachidonate (AA) on the activation of $p42^{ERK2}$, purified neutrophils were stimulated with either AA (12.5 μM), A23187 (1 μM) or DMSO (diluent control) and phosphorylation of $p42^{ERK2}$ was measured by gel retardation assay and western blotting as described in Chapter 2. The result from two experiments has shown that both AA and calcium-ionophore induced phosphorylation of $p42^{ERK2}$ (Figures 6.5 A and B). AA activated a transient phosphorylation of $p42^{ERK2}$ which was detected 1 minute after stimulation, was maximal within 2 minutes and was undetectable at 10 minutes.

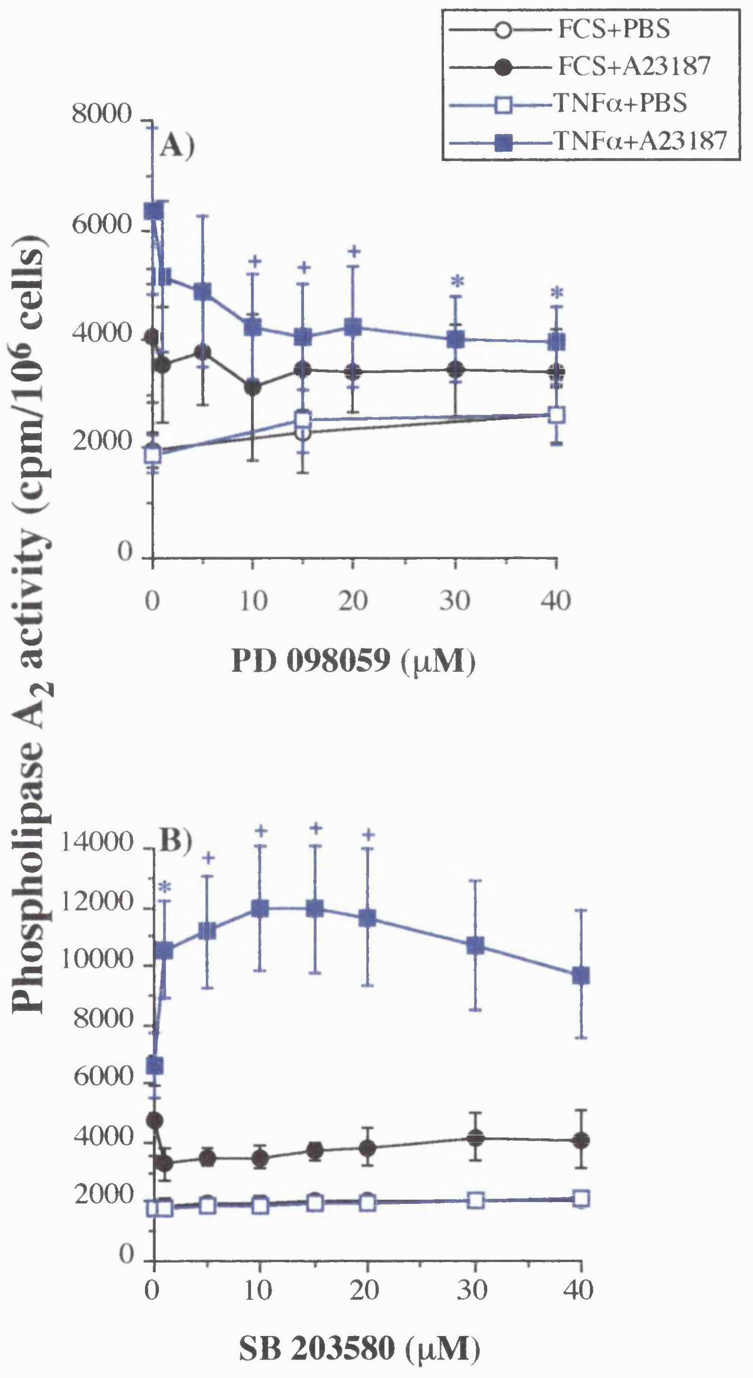



**Figure 6.3: Effect of MEK1 and p38 MAPK inhibitors on TNFα primed AA release in neutrophils stimulated with A23187**. Radiolabelled neutrophils were preincubated with different concentrations of either PD 098059 or SB 203580 before stimulation with TNFα (500 U/ml) followed by 1 μM A23187 as described in the Method section. The data shown are the mean ± 1 SE of 4 experiments, performed in duplicates. Note the difference in y-axis between A & B. There was a significant difference between samples incubated with or without the inhibitors when the absolute data were analysed by paired *t*-test ($^+P<0.05$, $^*P<0.01$).

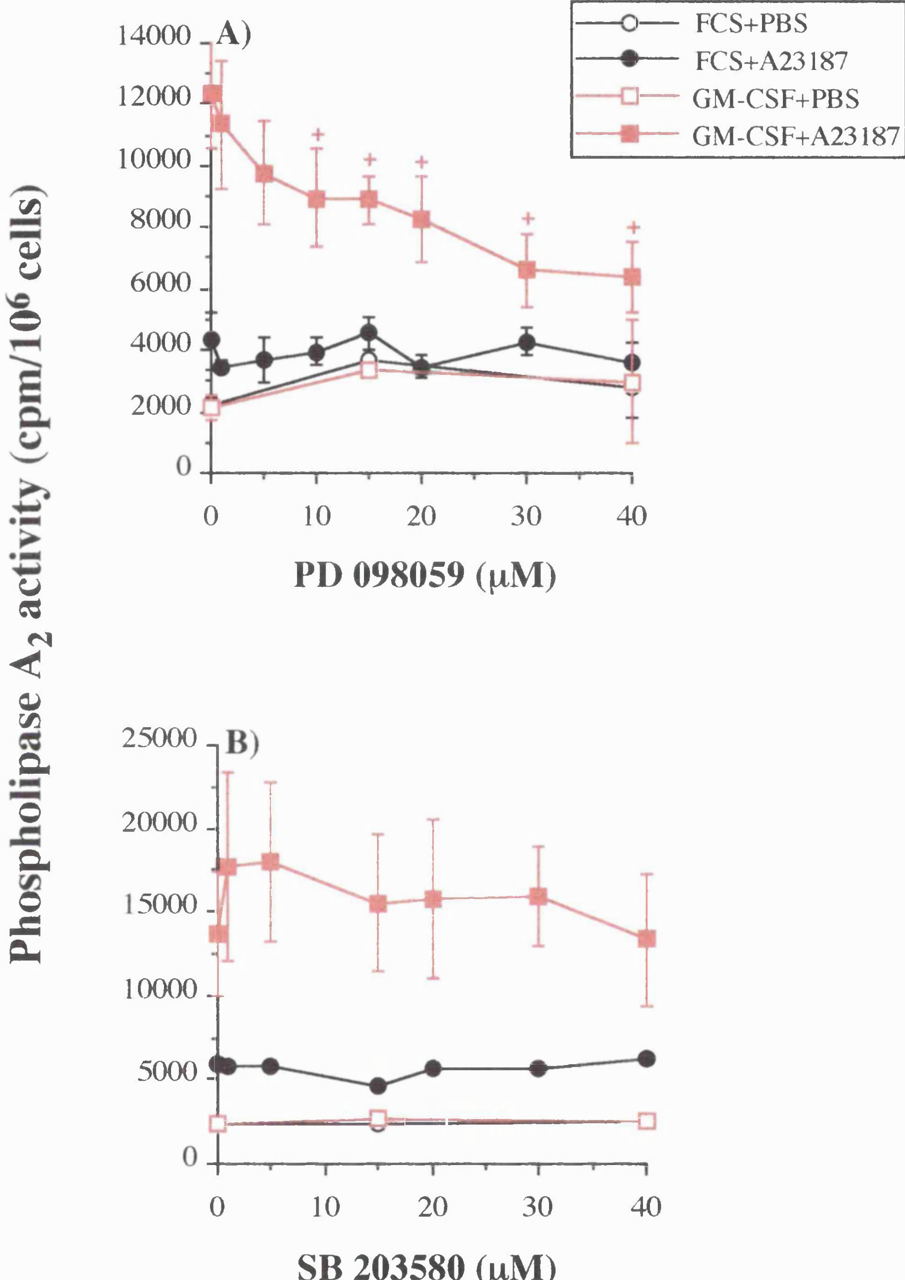



**Figure 6.4: Effect of MEK1 and p38 MAPK inhibitors on GM-CSF primed AA release in neutrophils stimulated with A23187.** Purified neutrophils were preincubated with different concentrations of either PD 098059 or SB 203580 before priming with GM-CSF (10 ng/ml) followed by 1μM A23187 as described in the Method section. The data shown are the mean ± 1 SE of A) 3 experiments and B) 4 experiments performed in duplicates. Note the difference in y-axis btween A & B. There was a significant difference between samples incubated with or without PD 098059 when the absolute data were analysed by paired *t*-test ($^{+}P<0.05$).

**Table 6.3: Effect of MEK inhibitor, PD 098059, on cytokine-mediated AA release in neutrophils stimulated by A23187**

| *PD 098059 (μM)* | *AA release* (% of control) | | |
|---|---|---|---|
| | *FCS* | *TNF α* | *GM-CSF* |
| 0 | 100 ± 0 | 100 ± 0 | 100 ± 0 |
| 1 | 79 ± 19 | 82 ± 8 | 79 ± 11 |
| 5 | 71 ± 22 | 66 ± 17 | 67 ± 14 |
| 10 | 80 ± 25 | 48 ± 5+ | 59 ± 8+ |
| 15 | 73 ± 24 | 42 ± 8+ | 46 ± 15+ |
| 20 | 86 ± 18 | 44 ± 8+ | 54 ± 8+ |
| 30 | 80 ± 21 | 38 ± 4* | 40 ± 7* |
| 40 | 74 ± 20 | 34 ± 4* | 39 ± 7* |

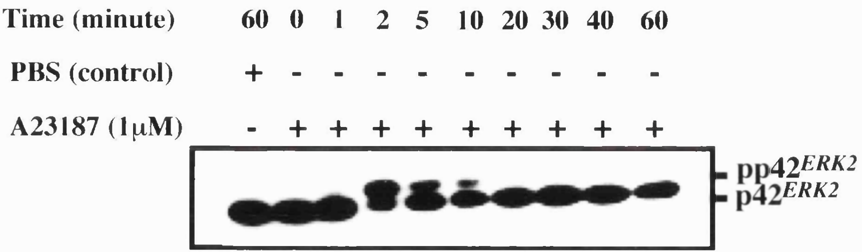
Neutrophils were preincubated with PD 098059 for 10 minutes at 37°C before priming with either TNFα (500 U/ml), GM-CSF (10 ng/ml) or diluent (0.01% FCS) for 20 minutes, followed by stimulation with A23187 (1μM) for 15 minutes. Basal release of arachidonate in unstimulated samples was subtracted from the A23187-stimulated values, and the data were expressed as a percentage of the relevant no inhibitor control (i.e. DMSO). The data shown are the mean ± 1SE of 4 experiments with TNFα, 3 experiments with GM-CSF and 7 experiments with FCS diluent control performed with duplicate samples. The significant differences between samples with or without PD 098059 are shown as: $^{+}P<0.05$ and $^{*}P<0.01$.

**Table 6.4: Effect of p38 MAPK inhibitor, SB 203580, on cytokine-mediated AA release in neutrophils stimulated by A23187**

| *SB 203580* ($\mu M$) | *AA release* (% of control) | | |
|---|---|---|---|
| | ***FCS*** | ***TNF α*** | ***GM-CSF*** |
| 0 | 100 ± 0 | 100 ± 0 | 100 ± 0 |
| 1 | 74 ± 13 | 187 ± 12* | 130 ± 12 |
| 5 | 91 ± 12 | 199 ± 19$^{+}$ | 138 ± 16 |
| 10 | 76 ± 23 | 211 ± 14* | ND |
| 15 | 80 ± 28 | 216 ± 27$^{+}$ | 115 ± 8 |
| 20 | 97 ± 21 | 206 ± 27$^{+}$ | 115 ± 9 |
| 30 | 112 ± 31 | 182± 22$^{+}$ | 127 ± 11 |
| 40 | 99 ± 21 | 158 ± 23 | 95 ± 9 |

Neutrophils were preincubated with SB 203580 for 30 minutes at 37°C before priming with TNFα (500 U/ml), GM-CSF (10 ng/ml) or diluent (0.01% FCS) for 20 minutes, followed by stimulation with A23187 (1μM) for 15 minutes. Basal release of arachidonate in unstimulated samples was subtracted from the A23187-stimulated values, and the data were expressed as a percentage of the relevant no inhibitor control (i.e. DMSO). The data shown are the mean ± 1SE of 4 experiments with TNFα, GM-CSF or FCS performed with duplicate samples. The significant differences between samples with or without SB 203580 are shown as: $^{+}P<0.05$ and $^{*}P<0.01$.
(ND=not done)

A)




B)

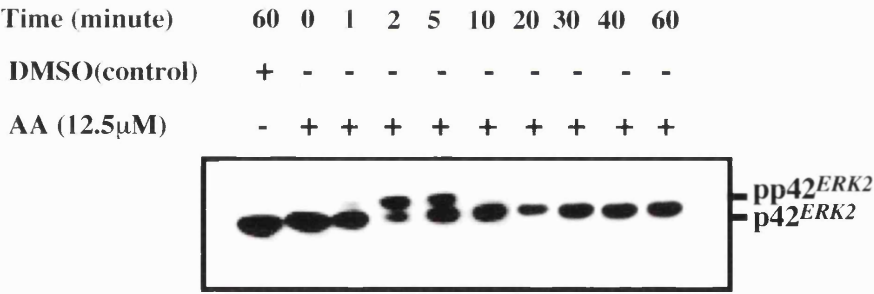



**Figure 6.5: Ability of calcium-ionophore and arachidonic acid to activate $p42^{ERK2}$ in neutrophils.** Neutrophils were stimulated with either calcium ionophore, A23187, (1μM) or AA (12.5 μM) for the time points indicated, before cell lysis. Samples were resolved on SDS/PAGE as described in the Materials and Method section. The autoradiographs represent the results of a single experiment that was performed twice.

