1.1 Introduction

The laboratory research described in this thesis was undertaken with the primary objective of identifying novel pharmacologic strategies for the control of the pro-inflammatory activities of human neutrophils *in vitro*, with particular emphasis on adenosine receptor agonists. The opening chapter is devoted to a literature review which is focussed on i) the pro-inflammatory activities of human neutrophils (including the recently-documented ability of these cells to synthesise pro-inflammatory cytokines *de novo*) ii) the involvement of calcium (Ca^{2+}) in initiating these activities iii) the anti-inflammatory spectrum, molecular mechanisms of action and limitations of corticosteroids and iv) the sub-types and distribution of adenosine receptors, particularly with respect to human neutrophils and their functions. This is followed by two chapters in which the effects of corticosteroids (chapter 2), as well as those of adenosine receptor agonists operative at the level of the A_1, A_{2A} and A_3 adenosine receptor sub-types (chapter 3) on pro-inflammatory activities of neutrophils are described. In chapter 4, the effects of an adenosine receptor agonist (CGS 21680) operative at the level of the sub-type A_{2A} receptor on Ca^{2+} fluxes and restoration of Ca^{2+} homeostasis in activated neutrophils are described, while an integrated assessment of the data is presented in the final chapter. Each chapter of results is a distinct entity with its own introduction, materials and methods, results and conclusions sections.

1.2 Literature Review

1.2.1 Origins of human neutrophils

Blood is the most readily obtained source of neutrophils and serves as the vehicle for their delivery to the various tissues from the bone marrow where they are produced. Neutrophils evolve from pluripotent stem cells under the influence of cytokines and colony stimulating factors. It is estimated that 400-500 stem cells are necessary to support haematopoiesis. The haematopoietic system not only produces enough neutrophils (1.3×10^{11} cells per 80 kg person per day) to carry out physiologic functions, but also has large reserve stores in the marrow which can be mobilised in

response to inflammation and infection. Approximately 8-14 days are required for a cell to move through the sequence of 4-6 cell divisions and complete maturation. After cell division is completed there are 3-4 days of neutrophil maturation. During this time the maturing cells can be released from the bone marrow into the blood under conditions of sufficient stress. These cells comprise the marrow neutrophil reserve and are 10 times as numerous as the neutrophils in the blood (Bainton, 1992).

Specific signals, including IL-1, IL-3, TNF-α, colony stimulating factors, the complement factors C3e, C5a and other cytokines mobilise neutrophils from the bone marrow which circulate in the unstimulated state. Under normal conditions 90% of the neutrophil pool is in the bone marrow, 2-3% in the circulation and the rest in the tissues. Up-regulation of the production of these signals during inflammatory stress increases the tempo of production and release of neutrophils from the bone marrow (Bainton, 1992; Goldstein, 1992; Greene, 1992).

The myeloblast is the first recognisable precursor cell and is followed by the promyelocyte which is characterised by the appearance of the classical lysozomal granules, known as primary or azurophilic granules. The promyelocyte divides and differentiates into the myelocyte, which, in addition to the primary granules, also contains secondary or specific granules (Bainton, 1992).

Following the myelocyte stage no further division takes place and during the final stages of maturation the cell passes through the metamyelocyte and then the band neutrophil phases. On maturation of the band form the nucleus becomes lobulated, consisting of up to four segments. The physiologic role of the multilobed nucleus is unclear, but may allow greater deformability during migration into tissue at sites of inflammation.

The circulating neutrophil pool exists in two dynamic compartments, freely flowing and marginated. The freely flowing pool consists of half of the neutrophils in the basal state and is composed of those cells that are in the blood and not in contact with the endothelium. Marginated neutrophils are in close physical contact with the endothelium. In the pulmonary circulation margination occurs because the

capillaries have about the same diameter as the mature neutrophils. Neutrophil fluidity and deformability are necessary for transit through the pulmonary bed. In the systemic post capillary venules, margination is mediated by chemoattractants and adhesion molecules by mechanisms which are described in later sections (1.3.1 and 1.3.2).

The lifespan of the neutrophil is estimated to be 24-48 hours (Bainton, 1992), after which the neutrophils undergo apoptosis (programmed cell death) and removal by the mononuclear phagocyte system, a process which is dependent on interactions between phosphatidylserine translocated from the inner to the outer plasma membrane of the apoptotic neutrophil and phospatidylserine receptors on monocytes/macrophages (Fadok *et al.*, 1998). Interactions between Fas ligand (FasL) on macrophages, as well as soluble FasL released by these cells, and the Fas "death receptor" on neutrophils may represent a mechanism of resolution of inflammation (Brown & Savill, 1999). Prolongation of the lifespan of the neutrophil occurs following migration of these cells out of the circulation to sites of inflammation and exposure to anti-apoptotic cytokines such as granulocyte/macrophage colony stimulating factor (GM-CSF) (Watson *et al.*, 1999).