Similarly, the response to calcium-ionophore was also transient, being maximal within 2 minutes and virtually undetectable at 20 minutes.

*ii) p38 MAPK*

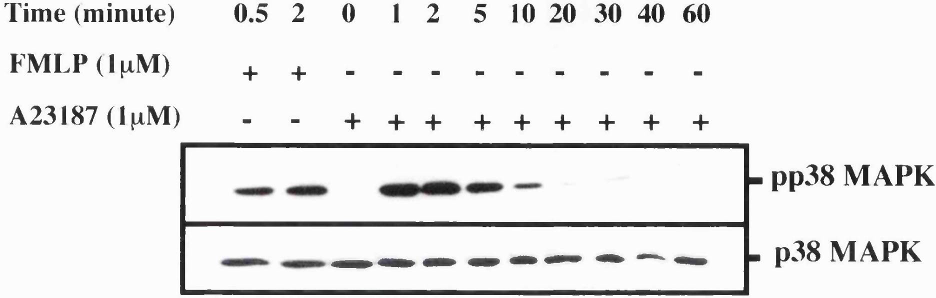
The effect of both AA and A23187 on phosphorylation of p38 MAPK was analysed by western blotting using 12.5% SDS/PAGE. The phosphorylated form of p38 MAPK did not show an electrophoretic mobility shift, therefore samples were loaded in duplicate and an anti-p38 antibody was used to detect for total p38 MAPK or phospho-specific p38 antibody was used to detect p38 MAPK which had been phosphorylated on threonine 180 and tyrosine 182. As shown in Figures 6.6 A, AA phosphorylates p38 MAPK and the phosphorylation persisted for up to 60 minutes. In contrast, p38 MAPK phosphorylation stimulated by calcium-ionophore was transient, being apparent within 1 minute of stimulation and decreasing within 10 minutes and being undetectable at 40 minutes (Figure 6.6 B).

### 6.3.3 Effect of kinase inhibitors on leukotriene production in human neutrophils

Specific inhibitors of MAP kinases have been widely used to investigate the involvement of $p42^{ERK2}$ and p38 MAPK in intracellular signal transduction pathways. Several studies have confirmed the selectivity of PD 098059 (MEK inhibitor) and SB 203580 (p38 MAPK inhibitor) against a variety of kinases (Chapter 5, Table 5.1 and Table 5.2). However, since SB 203580 has been developed from drugs that are inhibitors of cyclooxygenase and lipoxygenase, it was important to test the inhibitory action of these inhibitors on AA production as well as the downstream metabolite, leukotriene production.

A recent report has shown that both PD 098059 and SB 203580 directly inhibited cyclooxygenase-1 and -2 in human platelets and SB 203580 also inhibited thromboxane synthase (Börsch-Haubold *et al.*, 1998). In order to investigate the possible inhibitory effect of these inhibitors on leukotriene production in human neutrophils a specific inhibitor of 5-Lipoxygenase, MK-886 (Gillard *et al.*, 1989) was included in the reaction mixtures and the inhibitory effect of the PD 098059 and SB 203580 in the presence or absence of MK886 was investigated.

As SB 203580 had no inhibitory effect on $PLA_2$ activity, as demonstrated earlier in this chapter, therefore the effect of SB 203580 on the downstream enzymes was investigated.




B)

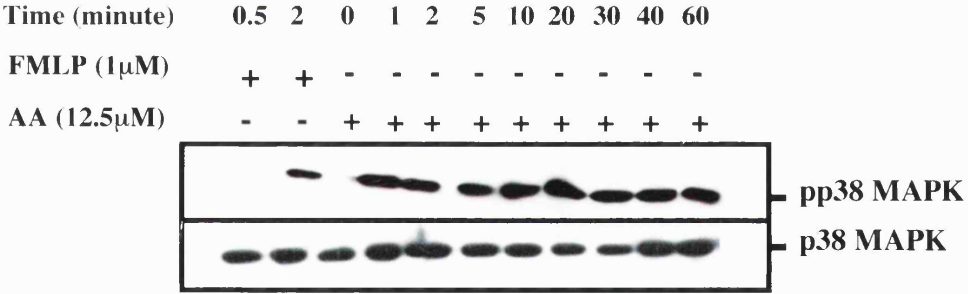



**Figure 6.6: Ability of calcium ionophore and arachidonic acid to activate p38 MAP kinase in neutrophils.** Neutrophils were stimulated with either calcium ionophore, A23187 (1μM), AA (12.5 μM) or FMLP (1μM) (positive control) for the time points indicated, before cell lysis. Samples were resolved on SDS/PAGE as described in the Materials and Method section. The autoradiographs represent the results of a single experiment that was performed twice.

Radiolabelled neutrophil samples were incubated with either 5-lipoxygenase inhibitor, MK-886 (200 nM), or DMSO (diluent control) prior to treatment of the cells with PD 098059 or SB 203580. Then samples were incubated with either 0.01% FCS (diluent control) or GM-CSF (10 ng/ml) for 20 minutes at 37°C followed by stimulation with 1 μM calcium-ionophore in the absence of BSA. As shown in Figure 6.7 A the unprimed leukotriene production was inhibited by 40 μM PD 098059 (7% ± 1% of the DMSO control). Leukotriene production was also blocked in the presence of MK886 (11% ± 4% of the DMSO control). In contrast, 40 μM PD 098059 partially inhibited the GM-CSF-primed leukotriene production (62% ± 4% of the DMSO control), and the primed response was also inhibited by MK886 (16% ± 1% of the DMSO control).

The above experiment was then repeated using the p38 MAPK inhibitor, SB 203580, instead of the PD 098059 compound. As shown in Figure 6.7 B in unprimed neutrophils, leukotriene production was blocked in the presence of MK 886 (16% ± 10% of the DMSO control), whereas SB 203580 (40 μM) partially inhibited leukotriene production (60% ± 10% of the DMSO control). Similarly, the GM-CSF primed response was also partially inhibited (62% ± 6% of the DMSO control), and the GM-CSF-primed response was also blocked by MK 886 (26% ± 2% of the DMSO control). These data showed that both compounds interfere with the metabolism of AA by 5-lipoxygenase, therefore the effect of PD 098059 and SB 203580 in $PLA_2$ assay was measured in the presence of MK886. These data confirm the finding by Börsch-Haubold *et al.* (1998), indicating the inhibitory effect of MAP kinase inhibitors on leukotriene production in platelets. These data showed that SB 203580 had an inhibitory effect on leukotrienes but not $PLA_2$ activity therefore, this inhibitor would not interfere with the $PLA_2$ assay.

Previously (Chapter 5), the superoxide assay were performed in the absence of MK886, whereas in this chapter the $PLA_2$ assay included MK886 to inhibit 5-lipoxygenase and demonstrated the maximal levels of AA production. Therefore the superoxide assays were repeated in the presence of MK886. Superoxide production was measured in purified neutrophils by the superoxide dismutase-inhibitable reduction of ferricytochrome *c*. Cells were preincubated with either MK-886 (200 nM final concentration) or 0.01% DMSO (diluent control) prior to treatment of cells with either PD 098059 or SB 203580. Then samples were primed with either 0.01% FCS or GM-CSF (10 ng/ml) for 30 minutes followed by stimulation with FMLP (1 μM) for 6 minutes at 37°C. As shown in Figure 5.8 A, PD 098059 had no inhibitory effect on the unprimed FMLP response (155% ± 19% of the DMSO control) in the absence of MK886. In another set of experiments, as shown in Figure 6.8 A, in the presence of MK886 there was also no inhibitory effect of PD 098059 on superoxide anion

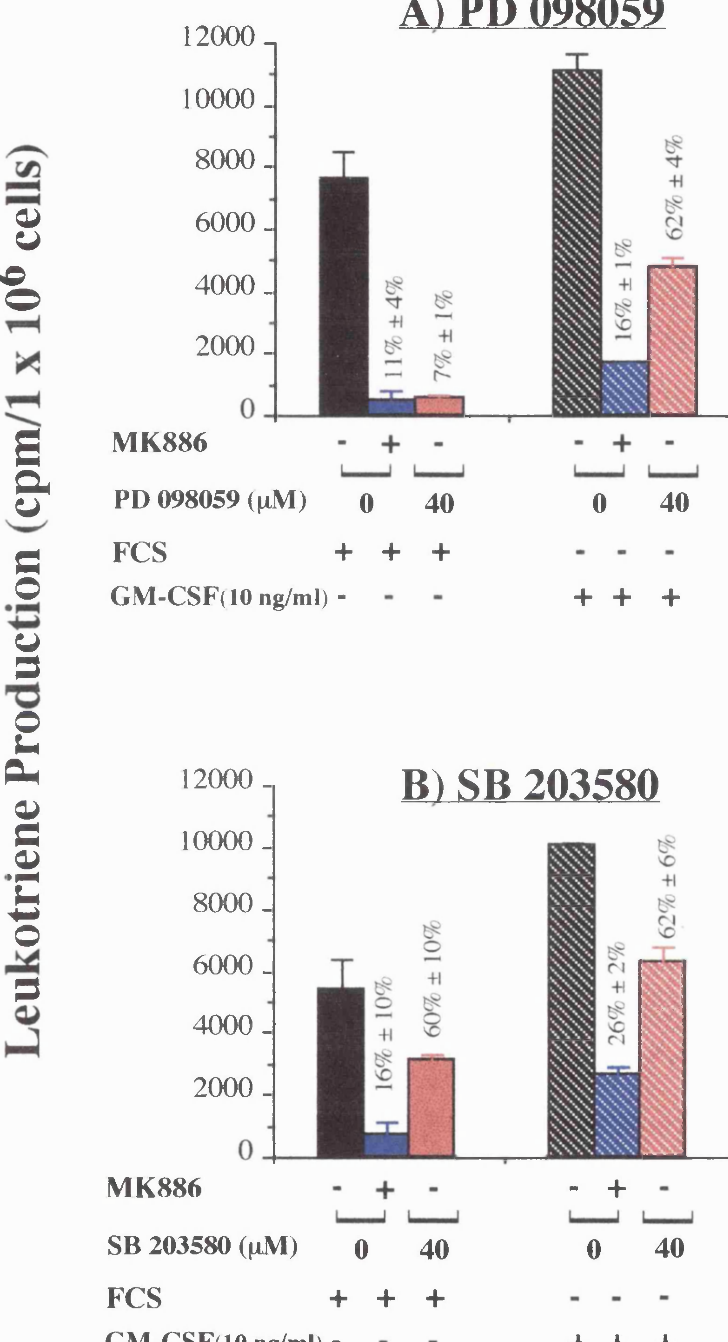



**Figure 6.7: Effect of PD 098059 and SB 203580 on leukotriene production in neutrophils.** The cells were preincubated with MK886 (200 nM) or DMSO control followed by either PD 098059, SB 203580 or DMSO control. Then samples were primed with 10 ng/ml GM-CSF or 0.01% FCS prior to stimulation with calcium-ionophore (1μM) as described in the Method section. The data shown are the mean ± range of one experiment performed in duplicate. The data also expressed as a percentage of no inhibitor (i.e. DMSO control)