Members of the ced-9-Bcl-2 family of apoptotic regulators have been shown to play a pivotal role in the regulation of apoptosis across multiple cell types and between species. Mediators that prolong the lifespan of neutrophils both *in vivo* and *in vitro* may exert these effects by induction of specific anti-apoptotic members of this family. Two anti-apoptotic members, Mcl-1 and A1, are abundantly expressed in neutrophils. An isoform of Mcl-1, (Mcl-1 S/Δ TM) is rendered similar to BH3 (containing only pro-apoptotic proteins) by deletion of BH1, BH2 and transmembrane regions. Both forms are expressed in neutrophils and thus co-expression of both pro- and anti-apoptotic isoforms of Mcl-1 may be an important regulatory event in the control of neutrophil apoptosis and interference with this balance may provide a strategy to control neutrophil mediated tissue injury (Bingle *et al.*, 2000).

1.2.2 Neutrophil granules

Notwithstanding the dynamic outer membrane and multilobed nucleus, the abundant cytoplasmic granules are the most striking structural features of neutrophils. These neutrophil granules have emerged as an extremely heterogeneous group of organelles and, according to the simplest classification, consist of four different groups distinguished on the basis of protein content, size and density. These are the primary (azurophil), secondary (specific) and tertiary (gelatinase) granules, and secretory vesicles (Witko-Sarsat *et al.*, 2000). As mentioned previously, primary and secondary granules are formed during the promyelocyte and myelocyte/ metamyelocyte stages respectively, while tertiary granules develop at the metamyelocyte/band cell stage (Le Cabec *et al.*, 1996). Secretory vesicles are the last to appear becoming evident in band and segmented cells (Borregard & Cowland, 1997). These different granule sub-types vary with respect to efficiency of mobilisation during neutrophil activation. The order of mobilisation is negatively correlated with the size and density of the granules. Secretory vesicles represent the most mobilisable of the neutrophil granule sub-types (Witko-Sarsat *et al.*, 2000), while the order of exocytosis observed in calcium ionophore (A 23187)-activated neutrophils was secretory vesicles, tertiary, secondary and primary granules, which was correlated with progressive increases in cytosolic Ca^{2+} (Sengelov *et al.*, 1993).

In addition to functioning as a mobilisable reservoir of membrane constituents, these various neutrophil granules contain an array of proteases and antimicrobial peptides and polypeptides (including several proteases) which participate in the migratory and antimicrobial activities of the neutrophil. The fact that several granule polypeptides are shared by the different granule sub-types (eg lysozyme and proteinase 3) is indicative of overlap between these (the granules), which may represent a continuum as opposed to clearly demarcated granule types (Witko-Sarsat *et al.*, 2000).

Primary granules

The majority of neutrophil proteases and antimicrobial peptides/polypeptides are stored in the primary granules. In contradistinction to the other granule sub-types, primary granules, perhaps reflecting their relative resistance to exocytosis, do not function as reservoirs of membrane receptors and polypeptides, although CD63 and

CD68 are present on the membranes of these granules, but the functional significance of this remains to be established (Saito *et al.*, 1991; Cham *et al.*, 1994). Myeloperoxidase and the defensins (a family of at least four peptides), also known as human neutrophil peptides (HNP1 to HNP4), small cationic, broad-spectrum antimicrobial peptides that contain six cysteines in disulphide linkage, are present in extremely high concentrations in primary granules (Elsbach & Weiss, 1992; Witko-Sarsat *et al.*, 2000). The constituents of neutrophil primary granules and their distribution are listed in Table 1.1 (page 7).

Although primary granules have not been considered to act as reservoirs of membrane components involved in the migratory, phagocytic and oxidant-generating activities of neutrophils, a very recent report describing translocation of Sialyl Lewis x from the membranes of primary granules to the plasma membrane during activation of these cells with chemoattractants suggests that this may not be the case (Suzuki *et al.*, 2000). These authors contend that primary granules may contribute to the up-regulation of Sialyl Lewis x, the ligand for the endothelial adhesion molecules, E- and P-selectins, on the cell surface following activation of neutrophils (Suzuki *et al.*, 2000).

Secondary granules

Lactoferrin and vitamin B_{12}-binding protein are unique to secondary granules, which are also notable for their high content of membrane-associated adhesion molecules, receptors for chemoattractants, cytokines and adhesion molecules, and cytochrome b558 (an integral component of the phagocyte superoxide-generating system, NADPH-oxidase). During neutrophil activation, mobilisation of secondary granules to the outer membrane is thought to cause sustained neutrophil activation by augmenting and/or replenishing these various pro-adhesive, -migratory and -oxidative polypeptides. The constituents of neutrophil secondary granules and their distribution are shown in Table 1.1 (page 7).

Tertiary and secretory granules

The tertiary and secretory granules also act as a reservoir for membrane polypeptides involved in neutrophil activation and function, albeit to a considerably lesser extent than secondary granules. Their major contribution to neutrophil

Table 1.1: Constituents of Neutrophil Primary and Secondary Granules

Primary Granules		Secondary Granules	
Membrane	***Matrix***	***Membrane***	***Matrix***
CD63	myeloperoxidase	cytochrome b558	lactoferrin
CD68	elastase	CD11b	vitamin B_{12} binding protein
Sialyl Lewis x	cathepsin G	FMLP receptor	lysozyme
	proteinase 3	TNF receptor	collagenase
	defensins	G-protein α-subunit	gelatinase
	azurocidin	laminin receptor	histaminase
	BPI*	fibronectin receptor	heparinase
	secretory PLA_2	thrombospondin receptor	$ß_2$-microglobulin
	lysozyme	vitronectin receptor	urokinase plasminogen activator
	ß-glucuronidase	urokinase plasminogen activator receptor	
	acid ß-glycerophosphatase		
	acid mucopolysaccaride		
	α-mannosidase		
	heparin-binding protein		
	sialidase		
	α_1-antiprotease inhibitor		
	ubiquitin protein		