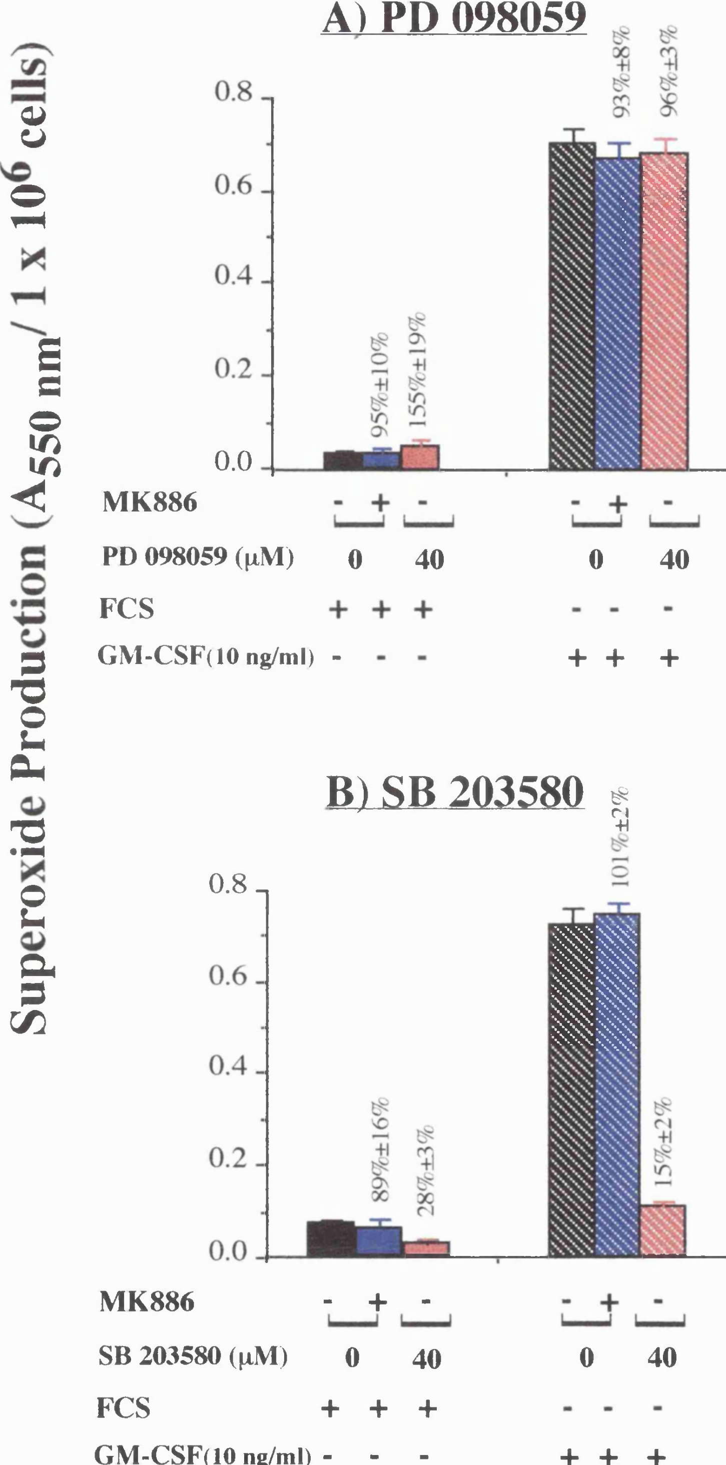



**Figure 6.8: Effect of MK886 on superoxide production in neutrophils.** Purified neutrophil were preincubated with MK886 (200 nM) or DMSO control followed by either PD 098059 for 10 minutes or SB 203580 for 30 minutes. Then samples were primed with 10 ng/ml GM-CSF or 0.01% FCS prior to stimulation with FMLP (1μM) as described in the Method section. The data shown are the mean ± SE of 3 experiment. The data also expressed as a percentage of no inhibitor (i.e. DMSO control).

production (95% ± 19% of the DMSO control). There was no significant inhibiton of GM-CSF primed superoxide generation in the presence of either PD 098059 (96% ± 3% of the DMSO control) or MK886 (93% ± 8% of the DMSO control). In confirmation of our previous finding in Chapter 5, Figure 6.8 B showed that SB 203580 had an inhibitory effect on either unprimed (28% ± 3% of the DMSO control) or primed (15% ± 2% of the DMSO control) superoxide generation. In the presence of MK886 there was no significant inhibition of either unprimed superoxide generation (89% ± 16% of the DMSO control), or primed response (101% ± 2% of the DMSO control). These data show that inhibition of 5-lipoxygenase did not inhibit priming of the respiratory burst and the experiments shown in Figure 6.8 confirm that the results were not different when MK886 was included in the assay.

### 6.3.4 Effect of a combination of PD 098059 and SB 203580 on arachidonate release in human neutrophils

As shown in Figure 6.7 and 6.8, PD 098059 did not completely inhibit AA release in neutrophils stimulated by either TNFα or GM-CSF. Therefore the effect of a combination of both PD 098059 and SB 203580 on AA release was investigated. Neutrophils were incubated with both inhibitors at 37°C. Then samples were treated with either 500 U/ml TNFα or 10 ng/ml GM-CSF for 20 minutes prior to stimulation by A23187 (1 μM) for 15 minutes. As shown in Figure 6.9 the inhibitory effect of both inhibitors was greater than each inhibitor alone. The inhibitory effect of PD 098059 and SB 203580 was apparent at 15 μM (78% ± 1%) when cells were primed with TNFα and was maximal at 40 μM (7% ± 1%). In contrast, the inhibitory effect in GM-CSF primed neutrophils was seen at 20 μM (86% ± 4%) and was maximal at 40 μM (17% ± 2%) (Table 6.5).

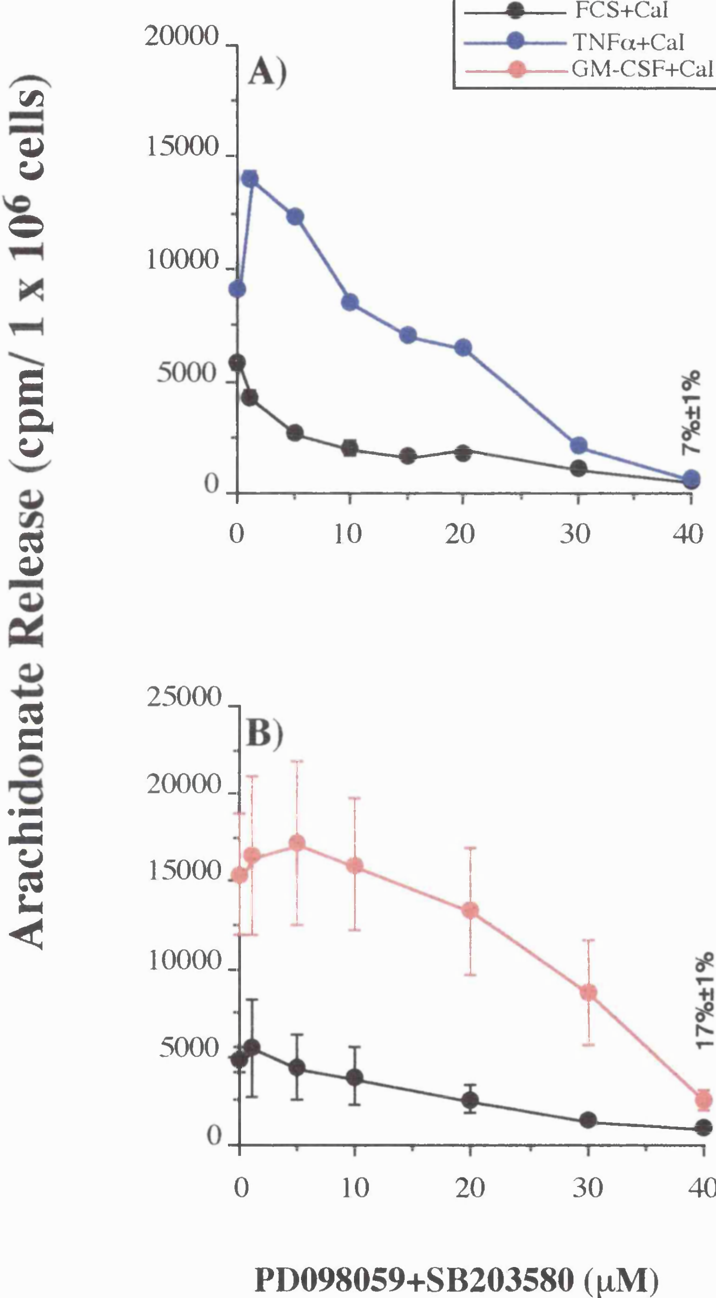



**Figure 6.9: Effect of a combination of both PD 098059 and SB 203580 on arachidonic acid release in neutrophils.** Radiolabelled neutrophils were preincubated with different concentrations of PD 098059 and SB 203580 before priming with either TNFα (500 U/Mml) or GM-CSF (10 ng/ml) for 20 minutes followed by activation with 1 μM CaI as described in the Method section. The data shown are the mean ± 1 SE of A) 2 experiments and B) 1 experiment performed in duplicates.

**Table 6.5: Effect of a combination of both PD 098059 and SB 203580 on cytokine-mediated AA release in neutrophils stimulated with A23187**

| *PD 098059* + *SB 203580* (*μM*) | *AA release* (% of control) | | |
|---|---|---|---|
| | *FCS* | *TNFα* | *GM-CSF* |
| 0 | 100 ± 0 | 100 ± 0 | 100 ± 0 |
| 1 | 92 ± 20 | 154 ± 1 | 106 ± 5 |
| 5 | 67 ± 16 | 135 ± 1 | 110 ± 5 |
| 10 | 56 ± 16 | 91 ± 3 | 103 ± 1 |
| 15 | 34 ± 5 | 78 ± 1 | 103 ± 2 |
| 20 | 42 ± 7 | 71 ± 1 | 86 ± 4 |
| 30 | 24 ± 3 | 23 ± 1 | 54 ± 6 |
| 40 | +15 ± 4 | 7 ± 1 | 17 ± 1 |

Neutrophils were preincubated with a combination of PD 098059 and SB 203580 for 20 minutes at 37°C before priming with TNFα (500 U/ml), GM-CSF (10 ng/ml) or diluent control (0.01% FCS) for 20 minutes, followed by stimulation with calcium ionophore (1μM) for 15 minutes. Basal release of arachidonate in unstimulated samples was subtracted from the calcium ionophore-stimulated values, and the data were expressed as a percentage of the relevant no inhibitor control (i.e. DMSO). The data shown are the mean ± range of 1 experiment with TNFα, 2 experiments with GM-CSF and 3-4 experiments with FCS diluent control performed with duplicate samples. The significant difference between samples in the presence or absence of the inhibitors are shown as: $^{+}P<0.05$

## 6.4 DISCUSSION

Previous data showed that the generation of AA is involved in priming of the FMLP-stimulated respiratory burst (Roberts *et al.*, 1996). The radiolabelled non-esterified AA that is released into the extracellular medium has been used as a measure of $PLA_2$ activity. The method was previously established in our lab and the release of AA from neutrophils was confirmed by thin layer chromatography (Roberts *et al.*, 1996).

Our data showed that FMLP-stimulated AA release was barely detectable, and this is in accord with a study showing that unprimed neutrophils were unable to produce leukotrienes in response to FMLP (Dahinden *et al.*, 1988). Treatment of neutrophils with cytokines caused an enhancement of FMLP-stimulated $PLA_2$ activity, in confirmation of previous works (Gasson, 1991; Durstin *et al.*, 1994; Roberts *et al.*, 1996). Incubation of neutrophils with different doses of PD 098059 prior to priming by either TNFα or GM-CSF followed by stimulation with FMLP caused partial inhibition of AA release. However, the doses of PD 098059 that has been shown to inhibit the phosphorylation of $p42^{ERK2}$ activity (10 μM) (Chapter 5, Figure 5.6 A) did not show an inhibitory effect on priming of the FMLP-stimulated respiratory burst (Figure 5.13). This observation suggests that the priming mechanism for the two enzyme systems are different. The fact that PD 098059 does not completely inhibit cytokine-mediated AA release suggests that priming might override the requirement for $p42^{ERK2}$ activation of $cPLA_2$ possibly by activation of a secondary signal transduction pathway or activation of a different $PLA_2$ isoform that is not inhibited by PD 098059. Furthermore, a study by Kramer *et al.* (1995) has demonstrated that the thrombin receptor agonist peptide SFLLRN ( this peptide mimics the effect of thrombin, causing platelets to secrete and aggregate), mediates activation of $cPLA_2$ in the absence of ERK, suggesting a role for p38 MAPK in phosphorylation of $cPLA_2$ in human platelets. In the present study, the involvement of p38 MAPK, in activation of $PLA_2$ was investigated by using the inhibitor of this kinase, SB 203580. There was no inhibitory effect of SB 203580 on cytokine-mediated AA release when neutrophils were stimulated by FMLP with the exception of the highest dose (40 μM) of SB 203580 which was 34% ± 18% and 61% ± 12% of the DMSO control when cells were primed with TNFα or GM-CSF, respectively. As shown in the previous chapter (Chapter 5), the dose of SB 203580 that fully blocked the FMLP-stimulated p38 MAPK activity (Figure 5.11) showed no inhibitory effect on primed or unprimed FMLP-stimulated $PLA_2$ activity (as shown in Figures 6.1 and 6.2). Failure of SB 203580 to inhibit $PLA_2$ activity stimulated by FMLP indicates that the inhibition of FMLP-stimulated p38 MAPK activity in the kinase assay shown in Figure 5.11 is not

due to any non-specific effect of the inhibitor. Our data indicate that p38 MAPK and $PLA_2$ are differentially sensitive to SB 203580. In contrast, both the primed and unprimed FMLP-stimulated respiratory burst activity was inhibited by SB 203580 in a dose dependent manner.