* Bactericidal permeability increasing protein

function is thought to be achieved through extracellular release of gelatinase which cleaves types IV (basement membrane) and V (interstitial tissues) collagen, thereby facilitating movement of the cells through basement membranes and into underlying tissue (Witko-Sarsat *et al.*, 2000). Secretory vesicles are thought to be endocytic in origin because they contain plasma proteins such as albumin. Interestingly, proteinase 3, a serine proteinase present in neutrophil primary granules, is also localised in the membrane of secretory vesicles, which are the most mobilisable compartment of neutrophils (Witko-Sarsat *et al.*, 1999). Secretory vesicles may also act as a reservoir of membrane components. The constituents of neutrophil tertiary granules and secretory vesicles are shown in Table 1.2 (page 9).

Secretory phospholipase A_2, a 14-kDa group II PLA_2, is also released by activated human neutrophils, and although of probable granule origin, its exact intracellular location remains to be established (Seeds *et al.*, 1998).

The mechanisms involved in membrane docking and fusion between cytoplasmic granules and the plasma/phagosome membrane appear to involve SNARE (soluble N-ethylmaleimide-sensitive fusion [NSF] factor attachment protein receptor) proteins (Martin-Martin *et al.*, 2000). These proteins have been found to mediate vesicle secretion in essentially all organisms investigated. According to the SNARE hypothesis, docking and fusion of vesicles with the plasma membrane is modulated by the specific interaction of vesicle proteins (v-SNARES) with target plasma membrane proteins (t-SNARES). Secretion of secondary granules appears to involve interaction between SNAP-23 (v-SNARE) on the granule membrane and syntaxin 6 (t-SNARE) on the plasma/phagosome membrane, while mobilisation of primary granules involves interaction of syntaxin 6 with an unidentified v-SNARE (Martin-Martin *et al.*, 2000).

1.3 Neutrophil Functions

With respect to protection against microbial pathogens, neutrophil activation is characterised by adhesion to vascular endothelium, trans-endothelial migration,

Table 1.2

Constituents of Neutrophil Tertiary Granules and Secretary Vesicles

Tertiary Granules		Secretary Vesicles	
Membrane	***Matrix***	***Membrane***	***Matrix***
cytochrome b558	gelatinase	cytochrome b558	plasma proteins
CD11b	lysozyme	CD11b	
FMLP receptor	$ß_2$-microglobulin	FMLP receptor	
diacylglycerol deacylating enzyme	acetyl transferase	urokinase plasminogen activator receptor	
urokinase plasminogen activator receptor			

chemotaxis, engulfment and intraphagocytic eradication of micro-organisms. Poorly regulated activation of these processes, such as that which may occur during inappropriate, hyperacute or chronic inflammatory responses does, however, present the potential hazard of neutrophil-mediated injury to bystander host cells and tissues. This is due to the largely indiscriminate, albeit highly effective, strategies used by neutrophils to eliminate microbial pathogens.

Recently, neutrophils have been shown to produce a range of pro-inflammatory cytokines, particularly IL-8 and TNF-α, raising the possibility of autocrine activation of these cells during inflammatory processes (Cassatella, 1999; Witko-Sarsat *et al.*, 2000).

1.3.1 Extravasation and chemotaxis

The purposeful movement of neutrophils out of the circulation and subsequent migration to sites of infection is dependent upon a complex, highly coordinated series of events involving localised activation of vascular endothelium, adherence of neutrophils, transendothelial migration and chemotaxis.

1.3.2 Adherence to vascular endothelium

The rapid transition of circulating neutrophils from a non-adherent state to a localised adherent state involves the sequential appearance of several families of adhesion molecules on vascular endothelium which initially retard and then immobilise neutrophils. These events are known as tethering and firm adhesion respectively and precede trans-endothelial migration and chemotaxis (Springer, 1994).

1.3.3 Tethering and rolling

This is the initial event in localised immobilisation of neutrophils on activated vascular endothelium and is mediated by L-selectin on neutrophils and by P- and E-selectins on activated endothelial cells (Witko-Sarsat *et al.*, 2000). L-selectin is constitutively expressed on neutrophils and its binding capacity is rapidly and transiently increased, possibly due to receptor oligomerisation, after activation of these cells (Li *et al.*, 1998).

Although the counter-receptor for L-selectin on vascular endothelium awaits precise characterisation, it has recently been reported that clustering of L-selectin on the neutrophil membrane during activation of these cells leads to recruitment of p38 mitogen-activated protein kinase (MAPK) with resultant alterations in cell shape, activation of $ß_2$-integrins, reactive oxidant production and release of secondary, tertiary and secretory granules (Smolen *et al.*, 2000).

P-selectin is contained in the Weibel-Palade bodies of vascular endothelial cells and is rapidly (within a few minutes) and transiently mobilised to the endothelial cell surface following stimulation with thrombin, histamine, PAF and reactive oxidants (Witko-Sarsat *et al.*, 2000). P-selectin interacts with Sialyl Lewis x-containing structures on the neutrophil plasma membrane (Suzuki *et al.*, 2000), and these interactions augment $ß_2$-integrin-mediated adhesion and increase the production of reactive oxidants (Ruchaud-Sparagano *et al.*, 2000).

E selectin appears on endothelial cells only 1-2 hours after exposure to the cytokines IL-1 and TNF-α and interacts with Sialyl Lewis x-containing counter-receptors on the neutrophil surface (Witko-Sarsat *et al.*, 2000). Engagement of E-selectin by neutrophils has been reported to increase $ß_2$-integrin-mediated adhesion, as well as reactive oxidant production by these cells, possibly by sensitising neutrophils for PAF-mediated mobilisation of intracellular Ca^{2+} (Ruchaud-Sparagano *et al.*, 2000).

The transient nature of selectin-mediated adhesive interactions is crucial because it allows neutrophils to sample the endothelium for the presence of triggers that can activate $ß_2$-integrins and allow the cascade to proceed. These triggers include chemoattractants such as IL-8, PAF and leukotriene B_4 (LTB_4) which are immobilised on proteoglycans on the luminal surface of endothelial cells (Witko-Sarsat *et al.*, 2000). If the intensity of these signals is weak, then the neutrophils will disengage from the endothelium. High intensity signalling, on the other hand, will result in activation of $ß_2$-integrins and firm adhesion, resulting in the cells coming to a complete halt and changing shape within seconds, acquiring a flattened, adherent morphology.

1.3.4 Firm adhesion

Firm adhesion of neutrophils to endothelial cells appears to involve exclusively the interaction of integrins of the $ß_2$ sub-group (CD11a, CD11b, CD11c/CD18) on neutrophils with the endothelial adhesion molecules, intercellular adhesion molecules-1 and –2 (ICAM-1 and ICAM-2), in contradistinction to monocytes, eosinophils, and lymphocytes which utilise the $\alpha_4/ß_1$ integrin to bind to vascular cell adhesion molecule-1 (VCAM-1) (Witko-Sarsat *et al.*, 2000). ICAM-2 is constitutively expressed, while ICAM-1 expression is increased on activation with IL-1 and TNFα and predominates in binding to inflamed endothelium.

$ß_2$-integrins (also known as CR3) are present on the surface of resting neutrophils, but are unable to bind to their physiological ligands, the ligand-binding capacity being acquired upon exposure to activation signals (eg chemoattractants such as PAF, FMLP, LTB_4, C5a and IL-8, as well as TNFα and GM-CSF). These chemoattractants and cytokines promote clustering and transition of a sub-population of $ß_2$-integrins to a high affinity state (Stewart & Hogg, 1996). These various signalling pathways that cause integrins to switch from an inactive to an active conformation differ with the type of pro-inflammatory agonist and are still incompletely characterised. Regulation of avidity of $ß_2$-integrins involves interaction of both α and ß chain cytoplasmic tails with the cytoskeleton (Van Kooyk *et al.*, 1999) and the membrane expression of cytokesin-1, a guanine nucleotide exchange protein that binds to the cytoplasmic portion of CD18 and up-regulates $ß_2$-integrin avidity (Kolanus *et al.*, 1996; Nagel *et al.*, 1998). In addition, the binding of chemoattractants and pro-inflammatory cytokines to neutrophils sets in motion a cascade of signalling events which ultimately leads to phosphorylation of serine/threonine residues in the cytoplasmic tail of CD18, also causing integrin activation (Capocidi *et al.*, 1998; Ghamberg *et al.*, 1998; Jones *et al.*, 1998; Blouin *et al.*, 1999).

The binding of activated $ß_2$-integrins to their counter-receptors on vascular endothelium, ICAM-1 and ICAM-2, mediates firm adhesion between neutrophils and endothelium. Firm adhesion is a transient event, and following weakening of integrin-mediated adhesion, neutrophils migrate into the interstitium.

1.3.5 Transendothelial migration

Migration of adherent neutrophils out of the vascular space is most evident at the borders of endothelial cells where discontinuities of tight junctions occur. Several types of adhesive interactions have been implicated in transendothelial migration. These are:

- P-selectin/Sialyl Lewis x interactions. P-selectin has been demonstrated to be highly expressed along endothelial borders and may focus neutrophil adhesion onto these regions (Burns *et al.*, 1999).
- CD31 (also known as PECAM-1, which, like ICAM-1 and ICAM-2, is a member of the Ig-superfamily) is expressed on the neutrophil surface and at the endothelial junction, and mediates neutrophil transendothelial migration by homophilic (CD31/CD31) interactions. Ligation of CD31 also causes activation of $ß_2$-integrins (Berman & Muller, 1995).
- junctional adhesion molecule (JAM, also a member of the Ig-superfamily) is also concentrated at inter-endothelial junctions, but is not present on neutrophils. Antibodies to JAM inhibit leukocyte transmigration *in vitro* (Martin-Padura *et al.*, 1998). The counter-receptor for JAM on neutrophils has not yet been characterised.
- induction of $ß_1$-integrin (CD49/CD29) expression in neutrophils has also recently been shown to be associated with transendothelial migration (Werr *et al.*, 2000a; Werr *et al.*, 2000b). The $\alpha_2/ß_1$-integrin (CD49b/CD29, VLA-2), which binds to collagen and laminin, is up-regulated following activation of $ß_2$-integrins (Werr *et al.*, 2000a; Werr *et al.*, 2000b).