This conclusion is confirmed when calcium-ionophore (A23187), a potent activator of $PLA_2$, was used as an agonist. PD098059 partially inhibited both the unprimed and cytokine-mediated AA release when stimulated by A23187 in a dose-dependent manner which was maximal at 10 μM and 15 μM PD 098059 when neutrophils were primed with either TNFα or GM-CSF, respectively. The doses of PD 098059 that showed inhibitory effects on A23187 stimulated $PLA_2$ activity corresponds to the doses (10-40 μM) that blocked the phosphorylation of $p42^{ERK2}$ to undetectable levels (Chapter 5, Figure 5.6 A). In contrast, SB 203580 did not inhibit cytokine-mediated primed AA release when cells were stimulated by A23187. These data suggests that priming pathways utilised by the respiratory burst and $PLA_2$ are different.

# CHAPTER 7

# A STUDY OF $PLA_2$ ACTIVITY IN PRIMITIVE HAEMOPOIETIC CELLS

## 7.1 INTRODUCTION

In previous Chapters the activity of $PLA_2$ and the mechanism of its priming was investigated in neutrophils. However, the role of $PLA_2$ in more immature myeloid cells and in other cell lineages has not been fully worked out. Mature T and B lymphocytes are reported not to express $cPLA_2$, whereas it is present at the protein level in immature lymphocytes and may have a role in antigen regulated clonal selection (Gilbert *et al.*, 1996). It is possible that in other immature haemopoietic cells and haemopoietic precursors, $PLA_2$ may have other roles such as regulation of cell division, differentiation and gene expression as suggested by Flati *et al.* (1996) that $cPLA_2$ may regulate STAT 1 activation in HeLa cells.

Primitive haemopoietic cells expressing CD34 antigen can be mobilised out of the bone marrow and into the circulation of using a combination of chemotherapy and growth factors, particularly G-CSF. These primitive cells have blast cell morphology, no respiratory burst activity, and their cell cycle profiles indicate that the majority of cells are in a quiescent $G_0$ stage of the cell cycle (Roberts and Metcalf, 1995; To *et al.*, 1994). The presence of $PLA_2$ mRNA in $CD34^+$ cells mobilised into the peripheral blood has been reported (Claesson *et al.*, 1996). In contrast, a study by Boyce *et al.* (1996) failed to show the expression of $cPLA_2$ mRNA in $CD34^+$ cells derived from cord blood.

Studies on primitive myeloid cells and the differentiation process have been restricted to growth factor-independent cell lines such as HL-60 cells which express $PLA_2$ protein (Xing *et al.*, 1994) which is active in an *in vitro* assay of permeabilised cells but intact cells release little AA when stimulated via functional receptors or receptor independent pathways (Billah *et al.*, 1986; Xing *et al.*, 1994).

The aim of this study was to screen a range of immature cells for their ability to express $PLA_2$ activity compared to that of primary myeloid progenitor cells and mature haemopoietic cells. The aim was to determine the effect of growth factors in priming the $PLA_2$ activity of these cells.

## 7.2 METHODS AND MATERIALS

Materials

- RPMI 1640 supplemented with L-glutamine (Gibco BRL, Paisley, UK)
- IMEM (Iscove's minimal essential medium) (Gibco BRL)
- Foetal calf serum (FCS), heat inactivated at 56°C for 30 minutes (Gibco BRL)
- 10 μg/ml *rh*GM-CSF (Recombinant human GM-CSF, expressed in *E.coli*; Hoechst, UK/Behringwerke, Marburg, Germany)
- *rh*Interleukin-2 and *rh*Interleukin-3, *rh*Interleukin-6 and stem cell factor (SCF), stock solutions were prepared at 10 μg/ml in sterile PBS containing 1% (v/v) FCS and stored at -20°C (all expressed in *E.coli*) (R & D Systems, Europe, Abingdon, Oxfordshire, UK)
- Retinoic acid (RA) at 10 mM stock solution in DMSO stored at -20°C (Sigma, Poole, Dorset)
- Dimethyl sulphoxide (DMSO) (Sigma)
- Nitroblue tetrazolium (NBT) (Sigma)
- Ficoll-Paque (density 1.077 g/ml) (Pharmacia Biotech, Uppsala, Sweden)
- Dynabeads M-450 CD14 (monocytes/macrophages) (Dynal, Bromborough, UK)
- Dynabeads M-450 CD3 (pan-T), CD19 (pan-B) (Dynal)
- Dulbecco's Phosphate Buffered Saline (PBS) (Gibco BRL, Paisley, UK)
- Anti-CD34 antibody HPCA (Becton Dickinson, Franklin Lakes, N.Y)
- Sterile tissue culture flasks (Becton, Dickinson Ltd, Cowley, Oxford)
- Cell lines (see Table 7.1)

Methods

### 7.2.1 Cell culture

All cell lines were maintained in suspension culture in RPMI 1640 supplemented with 10% (vol/vol) FCS and incubated at 37°C in a humidified atmosphere containing 5% $CO_2$.

*Factor independent cell lines*: These cells were subcultured twice a week and supplied with fresh medium to maintain the concentration between 2-5 x $10^5$ cells/ml (THP-1 cells were kept at 8 x $10^5$ cells/ml). Prior to analysis of AA release cultures of these cell lines were harvested by centrifugation and resuspended in $PBS^+$ supplemented with 10 mM glucose (PBSG).

**Table 7.1: Cell lines**

| Cell line | Tissue of origin | Reference |
|---|---|---|
| HL-60 | Acute promyelocytic leukaemia | Collins *et al.*, 1977 |
| THP-1 | Acute monocytic leukaemia | Tsuchiya *et al.*, 1980 |
| K562 | CML in blast transformation | Lozzio & Lozzio, 1975 |
| KG-1 | Acute myeloblastic leukaemia | Koeffler & Golde, 1978 |
| KG-1a | Acute myeloblastic leukaemia | Koeffler *et al.*, 1980 |
| Daudi | B cell lymphoma | Silverman *et al.*, 1982 |
| CTLL | T cells from C57bl/6 mouse | Gillis &Smith, 1977 |
| TF-1 | Erythroleukaemia | Kitamura *et al.*,1989 |

All cell lines are of human origin except where indicated

*Factor-dependent cell lines:* The IL-2-dependent T cell line, CTLL, and TF-1 myeloid cell line were maintained under the same conditions as factor independent cell lines but with addition of 2 ng/ml IL-2 or 5 ng/ml GM-CSF, respectively. Cells were subcultured three times a week and maintained at concentrations not exceeding 5 x $10^5$ cells/ml. Antibiotics were not routinely added to any of the above cell cultures. Before analysis of their responses to cytokines, the cells were removed from the medium containing growth factor by centrifugation, washed four times in growth factor free medium and incubated for 18 hours in medium plus 10% FCS to allow re-expression of growth factor receptors.

*In vitro differentiation of HL-60 cells*: The HL-60 cells were differentiated for 6 days in the presence of either 1.25% (v/v) DMSO or 1μM RA. Functional maturation was assessed by the nitroblue tetrazolium reduction (NBT) test. NBT, a water-soluble yellow dye, is reduced by superoxide to insoluble intracellular blue-black formazan by phagocytosing or membrane-stimulated granulocytes. Cells were incubated for 30 minutes at 37°C in 0.5 mg/ml NBT in RPMI medium containing 500 ng/ml PMA to stimulate the respiratory burst. Control samples were incubated without PMA to measure the resting level of NBT reduction. Morphologically mature HL-60 cells, but not the immature promyelocytes, reduce NBT when stimulated by PMA due to acquisition of a functional NADPH oxidase characteristic of mature phagocytes (Collins *et al.*, 1979; Roberts *et al.*, 1991). Positive cells containing black deposits of formazan were identified by light microscopy. The percentage of positive cells was calculated from a total of 300 cells in each sample. Cell viability was assessed by trypan blue exclusion (see Chapter 2 for details).

### 7.2.2 Isolation of monocytes from whole blood

On the day of experiment venous blood from healthy volunteers was drawn into 2.5 mM EDTA as anticoagulant, and diluted two fold with $PBS^-$ and then layered onto 10% Ficoll-paque (diluted in PBS) and centrifuged at 140 x g for 15 minutes. This step removed more than 90% of platelets and the cell pellet (containing white blood cells and erythrocytes) was resuspended in $PBS^-$ and layered onto full strength Ficoll-paque and centrifuged at 800 x g for 20 minutes. Mononuclear cells were collected from the interface and washed twice with $PBS^-$/2% FCS and the pellet was resuspended to a concentration of 10-20 x $10^6$ cells/ml. Then Dynabeads, CD14 and CD3, (were washed twice with PBS/FCS to remove the sodium azide) at concentration of 20 x $10^6$ beads (~50 μl ) were added to 1ml of cells and incubated at 4°C for 1 hour with gentle tilting and rotation. The beads were separated by using Magnetic Particle Concentrator (MPC) and the monocyte rich supernatant was taken into a fresh tube and washed twice with

PBS$^-$/2%FCS and finally cells were resuspended at 5x10$^6$/ml in PBSG. Cytospin preparations of the monocytes isolated by this method were shown to be more than 80% pure by Leishman staining and 99% viable by trypan blue exclusion (see Chapter 2 for details).

### 7.2.3 Purification of CD34$^+$ progenitor cells

CD34$^+$ peripheral blood progenitor cells were purified from 15 patients (4 with Hodgkin's disease, 4 with non-Hodgkin's lymphoma and 7 with myeloma) and 4 healthy normal donors after obtaining informed consent. Progenitor cells were first mobilised from the bone marrow of patients with chemotherapy followed by daily G-CSF (Watts *et al.*, 1997). Normal donors received G-CSF alone. White blood cells were isolated from the peripheral blood by apheresis and CD34$^+$ cells were purified on a CEPRATE SC immunoaffinity column (Cell Pro Inc, Bothwell, WA, USA) using a biotinylated mouse-anti-human CD34 monoclonal antibody and avidin coated beads (Watts *et al.*, 1997). Progenitor cell purity was assessed by flow cytometry using anti-CD34 antibody and by morphological analysis (Watts *et al.*, 1995). The purity of CD34$^+$ cells after affinity purification was 82% ± 10% (mean ± 1SD, n=19) with 2% ± 1% recognisable neutrophil precursors. The major cell contaminants were lymphocytes (7% ± 1%) and monocytes (6% ± 1%) which were removed, respectively, with anti-CD3 and CD19 magnetic beads or by adherence to plastic. Mobilised CD34$^+$ cells were cultured overnight in Iscove's minimal essential medium (IMEM) supplemented with 20% (v/v) FCS but in the absence of growth factors in a humidified atmosphere of 5% carbon dioxide in air (day 1). The final cell purity was 96% ± 1% blasts, 1% ± 1% promyelocytes, 3% ± 1% monocytes, and no lymphocytes were observed (Figure 7.1).

*In vitro* differentiation of CD34$^+$ selected cells was achieved by growth for up to 8 days in IMEM/20% FCS supplemented with SCF, IL-3 and IL-6 (all at 10 ng/ml), and antibiotics (penicillin 100U/ml and streptomycin 100 µg/ml). Differentiation was assessed by NBT test and 12% ± 2% (n=8) were positive on day 8 compared with 2% ± 1% (n=12) on day 1.

Before analysis of cellular responses to cytokines, cells were washed three times to remove ambient growth factors, and incubated for 18 hours in IMEM/20% FCS in order to allow the re-expression of growth factor receptors. The morphological maturity of cultured cells was assessed from cytocentrifuge preparations stained with Leishman's stain as described in Chapter 2 and the results are given in Figure 7.1.