1.3.6 Migration of neutrophils within interstitial tissues

Neutrophils migrate in tissues by haptotaxis, which is a process of directed movement along a gradient of immobilised, as opposed to soluble, chemoattractants (Witko-Sarsat *et al.*, 2000). These chemoattractants are bound to extracellular matrix components because of their negative charge, facilitating presentation to corresponding counter-receptors on neutrophils. Locomotion of the cells requires the continuous formation of new adhesive contacts at the cell front, while the rear of the cell detaches from the adhesive substrate (Lauffenburger & Horwitz, 1996). Although $ß_2$-integrins, acting in concert with $ß_1$- and $ß_3$-integrins, have been proposed to be the

major adhesins involved in neutrophil migration through the extracellular matrix (Witko-Sarsat *et al.*, 2000), others have reported that the $\alpha_2/ß_1$-(VLA-2) integrin is the principal receptor used by neutrophils for locomotion in extravascular tissue (Werr *et al.*, 2000a). This may, however, be an over-simplification, since the $\alpha_9/ß_1$-(VLA-9) integrin has been reported to be the dominant adhesin involved in the migration of neutrophils through human lung and synovial fibroblast barriers, implying a degree of tissue selectivity with respect to utilisation of neutrophil integrins (Shang *et al.*, 1999).

Interestingly, laminin-, fibronectin- and vitronectin receptors such as $\alpha_5/ß_1$ (VLA-5), $\alpha_6/ß_1$ (VLA-6) and $\alpha_v/ß_3$ are mostly stored in neutrophil granules and are rapidly expressed on the plasma membrane following exposure of the cells to chemoattractants and during transendothelial migration (Roussel & Gingras, 1997).

1.4 Antimicrobial Mechanisms of Neutrophils

Neutrophils utilise oxygen-dependent and –independent mechanisms to eradicate microbial pathogens. The latter have been mentioned briefly elsewhere (1.2.1) and this section will focus only on oxygen-dependent antimicrobial systems.

1.4.1 NADPH oxidase

The superoxide (O_2^-)-generating system of phagocytes, NADPH oxidase, is a multi-component, membrane-associated electron transporter. NADPH oxidase comprises resident membrane components and translocatable cytosolic components which come together in the membrane (outer membrane and phagosome membrane) during neutrophil activation to form the fully functional oxidase. Over the past decade the membrane-bound and cytoplasmic components of the phagocyte NADPH oxidase have been identified, cloned and sequenced and the molecular/biochemical mechanisms by which they are assembled into a functional electron-transporting complex have been partly elucidated.

To date six oxidase components have been described *viz* cytochrome b_{558} which is a membrane-bound heterodimer consisting of $gp91^{phox}$ and $p22^{phox}$ (where p and gp =

protein/polypeptide and glycoprotein respectively and *phox* = phagocyte oxidase, while the numbers = molecular weight in kiloDaltons) which is associated with the GTP-binding protein, Rap1A, and four cytosolic components ($p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and Rac2, the latter being a member of the Ras superfamily of GTP-binding proteins of molecular weight approximately 21 kDa as is also the case with Rap1A) (Leusen *et al.*, 1996; Clark, 1999; Segal *et al.*, 2000).

Upon activation of the oxidase by receptor-mediated (chemoattractants, opsonized particles, TNFα) and non-receptor-mediated (phorbol esters, long chain unsaturated fatty acids, calcium ionophores) stimuli of membrane-associated oxidative metabolism, the cytoplasmic components of the oxidase, $p40^{phox}$, $p47^{phox}$ and 67^{phox} translocate *en bloc* to the plasma/phagosome membrane where they interact with membrane-bound cytochrome b_{558} inducing a conformational change in the oxidase which promotes interaction of NADPH with gp 91^{phox} (Segal *et al.*, 2000). Rac2 translocates independently (Segal *et al.*, 2000). NADPH is then oxidised to $NADP^+$ and electrons are transported down a reducing potential gradient to flavin adenine dinucleotide (FAD) and then apparently to the two non-identical haem groups of cytochrome b_{558}. The final step in the electron transport chain occurs when oxygen accepts an electron and is converted to the superoxide radical according to the following reaction:

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

Cytochrome b_{558}

The membrane-bound $gp91^{phox}/p22^{phox}$ heterodimer is designated cytochrome b_{558} because of its optical spectrum with an absorbance peak at 558 nm; this cytochrome also has an unusually low mid-potential at –245 mV which enables the direct transfer of electrons to molecular oxygen (Leusen *et al.*, 1996). A flavin (FAD) and two haem moieties (redox centres) are contained within the heterodimer such that the catalytic core to transfer electrons from NADPH to molecular oxygen is contained entirely within cytochrome b_{558}. In resting neutrophils the cytochrome in association with Rap1A is localised in the membranes of secondary and tertiary granules, as well as in those of secretory vesicles. During neutrophil activation, coalescence of these granules with the plasma membrane results in redistribution of the oxidase (Leusen

et al., 1996). Although the exact nature of the interaction between Rap1A and cytochrome b_{558} during oxidase activation remains to be established, it does appear that translocation and binding to cytochrome b_{558} of Rap1A in its GTP-bound form positively regulates NADPH oxidase activity, while the GDP-bound form negatively affects oxidase activity (Segal *et al.*, 2000).