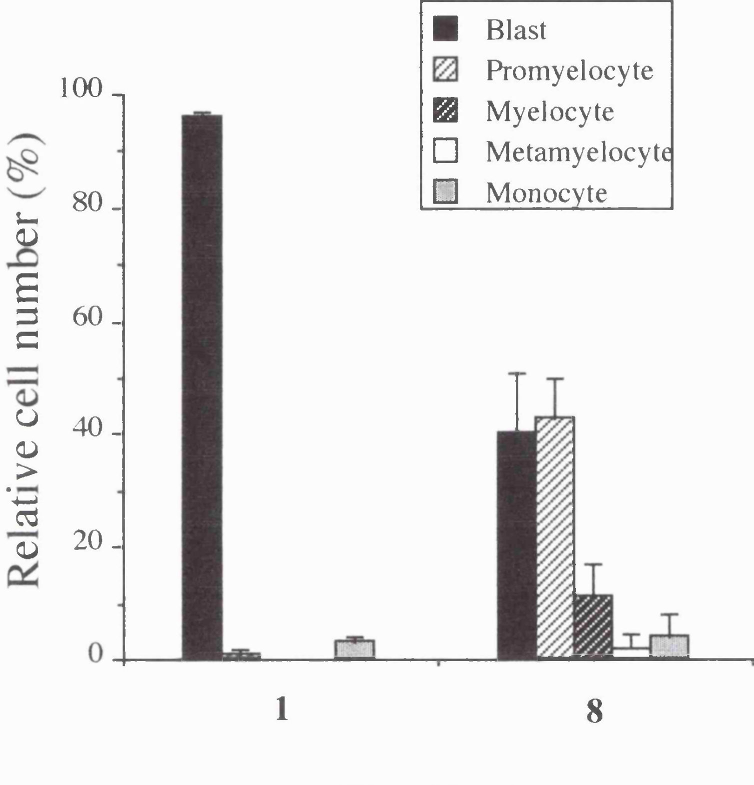



**Figure 7.1: Morphological maturity of cultured cells assessed by cytocentrifuge preparations stained with Leishman's stain.**

### 7.2.4 Phospholipase $A_2$ assay

The incorporation and release of [$^3$H]-AA from the cells were analysed as described in detail in Chapter 2. Cells were incubated with [$^3$H]-AA at a final concentration of 0.5 μCi/ml plus 0.01% FCS at 37°C for two hours with occasional mixing. Purified human neutrophils and monocytes (for purification of neutrophils from peripheral blood see Chapter 2) at concentration of 5 x $10^6$ cells/ml in PBSG (5 mM) were incubated at room temperature. Mobilised CD34$^+$ cells at a concentration of 2.5 x $10^6$ cells/ml in 50% IMEM/50% PBSG (10 mM) and cell lines at a concentration of 5 x $10^6$ cells/ml in 50% RPMI supplemented with 50% PBSG (10 mM) were incubated at 37°C for one hour followed by one hour at room temperature. The radiolabelled cells were centrifuged (180 x g for 7 minutes) and the supernatants removed. The cell pellets were washed a further three times in PBSG, and finally resuspended at 2 x $10^6$ cells/ml in PBSG. 0.5 ml aliquots of the cell suspensions were incubated with 200 nM MK886 to inhibit the conversion of AA to leukotrienes via the action of 5-lipoxygenase. Cells were then incubated with the following cytokines and growth factors for 10 minutes; GM-CSF, IL-3 both at (10 ng/ml) or growth factor diluent (0.01% FCS); or PMA (500 ng/ml) or DMSO diluent (0.01%). Replicate samples were then stimulated for 20 minutes with or without 1 μM calcium ionophore, A23187, in the presence of fatty acid-free bovine serum albumin (BSA, final concentration of 1 mg/ml) which was added to trap the AA that was released and prevent re-esterification (see Chapter 2, Figure 2.1, for the priniciple of the $PLA_2$ assay). The reaction was terminated by placing the samples on ice for 10 minutes. The samples were then centrifuged at 12000 x g for 4 minutes and aliquots of the supernatants were assayed for radioactivity by liquid scintillation spectroscopy.

### 7.2.5 Statistical analysis of Data

Unless otherwise stated the data are the mean ± 1 SE of the number of experiments given in the text. Statistical analysis was performed by Student's paired *t*-test.

## 7.3 RESULTS

### 7.3.1 Detection of $PLA_2$ activity in immature haemopoietic cells in response to PMA, A23187, and a combination of both agonists

Phospholipase $A_2$ activity was quantitated by measuring the release of radioactivity into the supernatant from cells which had been radiolabelled with $^3$H-AA and then stimulated with either PMA (500 ng/ml) or A23187 (1 μM), or sequential addition of both agonists. Then the radioactivity which has been released was expressed as a percentage of the total cellular radioactivity, in order to control for the differences in the amount of $^3$H-AA incorporated by the different cell types.

*i) Immature myeloid cell lines*

Studies performed on TF-1 cells showed small, but significant release of AA when cells were stimulated by A23187 alone which was 4.0% ± 0.9% , whereas PMA alone did not elicit AA release. Stimulation of cells with PMA followed by A23187 showed a significant release of AA which was 15.6% ± 3.3%. Similarly, KG-1 and THP-1 cells showed similar pattern of AA release as TF-1 cells. When cells were stimulated by PMA followed by A23187 the AA release was 19.4% ± 4% for KG-1 cells and 7.2% ± 1.3% for THP-1 cells. These cells showed levels of arachidonate release which was comparable to that seen in mature neutrophils and monocytes (Table 7.2). Another myeloid cell line, KG-1a showed small AA release in response to A23187. This response was significantly increased when cells were stimulated by PMA alone (9.2% ± 1.2% ). The release of AA in KG-1a cells was further increased when cells were stimulated by PMA followed by A23187 (13.6% ± 6.3%). K562 and HL-60 cells showed little $PLA_2$ activity, which was not increased in response to either A23187, PMA or a combination of both agonists (Table 7.2).

*ii) Immature lymphocytic cell lines*

The growth factor independent cell line Daudi cells showed little AA release in response to either A23187, PMA or combination of both agonists. Similarly, growth factor-dependent CTLL cells showed very little AA release in response to either agonists as shown in Table 7.2

**Table 7.2: Activation of arachidonic acid release from immature haemopoietic cells compared with mature and CD34+ cells**

| Cell type (n) (Total radioactivity cpm/10[6] cells) | AA release stimulated by DMSO + PBS | DMSO + A23187 | PMA + PBS | PMA + A23187 |
|---|---|---|---|---|
| | (% of cellular radioactivity) | | | |
| **Cell lines:** | | | | |
| **TF-1 (4)** (34177±19445) | 2.5±0.5 | 4.0±0.9+ | 2.8±0.4 | 15.6±3.3* |
| **KG-1 (3)** (49698±7700) | 2.9±0.3 | 4.8±0.5 | 2.9±0.6 | 19.4±4.0* |
| **KG-1a (3)** (64612±4665) | 1.9±0.3 | 2.8±0.3 | 9.2±1.2$^+$ | 13.6±6.3$^+$ |
| **THP-1 (4)** (52702±6375) | 1.3±0.2 | 7.1±2.0 | 1.3±0.2 | 7.2±1.3$^+$ |
| **K562 (3)** (74462±13253) | 2.3±0.4 | 2.6±0.4 | 2.2±0.5 | 2.7±0.5 |
| **HL-60 (6)** (151557±14078) | 1.0±0.3 | 1.1±0.3 | 0.9±0.3 | 1.3±0.4 |
| **Daudi (1)‡** (98344±4739) | 1.8,1.7 | 1.9,1.9 | 1.6,1.9 | 1.9,2.1 |
| **CTLL (3)** (90282±3825) | 2.1±0.5 | 2.1±0.2 | 2.0±0.2 | 2.3±0.4 |
| **Primary cells:** | | | | |
| **Neutrophils (6)** (75047±8138) | 2.3±0.4 | 6.9±1.1 | 8.8±1.1 | 19.4±1.3* |
| **Monocytes (3)** (42108±13749) | 6.2±1.0 | 14.6±4.5 | 19.8±2.0$^+$ | 26.7±2.0$^+$ |
| **CD34$^+$** day 1 **(6)** (129709±32696) | 5.3±1.1 | 6.7±1.6 | 6.1±1.0 | 23.2±5.7$^+$ |
| **CD34$^+$** day 8 **(6)** (108538±24802) | 2.4±0.2 | 6.2±0.5* | 3.3±0.2$^+$ | 22.4±1.4* |

Cells were radiolabelled with [$^3$H]-AA, stimulated with either PMA (500 ng/ml) or DMSO diluent, followed by 1 µM A23187 or PBS control, and AA release was measured as described in the Method section. The data shown are the mean±1SE of the number (n) of experiments. The significance of difference from DMSO controls was $^+P$<0.05 and $^*P$<0.01. ‡Individual values of duplicate samples of Daudi cells are shown

*iii) Mature neutrophils, monocytes and CD $34^+$ stem cells*

Further studies were then carried out in mature neutrophils and monocytes. Both cell types showed AA release in response to A23187 which was greater in monocytes (14.6% ± 4.5%) than in neutrophils (6.9% ± 1.1%). As shown in Table 7.2, PMA also increased AA release in these mature cells which was 19.8% ± 2.0% in monocytes and 8.8% ± 1.1% in neutrophils. The combination of both agonists significantly increased $PLA_2$ activity to 26.7% ± 2.0% in monocytes and 19.4% ± 1.3% in neutrophils.

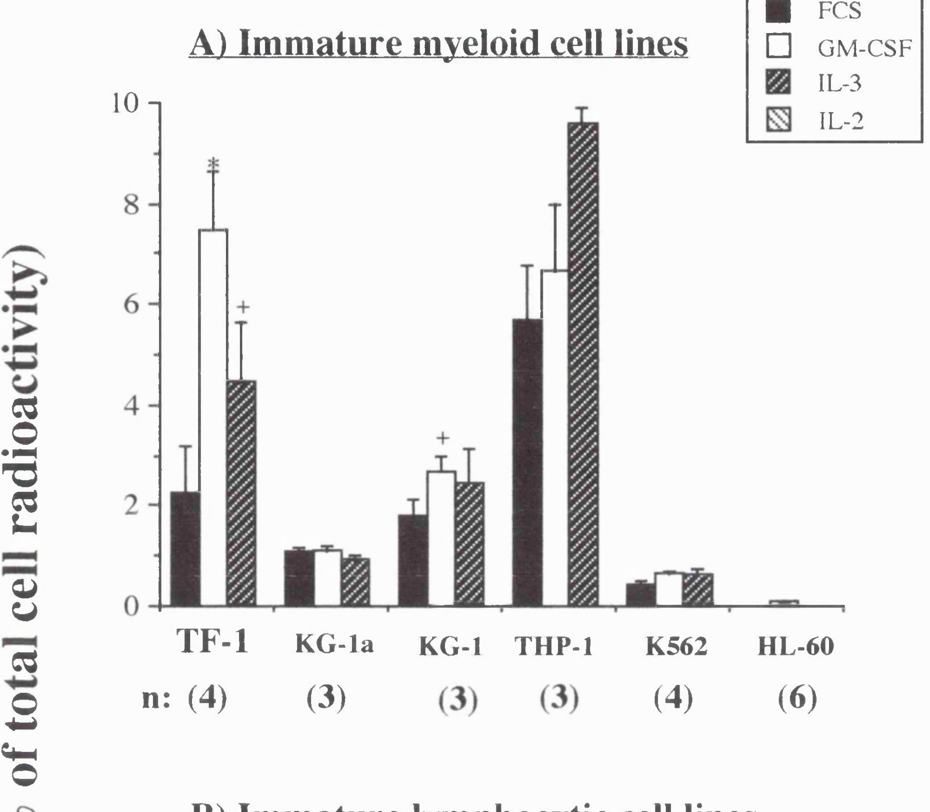
In collaboration with Dr Pamela Roberts, the activity of $PLA_2$ was investigated in primary $CD34^+$ haemopoietic cells which were mobilised into the circulation (Watts *et al.*, 1997). These cells showed a significant release of AA in response to A23187 (6.7% ± 1.6%), PMA (6.1% ± 1.0%) and a combination of both PMA and A23187 (23.2% ± 5.7%) (Table 7.2). In addition, these cells were also cultured for 8 days, and their AA release in response to A23187 was 6.2% ± 0.5%, PMA was 3.3% ± 0.2% and combination of both was 22.4% ± 1.4%.

### 7.3.2 Cytokine-mediated priming of $PLA_2$ activity stimulated by A23187 in haemopoietic cells

The activity of $PLA_2$ was measured by the release of $^3H$-AA when cells were stimulated with either GM-CSF, or IL-3 (both at 10 ng/ml) or diluent (0.01% FCS), followed by stimulation with A23187. Then the released radioactivity was expressed as a percentage of total cellular radioactivity.

*i) Immature myeloid cell lines*

Previous studies have shown that $PLA_2$ activity in mature phagocytes stimulated with either PMA or A23187, is enhanced (primed) when cells were incubated (10-20 minutes) with growth factors such as GM-CSF (DiPersio *et al.*, 1988; Durstin *et al.*, 1994; Roberts *et al.*, 1996). Therefore the effect of priming on AA release in immature haemopoitic cells was investigated and compared to that of the mature phagocytes. The data in Figure 7.2 A show the effect of cytokine-mediated AA release stimulated by A23187. Each cell type showed a different pattern of response. In TF-1 cells, both GM-CSF and IL-3 significantly primed AA release when cells were stimulated by A23187. Similarly, the KG-1 and THP-1 cells showed AA release when primed with either GM-CSF or IL-3. In contrast, KG-1a, K562 and HL-60 cells did not show enhanced AA release when cells were primed with GM-CSF or IL-3 prior to



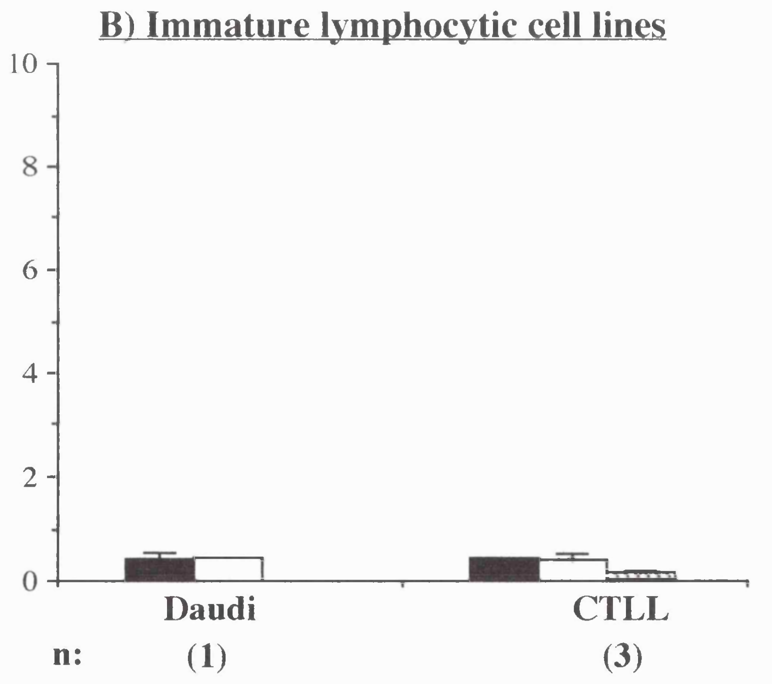




**Figure 7.2: Comparison of $PLA_2$ activity in immature haemopoietic cell lines.** Suspension of cells were incubated for 10 minutes with either growth factor diluent (0.01% FCS), GM-CSF (10 ng/ml), IL-3 (10 ng/ml) or IL-2 (20 ng/ml) and then stimulated with 1 μM A23187 for 15 minutes. Arachidonate release was measured as described in the Methods and Materials. The number (n) of replicate experiments performed are indicated. The statistical significance of the difference between cytokine and diluent mediated priming are shown as: $^{+}P<0.05$, $^{*}P<0.01$

stimulation with A23187. The magnitude of cytokine-mediated priming between cell types was expressed by standardising the data as follows. The arachidonate release in resting cells (i.e. without A23187 stimulation) was subtracted from the A23187-stimulated value and the data for cytokine primed cells was then expressed as a percentage of the respective FCS diluent control. Thus the priming of AA release by GM-CSF was 431% ± 109% in TF-1 cells (n=4), 155% ± 18% in KG-1 cells (n=3), and 147% ± 34% in THP-1 cells (n=4) (Table 7.3).