Cytosolic components of NADPH oxidase

As is the case with cytochrome b_{558}, the cytosolic components of the oxidase are functionally inert in unstimulated neutrophils. Three of the cytosolic components *viz* $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$ exist as a cytoplasmic trimolecular complex in the resting state. These interactions are achieved in part through non-covalent links between SH3 and proline rich domains. $P47^{phox}$ and $P67^{phox}$ each possess two SH3 domains, while at least one is present on $p40^{phox}$ (Leusen *et al.*, 1996; Segal *et al.*, 2000). The SH3 domain of $p40^{phox}$ interacts with $p47^{phox}$ and its last 36 amino acids interact with $p67^{phox}$, while the C-terminal SH3 domain of $p67^{phox}$ is crucial for the interaction with both $p40^{phox}$ and $p47^{phox}$ (Leusen *et al.*, 1996). Interestingly, in unstimulated phagocytes $p47^{phox}$ appears to exist in a closed, inactive form in which the SH3 domains are masked via an intra-molecular interaction with the C-terminal region of the polypeptide. As described below, conformational alterations in $p47^{phox}$ result in unmasking of these SH3 regions, which is a key event in activation of NADPH oxidase (Shiose & Sumimoto, 2000).

Activation of NADPH oxidase

Activation of NADPH oxidase through receptor-mediated signalling by chemoattractants involves modification of the GDP-binding state of G-proteins, thus the activated receptor catalyses exchange of GDP for GTP by both the G-protein α subunit and low molecular weight G-proteins of the Ras, Rho and ARF (ADP-ribosylation factor) families. This process leads to the serial activation of phospholipases (PL) C and D and the generation of lipid second messengers. Several converging pathways (protein tyrosine kinases/phosphatidylinositol 3-kinase; Ras/Rho; PLD) activate the serine kinase Raf, which together with the serine/threonine kinase, protein kinase C (activated by PLC/PLD-derived diacylglycerol), activates MAP kinases which in turn cause the phosphorylative

activation of cytosolic PLA_2 (reviewed by Alonso *et al.*, 1998).

Signalling triggered by cross-linking and activation of Fcγ receptors on the other hand involves receptor-mediated, early activation of Src-protein tyrosine kinases and recruitment of various downstream effectors including phosphatidylinositol 3-kinases, PLC and the MAP kinase cascade, resulting in activation of cytosolic PLA_2 and PKC, as well as other kinases (Alonso *et al.*, 1998).

With respect to activation of NADPH oxidase in neutrophils and other phagocytes, the key events are activation of kinases, particularly protein kinase C, and cytosolic PLA_2 (Shiose & Sumimoto, 2000). Phosphorylation of $p47^{phox}$ at several serine residues in the SH3-containing C-terminal region alters the conformation of the polypeptide. This in turn leads to unmasking of the SH3 domains enabling weak interactions of $p47^{phox}$ with $p22^{phox}$ (Segal *et al.*, 2000; Shiose & Sumimoto, 2000). However, this event alone is insufficient for efficient activation of NADPH oxidase, a second complementary/synergistic mechanism being required. This is provided by low concentrations of arachidonic acid, generated during cleavage of membrane phosphatidylcholine by cytosolic PLA_2. Arachidonic acid, by mechanisms which remain to be fully elucidated, maximises the interactions of phosphorylated $p47^{phox}$ with $p22^{phox}$, resulting in complete activation of the oxidase (Shiose & Sumimoto, 2000). Phosphorylative and arachidonic acid-mediated unmasking of SH3 domains in $p47^{phox}$ also intensify the interactions of this polypeptide with $p67^{phox}$. Phosphatidic acid, generated during hydrolysis of membrane phospholipids by PLD, also activates $p47^{phox}$, presumably by the same mechanism as does arachidonic acid (Erickson *et al.*, 1999). However, whether phosphatidic acid also synergises with phosphorylation of $p47^{phox}$ in activation of the oxidase remains to be established.

Although the exact mechanism by which $p47^{phox}$ (in combination with $p67^{phox}$/$p40^{phox}$) interacts with $p22^{phox}$ and initiates the electron-transporting activity of the oxidase is incompletely understood, it has been proposed that the interaction of the trimolecular complex of cytosolic polypeptides with cytochrome b_{558} on the cytoplasmic side of the membrane induces a conformational change in the cytochrome that permits binding

of NADPH to gp 91^{phox} and initiation of electron flow in the cytochrome (Leusen *et al.*, 1996; Segal *et al.*, 2000).