*ii) Differentiated HL-60 cells*

HL-60 cells differentiated with either 1μM retinoic acid (RA) for 6 days (80% NBT positive) or 1.25% (v/v) DMSO for 5 days (51% NBT positive) were stimulated with either GM-CSF (10 ng/ml), IL-3 (10 ng/ml) or diluent control (0.01% FCS) for 10 minutes followed by stimulation with A23187. The data in Figure 7.3 show that there was little $PLA_2$ activity in undifferentiated HL-60 cells. This activity was increased when HL-60 cells were differentiated with RA (Figure 7.3). The AA release from HL-60 cells differentiated with DMSO was enhanced. The magnitude of priming with GM-CSF was 244% ± 50% for HL-60 cells induced with RA and, 154% ± 9% for HL-60 cells induced by DMSO. Similarly, the magnitude of priming with IL-3 was 154% ± 11% for RA differentiated HL-60 cells, and 92% ± 13% for DMSO differentiated HL-60 cells.

*iii) Immature lymphocytic cell lines*

The activity of $PLA_2$ in lymphocytic cell lines was investigated when cells were primed with growth factors. In a single experiment, priming of $PLA_2$ activity with growth factors had no effect on AA release in Daudi or CTLL cells (Figure 7.2 B). Because myeloid growth factors may not be appropriate priming agents for these cells, CTLL cells were primed with 20 ng/ml IL-2 for 10 minutes prior to stimulation with 1 μM A23187 for 15 minutes. There was no priming of AA release when CTLL cells were stimulated by IL-2 (62% ± 21% of the control, $P>0.05$, n=4).

*iv) Mature neutrophils and monocytes*

Experiments on mature neutrophils showed significant increase in AA release when cells were primed with GM-CSF (516% ± 55%, $P<0.01$, n=6) whereas, the

**Table 7.3: Comparison of the cytokine-mediated priming of growth factor-dependent and -independent haemopoietic cells**

| *Cell types (n)* | *AA release* (% priming) *FCS* | *GM-CSF* | *IL-3* |
|---|---|---|---|
| TF-1 (4) | 100% | 431% ± 109% * | 251% ± 34%* |
| KG-1 (3) | 100% | 155% ± 18% | 130% ± 13% |
| KG-1a (3) | 100% | 107% ± 7% | 81% ± 10% |
| THP-1 (4) | 100% | 147% ± 34% | 188% ± 28% |
| K562 (3) | 100% | 115% ± 9% | 119% ± 7% |
| HL-60 (6) | 100% | 104% ± 8% | ND |
| HL-60+RA (3) | 100% | 244% ± 50% | 154% ± 11% |
| HL-60+DMSO (3) | 100% | 154% ± 9% | 92% ± 13% |
| Daudi (1)‡ | 100% | 83%, 88% | 50%, 55% |
| CTLL (3) | 100% | 53% ± 18% | ND |
| Neutrophils (6) | 100% | 516% ± 55%* | 112% ± 15% |
| Monocytes (3) | 100% | 194% ± 73% | 165% ± 68% |
| CD34+ (5) | 100% | 148%±23% | 140%±19% |

Cell suspensions were preincubated with either GM-CSF (10 ng/ml), IL-3 (10 ng/ml) or growth factor diluent (0.01% FCS) for 10 minutes prior to stimulation with A23187 (1 μM) for 15 minutes. For each experiment the AA release in resting cells was subtracted from the A23187-stimulated value and the data for cytokine primed cells was then expressed as a percentage of the respective FCS diluent control. The data shown are the mean ± 1 SE of the number of experiments shown in parentheses. The significance of the difference between the samples primed with cytokines and their respective FCS control is shown as: $^{+}P<0.05$, $^{*}P<0.01$. ND=not done

‡Individual values for duplicate samples of Daudi cells are shown.

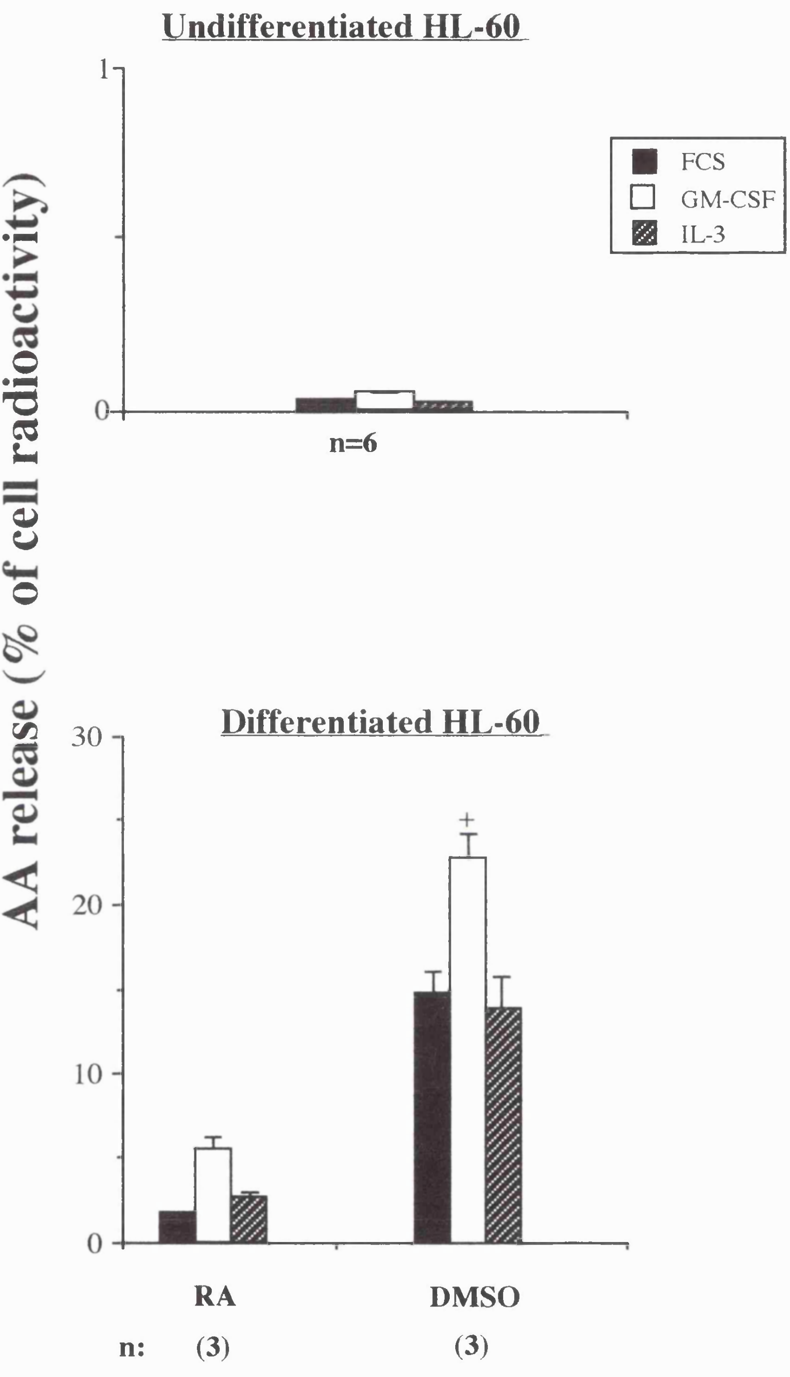



**Figure 7.3: The activity of $PLA_2$ in HL-60 cell lines.** Undifferentiated or differentiated HL-60 cells (80% NBT positive when differentiated with RA and 51% NBT positive when differentiated with DMSO) were incubated for 10 minutes with either growth factor diluent (0.01% FCS), GM-CSF (10 ng/ml), or IL-3 (10 ng/ml) and then stimulated with 1 μM A23187 for 15 minutes. Arachidonate release was measured as described in the Methods and Materials. The number (n) of replicate experiments performed are indicated. The statistical significance of the difference between cytokine and diluent mediated priming are shown as: $^{+}P<0.05$.

response to IL-3 was 112% ± 15% ($P$>0.05, n=6) of the FCS control (Figure 7.4). In contrast, GM-CSF (194% ± 73%, $P$>0.05, n=3) and IL-3 (165% ± 68% $P$>0.05, n=3) both equally primed AA release in monocytes (Table 7.3)

*v) Primary CD34$^+$ stem cells*

The effect of stimulation with growth factors on A23187-stimulated AA release is shown in Figure 7.5. Cells were preincubated with 500 ng/ml PMA, 100 ng/ml SCF, 10 ng/ml GM-CSF or diluent control (0.01% FCS) prior to stimulation with 1μM A23187 for 20 minutes. The release of AA from A23187-stimulated cells which were preincubated with growth factor diluent (FCS) remained relatively constant over the culture period. There was no significant priming in response to GM-CSF and IL-3 in day 1 cells but significant priming to SCF was observed. The effect of SCF was seen to decline with culture and by day 8 no significant SCF-responses were seen. In contrast, the priming effect of GM-CSF and IL-3 on A23187-stimulated $PLA_2$ activity increased during the culture period to levels that were significantly greater than those seen with diluent (Figure 7.5). The increase in GM-CSF and IL-3 response was concomitant with the increase in more mature phagocytic cells in the culture, whereas the SCF response declined in parallel with the number of blast cells (Figure 7.1).

### 7.3.3 Activation of $p42^{ERK2}$ in CD34$^+$ cells

To investigate whether GM-CSF, SCF and PMA could activate MAP kinase in primary CD34$^+$ stem cells, cells were stimulated with either 500 ng/ml PMA, 10 ng/ml GM-CSF or 100 ng/ml SCF and phosphorylation of $p42^{ERK2}$ was measured by gel retardation assay and western blotting (see Chapter 2 for details). As shown in Figure 7.6 both PMA and SCF activated $p42^{ERK2}$, but with different kinetics. Activation of $p42^{ERK2}$ by SCF and PMA was rapid, being apparent within 1 minute and maximal within 2-3 minutes of stimulation (Figure 7.6 A). However, responses to SCF were transient and were virtually undetectable at 11 minutes post-stimulation (Figure 7.6 B), whereas the responses to PMA persisted at these time intervals. In contrast, no response to GM-CSF was apparent in day 1 CD34$^+$ cells (Figure 7.6 B). These data are consistent with ability of PMA and SCF, and the inability of GM-CSF, to prime $PLA_2$ activity in these primitive cells.

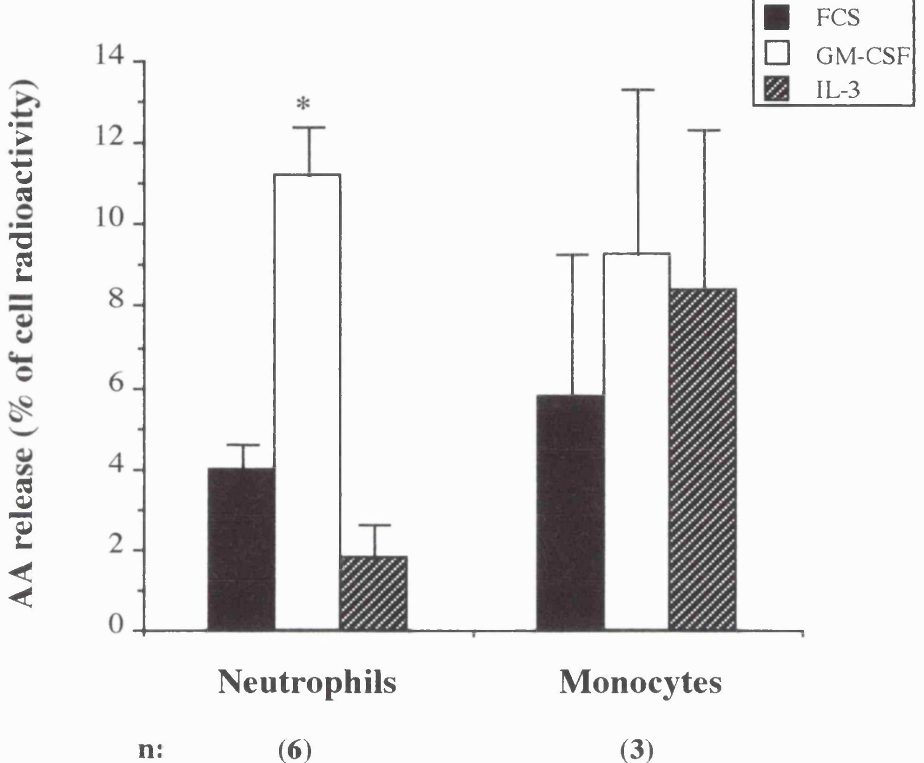



**Figure 7.4: The activity of $PLA_2$ in purified human neutrophils and monocytes.** Suspension of cells were incubated for 10 minutes with either growth factor diluent (0.01% FCS), GM-CSF (10 ng/ml), or IL-3 (10 ng/ml) and then stimulated with 1 μM A23187 for 15 minutes. Arachidonate release was measured as described in the Methods and Materials. The number (n) of replicate experiments performed are indicated. The statistical significance of the difference between cytokine and diluent mediated priming are shown as: $^{+}P<0.05$.

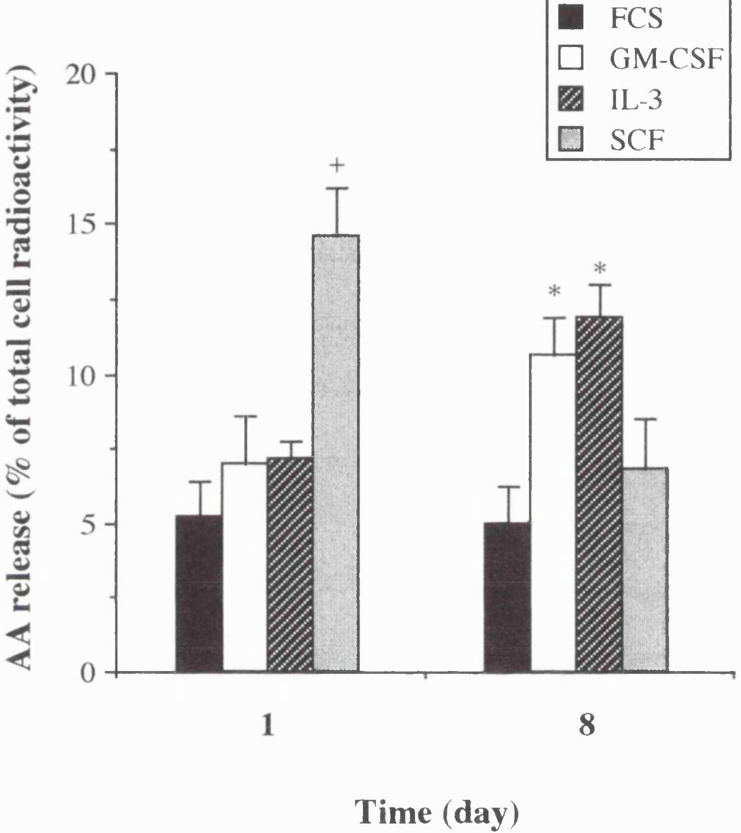



**Figure 7.5: Regulation of phospholipase $A_2$ activity during differentiation of $CD34^+$ cells**. Freshly purified cells were incubated for up to 8 days with IL-3, IL-6 and SCF (all at 10 ng/ml). Following an 18 hour incubation without growth factors, cells were primed with either IL-3 (10 ng/ml), GM-CSF (10 ng/ml), SCF (100 ng/ml) or FCS diluent (0.01%), followed by activation with 1$\mu$M A23187 for 15 minutes and AA release was measured. The data shown are the increment in AA release due to A23187 stimulation following subtraction of AA release in control samples stimulated without A23187, and are the mean ± 1SE of 3-8 experiment. The statistical significance of the differences between cytokine and diluent-treated cells are shown as: $^+P<0.05$, $^*P<0.01$

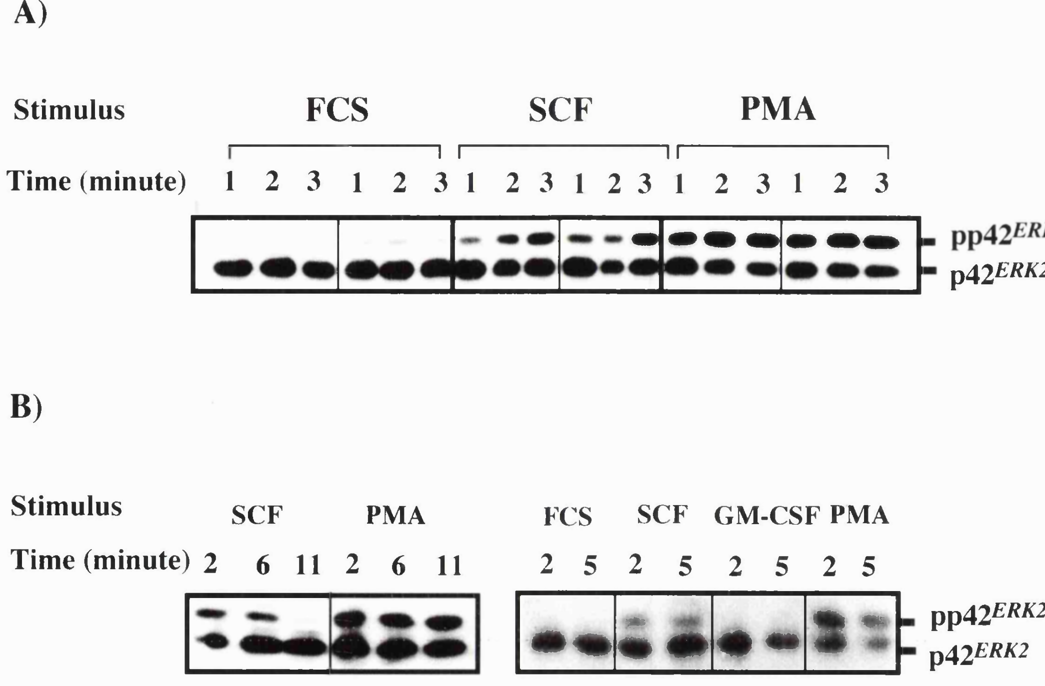



**Figure 7.6: Activation of p42$^{ERK2}$ in primary CD34$^{+}$ cells measured by gel retardation assay and western blotting.** A) Cells were stimulated with either cytokine diluent (0.01% FCS), SCF (100 ng/ml), or PMA (500 ng/ml) for the times indicated. B) Cells were incubated with cytokines as in (A) as well as GM-CSF (10 ng/ml), for the times indicated . The autoradiographs shown in (A) are from a single experiment that was performed in duplicate and (B) are from two experiments.

### 7.3.4 Effect of PD 098059, MEK1 inhibitor, on $PLA_2$ activity in $CD34^+$ cells

In order to investigate whether activation of MAP kinase was required for $PLA_2$ activity in $CD34^+$ cells, the effect of PD 098059 on AA release was studied. Figure 7.7 A confirms that 30 μM PD 098059 inhibited the activation of $p42^{ERK2}$. Then $CD34^+$ cells were incubated for 15 minutes with either 30μM PD 098059 or DMSO diluent control, and AA release after priming with growth factors and activation with A23187 was measured as described in the Method section. Figure 7.7 B shows that PD 098059 partially inhibited AA release in day 1 cells primed with either growth factor diluent (to 59% ± 36% of DMSO control, n=4), SCF (to 47% ± 6% of the control, n=4) or PMA (to 36% ± 12% of control, n=4). Similarly, in 3 experiments PD 098059 partially inhibited AA release in day 8 cells primed by diluent (to 66% ± 8% of the DMSO control), GM-CSF (to 45% ± 8% of control), and PMA (to 57% ± 8% of control).

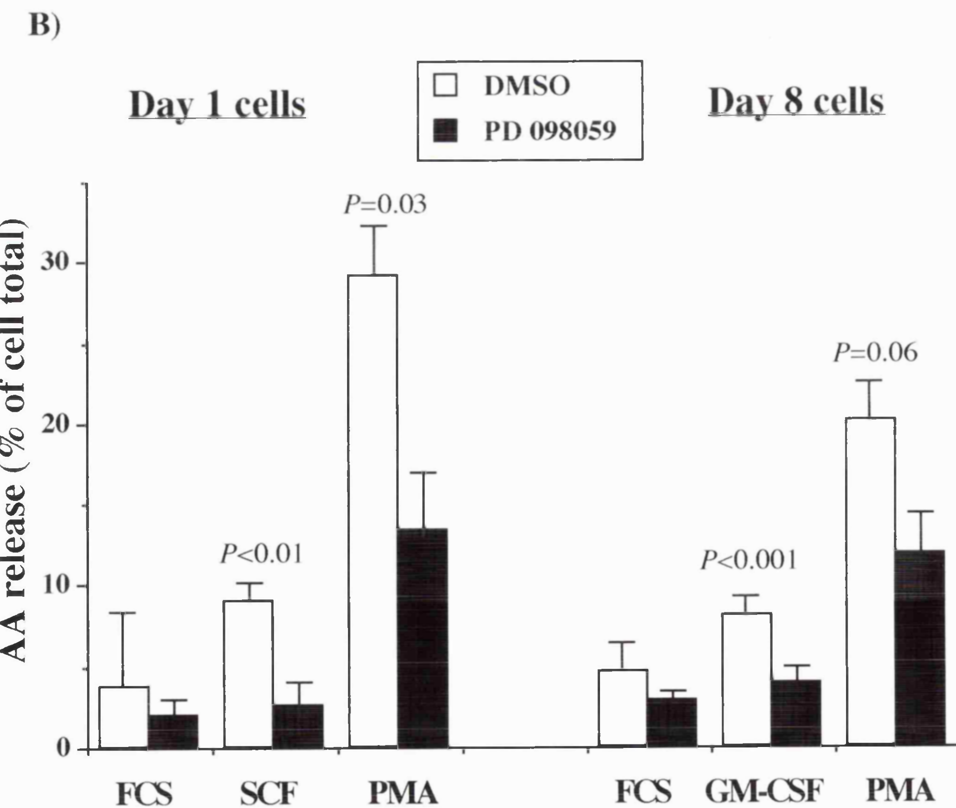

A)
GM-CSF - - + + - + - +
PD 098059 - + - + - - + +
pp42ERK2
p42ERK2
B)
Day 1 cells
DMSO
PD 098059
Day 8 cells
AA release (% of cell total)
0
10
20
30
P<0.01
P=0.03
P<0.001
P=0.06
FCS
SCF
PMA
FCS
GM-CSF
PMA

**Figure 7.7: Effect of MEK1 inhibitor, PD 098059, on $CD34^+$ cells.**
A) Cells cultured for 8 days in IL-3, IL-6, and SCF were incubated for18 hours in the absence of growth factors to allow re-expression of growth factor receptors. Cells were then preincubated with 30$\mu$M PD 098059 or DMSO diluent for 30 minutes prior to stimulation with GM-CSF for 7 minutes, and $p42^{ERK2}$ activation was measured by gel retardation assay. Data shown are from two separate experiments. B) Effect of PD 098059 on $PLA_2$ activity of primary $CD34^+$ cells (day 1) or day 8 cultured cells. Cells were preincubated for 30 minutes with 30$\mu$M PD 098059 or DMSO diluent, and AA release was measured after 10 minutes priming with 100 ng/ml SCF, 10 ng/ml GM-CSF or 500 ng/ml PMA, followed by activation with 1$\mu$M A23187 for 20 minutes. Basal release of AA in unstimulated samples was subtracted from the A23187-stimulated values, and the data expressed as the percentage of incorporated cellular radioactivity. The data shown are the mean ± 1SE of four experiments. The significance of the difference between the samples with PD 098059 and DMSO control is shown as *P*.

## 7.4 DISCUSSION

This study has demonstrated that immature myeloid cell lines, K562 and HL-60 have very little $PLA_2$ activity when cells were stimulated with the most potent agonists A23187 and PMA. In contrast, TF-1, THP-1, KG-1 and KG-1a cells showed more $PLA_2$ activity which was comparable with that of mature neutrophils and monocytes. TF-1 and THP-1 cells showed significantly high levels of AA release in response to A23187 and in combination with PMA. KG-1 and KG-1a cells also showed significant increase when cells where stimulated by PMA followed by A23187.