With respect to the other cytosolic components of the oxidase, $p40^{phox}$ does not appear to be involved in oxidase activation/activity, but rather contributes to maintaining the latent trimolecular complex in an inactive state. Activated (GTP-bound) Rac 2 binds to $p67^{phox}$, an interaction which is essential for the activity of NADPH oxidase, and is essential for translocation of $p47^{phox}$ and $p67^{phox}$ (Leusen *et al.*, 1996). Unlike $p47^{phox}$, which undergoes phosphorylation at multiple (9-10) serine residues in the distal portion of the C-terminal region, probably involving several kinases, $p67^{phox}$ undergoes discreet MAP kinase-mediated phosphorylation of a single amino acid (threonine-233) which is followed by translocation to the membrane during oxidase activation (Forbes *et al.*, 1999).

$p47^{phox}$ and $p67^{phox}$, both of which are essential for NADPH oxidase activation, have distinct roles in the regulation of electron flow in cytochrome b_{558}. $p67^{phox}$ facilitates electron flow from NADPH to the flavin centre resulting in the reduction of FAD, while $p47^{phox}$ is required for electron flow to proceed beyond the flavin centre to the haem groups in cytochrome b_{558} and then to molecular oxygen (Leusen *et al.*, 1996).

Superoxide-derived antimicrobial oxidants

Although the primary consequence of assembly of the NADPH oxidase complex is the univalent reduction of molecular oxygen to $O_2^{\cdot}$, this oxygen-derived radical is a weak and unstable antimicrobial oxidant. Importantly, however, $O_2^{\cdot}$ functions as the precursor of a series of potent microbicidal oxidants.

Hydrogen peroxide (H_2O_2) is formed by spontaneous or enzymatic dismutation (by superoxide dismutase) of $O_2^{\cdot}$:

$$O_2^{\cdot} + O_2^{\cdot} + 2H^+ \longrightarrow H_2O_2 + O_2$$

The antimicrobial potential of H_2O_2 is dramatically potentiated by the granule enzyme, myeloperoxidase (MPO), which utilises this oxidant to oxidise chloride to the extremely potent oxidising agent hypochlorous acid (HOCl):

$$H_2O_2 + Cl^- \xrightarrow{MPO} H_2O + HOCl$$

It has also been proposed that neutrophils transform H_2O_2/O_2^- to hydroxyl radical (•OH, the most potent oxidant known in biological systems) by the iron-catalysed Haber-Weiss reaction:

$$H_2O_2 + O_2^- \xrightarrow{Fe^{2+} / Fe^{3+}} \bullet OH + OH^- + O_2$$

However, production of •OH by neutrophils via this mechanism has only been demonstrated *in vitro* in the presence of added iron, and appears to be of little or no biological relevance given that under physiological conditions, iron in and around neutrophils is tightly complexed to binding proteins (Ramos *et al.*, 1992). An alternative, transition metal-independent pathway of •OH generation has, however, been described in neutrophils and involves the interaction of O_2^- and HOCl (Ramos *et al.*, 1992):

$$HOCl + O_2^- \longrightarrow \bullet OH + O_2 + Cl^-$$

Although described several years ago, to my knowledge the biological significance of this pathway has not yet been resolved.

Neutrophils have also been reported to generate significant amounts of singlet oxygen [O_2 ($^1\Delta g$)], a highly reactive, diffusible and long-lived electronically excited state of molecular oxygen. These cells apparently transform up to 20% of oxygen consumed by NADPH oxidase to singlet oxygen by a MPO-dependent pathway involving interaction of H_2O_2 and HOCl (Steinbeck *et al.*, 1992):

$$H_2O_2 + OCl^- \longrightarrow O_2\ (^1\Delta g) + H_2O + Cl^-$$

These various phagocyte-derived oxidants acting directly or through more stable intermediates (eg chloramines and chloramides in the case of HOCl) are powerful antimicrobial agents. They are, however, indiscriminate, and, if released

extracellularly during hyperacute and/or chronic activation of phagocytes, they pose the potential threat of oxygen toxicity to bystander host cells and tissues in the vicinity of inflammatory reactions. In this regard they are potentially cytotoxic for eukaryotic cells, as well as being potential carcinogens, pro-proteolytic, pro-adhesive and immunosuppressive (reviewed by Anderson, 1995).

Other activities of NADPH oxidase

Apart from its primary role in generating superoxide, the NADPH oxidase of activated phagocytes also regulates membrane potential in these cells as a consequence of the vectorial, outward transport of electrons across the plasma membrane. The oxidase also functions as a H^+ channel. These activities are important in restoring Ca^{2+} homeostasis in activated neutrophils and are discussed in more detail in section 1.6.2 (page 26).

1.4.2 Nitric oxide synthase

The production of nitric oxide (NO) within phagocytes is an important component of host defences against microbial infection. Although NO *per se* is only weakly antimicrobial, its microbicidal activity is considerably enhanced by reaction with O_2^- to yield the highly reactive anion, peroxynitrite (Koppenol, 1998).

While the role of NO in rodent phagocytes is well-established, the involvement of inducible NO synthase (iNOS) in the intrinsic antimicrobial function of human neutrophils is less clear with many investigators having failed to detect NO production by these cells. Recently, however, Webb *et al.* (2001) have reported that neutrophils isolated from the peripheral blood of healthy adult humans are not primed for iNOS activity. Production of NO by these cells requires $ß_2$-integrin/Fcγ receptor-independent adhesion together with exposure to the cytokines IL-1, IFN-γ and TNF-α for extended incubation times (3 hours). Under these conditions iNOS was detectable in up to 36% of neutrophils (Webb *et al.*, 2001).