The lymphocytic cells, Daudi and growth factor-dependent CTLL cells did not show any $PLA_2$ activity in response to either agonist. Priming of these cells with either GM-CSF or IL-3 (myeloid growth factors) had no effect on AA release, therefore CTLL cells were primed with IL-2 followed by stimulation by A23187. These results showed that IL-2 had no effect on $PLA_2$ activity in CTLL cells (62% ± 21%, $P$>0.05, n=4). The lack of responsiveness of Daudi and CTLL cells, to GM-CSF was likely due to their lack of GM-CSF receptor expression, and the lack of response to IL-2 indicates that the mechanism by which IL-2 induces proliferation of CTLL cells is not via $PLA_2$ activation.

Mature neutrophils and monocytes showed an enhanced AA release in reponse to A23187, PMA and in combination. The $PLA_2$ activity was increased when these cells were incubated with GM-CSF prior to stimulation with A23187. The magnitude of priming of neutrophils with GM-CSF was greater (516% ± 55%, $P$<0.01, n=6) compared to that of monocytes (194% ± 73%, $P$>0.05, n=3), whereas the response to IL-3 was greater in monocytes (165% ± 118%, $P$>0.05, n=3) compared to that of mature neutrophils (112% ± 15%, $P$>0.05, n=6) confirming the fact that neutrophils do not express IL-3 receptors.

Similarly, primary $CD34^+$ stem cells showed $PLA_2$ activity which was measured in intact cells by the extracellular release of AA using radiometric assay. $PLA_2$ activity of $CD34^+$ cells stimulated by A23187 was of similar magnitude as that in similarly-stimulated neutrophils and monocytes. This study further investigated the changes in $PLA_2$ activity during *in vitro* differentiation of $CD34^+$ stem cells with IL-3, IL-6 and SCF. There was a minor decrease in $PLA_2$ activity over time in the responses to PMA + A23187, and this contrasts with significant increase in $PLA_2$ activity during HL-60 cell differentiation that was previously reported (Billah *et al.*, 1986). The A23187-stimulated AA release from freshly purified $CD34^+$ stem cells was enhanced by preincubation with SCF but not significantly by GM-CSF or IL-3. This differential activation by SCF was confirmed in experiments where SCF was able to activate $p42^{ERK2}$ whereas no responses to GM-CSF were seen. The selective activation of

CD34+ cells by SCF at this early stage of differentiation is consistent with SCF being an early-acting factor for haemopoiesis (Broudy, 1997). The $PLA_2$ response to growth factors changed when $CD34^+$ stem cells were induced to differentiate. The A23187-stimulated AA release ceased to be enhanced by SCF, but was able to respond to both IL-3 and GM-CSF. This may reflect either a change in growth factor receptor expression or changes in signal transduction pathways.

The $PLA_2$ activity in primary $CD34^+$ stem cells was partially inhibited when cells were incubated with PD 098059, whether cells were primed with SCF or unprimed (30-50% of the control) suggesting the involvement of MEK1 in $PLA_2$ activation. However, inhibition of PLA2 by PD 098059 was only partial indicating the possibility for another pathway of activation. Indeed, recent evidence showed that in platelets p38 MAPK and other kinases have a role in the activation of $PLA_2$ in certain cell types (Börsch-Haubold *et al.*, 1998).

In conclusion this study showed that each cell type has a different pattern of response to agonists or when they were primed by growth factors. There was not a clear relationship between the growth factor-dependent and –independent cells in their ability to release AA in response to agonists. Pri mary $CD34^+$ stem cells also express $PLA_2$ activity indicating that expression in cell lines is not an aberrant phenomenon. The regulation of $PLA_2$ activity in primary $CD34^+$ stem cells by SCF which promotes the maintenance of these cells, supports the notion that $PLA_2$ activity has a role in the physiology of human stem cells.

# CHAPTER 8

# GENERAL DISCUSSION

AA is a long chain, polyunsaturated fatty acid which is esterified in the fatty acyl chains of glycerophospholipids at the *sn*-2 position. AA and related fatty acids are involved in a series of biological events that amplify/regulate cell activation and inflammatory responses. For example, AA has been shown to induce degranulation, regulate chemotaxis, increase expression of CR3 receptors and activate the oxygen-dependent respiratory burst in neutrophils. It has been shown that AA stimulates the activity of NADPH oxidase by increasing the number of active enzyme complexes and its affinity for the NADPH (Rubinek and Levy, 1993; Sumomoto *et al.*, 1994).

In Chapter 3 the activity of unprimed NADPH oxidase and $PLA_2$ in neutrophils from patients with SCD, both in steady state and during painful vaso-occlusive crises was investigated. This study further investigated the effect of cytokine-mediated priming on these two enzymes and showed increased basal $PLA_2$ activity in neutrophils in steady state of SCD which indicates the activation of neutrophils in steady state of the disease. However, using a whole blood assay there was no concomitant increase in basal NADPH oxidase activity. There is evidence for activation of neutrophil NADPH oxidase when in contact with sickled red blood cells (Hofstra *et al.*, 1996), however this study used purified neutrophils *in vitro* . It is possible that neutrophil $PLA_2$ may also be activated in the same way and this would be an area for future investigation.

A previous study has shown that $PLA_2$ activity is associated with GM-CSF primed superoxide production (Roberts *et al.*, 1996). Therefore I investigated the effect of both GM-CSF and TNF$\alpha$ on priming of NADPH oxidase and $PLA_2$ when neutrophils from patients with SCD were stimulated by FMLP or calcium ionophore. I showed that production of both $H_2O_2$ and AA in primed cells was only 50% of control values. The defective $H_2O_2$ production in SCD patients during painful crisis was even more pronounced (30% of control). The inability of neutrophils from patients with SCD to fully prime when stimulated with cytokines may render these patients susceptible to infection. As the defective priming of neutrophil $H_2O_2$ production in SCD patients was not due to inherent defects of the NADPH oxidase, further mechanisms that might cause altered neutrophil function in SCD were studied in Chapter 4. Racial differences in neutrophil function and poor splenic function were excluded as no defects in neutrophil $H_2O_2$ production were observed in either healthy Afro-Caribbean subjects or splenectomised patients. The defective priming seen in patients with SCD was not reproduced in patients with rheumatoid arthritis, indicating that the defect was not due to the general inflammatory reaction but likely to be associated with events in the circulation. To investigate whether the defective priming of $H_2O_2$ production in neutrophils from SCD patients was due to the severe anaemia associated with the disease, a similar study was carried out in neutrophils from a

group of patients with iron-deficiency anaemia. However, there was no evidence for reduced $H_2O_2$ in these patients. Finally, the effect of haemolytic conditions associated with SCD on priming of $H_2O_2$ production was investigated in patients with β-thalassaemia intermedia. The data showed reduced cytokine-mediated priming of $H_2O_2$ production in neutrophils from these patients, indicating that intravascular haemolysis may be a contributing factor in defective priming of neutrophils. Therefore in a study, sickle cell plasma was replaced with plasma from a healthy individual. The defective priming of $H_2O_2$ production was only partially reversed, indicating that a component of the defect was of cellular origin. In addition, normal cells suspended in plasma from patients with SCD in steady state showed reduced $H_2O_2$ production. Similarly, when diluted plasma from patients in crisis was used a reduction of $H_2O_2$ production was also observed. These data indicate both cellular and plasma defects associated with SCD. The mechanism whereby haemolysis may lead to defective neutrophil function is an area for further studies.

Priming of the respiratory burst and $PLA_2$ may occur at different sites, either in the agonist signal transduction pathways to these enzymes or in the components of the NADPH oxidase. In Chapter 5 and 6 the involvement of $p42^{ERK2}$ and p38 MAP kinase pathways in the activation of NADPH oxidase and $PLA_2$ in unprimed and primed human neutrophils was investigated by using the two specific inhibitors of $p42^{ERK2}$ and p38 MAPK. The results presented in this thesis indicate that $p42^{ERK2}$ is phosphorylated by FMLP and this was inhibited by 10 μM PD 098059 whereas, the FMLP-stimulated respiratory burst activity was not significantly inhibited by PD 098059 at this dose. In comparison, SB 203580 showed an inhibitory effect on the unprimed FMLP-stimulated respiratory burst (18% ± 7% of the control with 40 μM SB 203580, $P<0.05$, n=3) but not basal activity suggesting the involvement of p38 MAPK in activation of the neutrophil NADPH oxidase. Indeed, the rapid stimulation of p38 MAPK activation by FMLP shown in the kinase assay using MAPKAPK-2 as the downstream substrate, was similar to the time course of superoxide generation elicited by FMLP, which upholds the hypothesis that p38 MAPK activation is necessary for stimulation of the respiratory burst. In the present study, the involvement of p38 MAPK, in the activation of $PLA_2$ was investigated by using the inhibitor of this kinase, SB 203580. These studies showed that p38 MAPK is unlikely to be involved in the activation of $PLA_2$ as there was no inhibitory effect of SB 203580 on cytokine-mediated AA release when neutrophils were stimulated by FMLP. This conclusion is confirmed when calcium-ionophore (A23187), a potent activator of $PLA_2$, was used as an agonist and SB 203580 also failed to block this activation. Failure of SB 203580 to inhibit $PLA_2$ activity stimulated by FMLP indicates that the inhibition of FMLP-stimulated p38 MAPK activity in the kinase assay is not due to

any non-specific effect of the inhibitor. Our data indicate that p38 MAPK and $PLA_2$ are differentially sensitive to SB 203580. Of interest, I have found that although SB 203580 does not inhibit either FMLP or A23187 activity of $PLA_2$ it was an inhibitor of leukotriene production which occurs downstream of $PLA_2$ activation, and this compound may therefore be a very useful antiinflammatory agent. In contrast, both the primed and unprimed FMLP-stimulated respiratory burst activity was inhibited by SB 203580 in a dose dependent manner.

Incubation of neutrophils with different doses of PD 098059 prior to priming by either TNFα or GM-CSF followed by stimulation with FMLP caused partial inhibition of AA release. This observation suggests that the priming mechanism for NADPH oxidase and $PLA_2$ are different. PD 098059 also inhibited in a dose-dependent manner both the unprimed and cytokine-mediated AA release when stimulated by A23187 which was maximal at 10 μM and 15 μM PD 098059 when neutrophils were primed with either TNFα or GM-CSF, respectively. The doses of PD 098059 that showed inhibitory effects on A23187 stimulated $PLA_2$ activity corresponds to the doses (10-40 μM) that blocked the phosphorylation of $p42^{ERK2}$ to undetectable levels. In contrast, SB 203580 did not inhibit cytokine-mediated primed AA release when cells were stimulated by A23187. These data also suggest that the priming pathways utilised by the respiratory burst and $PLA_2$ are different. At this stage it is not known which isoform of $PLA_2$ is involved in mediating priming by GM-CSF, although experiments carried out in these chapters showed calcium-dependent AA release, and this was further increased by preincubation of the cells with phorbol ester, PMA. These are characteristics shared by $cPLA_2$ isoform however it has also been reported that $iPLA_2$ isoform was also present in human neutrophils (Larsson *et al.*, 1998). The identification of the isoforms involved would be an area for further work.

Finally, in Chapter 7 the $PLA_2$ activity of immature myeloid cells was studied. TF-1 and THP-1 cells showed high levels of AA release in response to A23187 and in combination with PMA. KG-1 and KG-1a cells also showed an enhanced AA release when cells were stimulated with A23187 followed by PMA. In contrast, lymphocytic cell lines, Daudi and CTLL, did not show any $PLA_2$ activity in response to either agonist. Of myeloid cells only TF-1, THP-1 and KG-1 cell lines showed $PLA_2$ activity when primed by GM-CSF and IL-3 which are known to regulate proliferation of haemopoietic cells. In addition, mature neutrophils and monocytes both showed increased $PLA_2$ activity when primed with GM-CSF. When this study was carried out in primary $CD34^+$ stem cells their $PLA_2$ activity was of similar magnitude as that in similarly-stimulated mature neutrophils and monocytes. This degree of $PLA_2$ activity in primary $CD34^+$ stem cells was relatively unexpected as these are highly immature. The AA release in these cells was selectively enhanced by SCF but not significantly by

GM-CSF or IL-3 confirming that SCF is important as a regulatory factor in these cells. This was further confirmed when we showed that the $PLA_2$ response to growth factors was changed when primary $CD34^+$ cells were differentiated and SCF responses were also lost, concomitantly with the disappearance of blast cells from the cultures. The activation by SCF was confirmed where SCF was able to activate $p42^{ERK2}$ in the primary $CD34^+$ stem cells whereas no response to GM-CSF was observed. The $PLA_2$ activity was only partially inhibited in the presence of PD 098059 in primary $CD34^+$ stem cells indicating the possibility for another pathway of activation such as p38 MAPK in addition to $p42^{ERK2}$ and this would be an area for further investigation.

In conclusion, I have found that $PLA_2$ can be activated in both the most primitive and in fully mature myeloid cells. The role of $PLA_2$ in such early cells is completely unknown, but may have importance in signalling pathways such as activation of protein kinase C or calcium mobilisation, and in regulating gene expression.

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# PUBLICATIONS

## ABSTRACTS

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## PAPERS

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