1.4.3 Cytokine production by neutrophils

Until fairly recently neutrophils, largely because of their perceived limited biosynthetic capability, were not considered to be cytokine-producing cells. This perception was compounded by the relatively short lifespan of neutrophils *in vitro*, difficulties in identifying the origins of cytokines (measured by enzyme-based immunoassays) due to the presence of major cytokine-producing, contaminating cells such as monocytes in "pure" neutrophil suspensions, as well as the potential of neutrophils to passively acquire cytokines from other cell types during neutrophil purification procedures. However, with the acquisition of procedures which enable acquisition of ultra-pure neutrophil suspensions (flow cytometry), as well as intracellular detection of cytokine messenger RNA (mRNA), a considerable body of evidence has accumulated which clearly demonstrates that the human neutrophil is a source of various pro-inflammatory cytokines, chemokines and growth factors. The concept that neutrophils can be a source of cytokines has only recently emerged and has been reviewed in considerable detail by Cassatella (1999) and Witko-Sarsat *et al.* (2000).

Although the production of cytokines by neutrophils is striking in its diversity (as shown in Table 1.3, page 22), IL-8 and TNF-α appear to be the major cytokines produced by these cells, suggesting the potential for autocrine regulation of neutrophil function (Cassatella, 1999). TNF-α enhances neutrophil adhesion, sensitises (primes) for and activates reactive oxidant production by adherent cells, and induces release of granule enzymes by these cells (Witko-Sarsat, 2000).

Interleukin-8, a potent chemoattractant for neutrophils and inducer of degranulation, is the most abundantly secreted cytokine by neutrophils (Witko-Sarsat, 2000). Agents which activate cytokine production by neutrophils are shown in Table 1.4 (page 23).

Neutrophils, as is the case with other types of inflammatory cells, are also subject to negative modulation by cytokines. Those cytokines which suppress the pro-inflammatory activities of neutrophils include IL-4, IL-10 in particular, and IL-13 (Witko-Sarsat, 2000).

Table 1.3: Cytokines Expressed by Neutrophils

Cytokines that are expressed by neutrophils *in vitro*	**Cytokines that are not expressed by neutrophils *in vitro***
Pro-inflammatory cytokines TNF-α IL-1-α and -ß IL-12	T-cell-derived cytokines IL-10 IL-13
Anti-inflammatory cytokines IL-1 receptor antagonist (IL-1Ra)	Other cytokines RANTES MCP-2, MCP-3
Chemokines IL-8 Growth-related gene product-α (GRO-α) Macrophage infiltrating protein-1α (MIP-1α), MIP-1ß Cytokine-induced chemoattractants (CINC)	
Other cytokines and growth factors Interferon-α (IFN-α), IFN-ß Granulocyte colony-stimulating factor (G-CSF) Fas ligand (FasL), CD30 ligand (CD30L) Vascular endothelial growth factor (VEGF) Hepatocyte growth factor (HGF)	
Release under certain conditions Macrophage-CSF (M-CSF), IL-3, GRO-ß IL-18 (IFN-γ inducible factor) TGF-α Oncostatin (OSM) and neurotrophins	
Secretion still debated IL-6, monocyte chemotactic protein-1 (MCP-1), granulocyte-macrophage CSF (GM-CSF), stem cell factor (SCF), and IFN-γ	

Adapted from Cassatella, 1999

Table 1.4: Agents which Trigger Cytokine Production by Neutrophils

Cytokines and growth factors
TNF-α, IL-1α, IL-1ß,
IL-4, IL-13, IL-10,
GM-CSF,
TGF-ß
Chemoattractants
FMLP
Surface molecules
Anti-CD32 (FcγRII) and anti-CD16 (FcγRIII) antibodies
Particulate agents
Calcium microcrystals
Urate microcrystals
Other agents
Calcium ionophores
PMA, concanavalin A
ANCA
Matrix protein (fibronectin, laminin)
Bacteria and related products
LPS
Staphylococcus aureus
Yersinia enterocolitica
Listeria monocytogenes
Fungi and related products
Candida
Saccharomyces cerevisiae
Zymosan
Protozoa
Plasmodium falciparum
Viruses
Epstein-Barr virus

Adapted from Cassatella, 1999

The discovery that neutrophils are cytokine-producing cells may have important implications for neutrophil-directed anti-inflammatory chemotherapy, suggesting that combinations of those agents which suppress protein synthesis-independent, early activatable pro-inflammatory activities (such as oxidant production, synthesis of eicosanoids/prostanoids and granule enzyme release) together with agents which suppress protein synthesis-dependent, later-occurring activities (such as cytokine production) may be maximally effective.

1.5 Phospholipase A_2-Derived Mediators of Inflammation

Neutrophil activation is accompanied by Ca^{2+}-dependent activation of PLA_2 with resultant cleavage of the integral membrane phospholipid, phosphatidylcholine, to the primary hydrolysis products arachidonic acid and lysophosphatidylcholine (LPC). As mentioned above, arachidonic acid, through its direct interaction with $P47^{phox}$, mediates activation of NADPH oxidase, while LPC possesses a range of pro-inflammatory activities (pro-oxidative, pro-adhesive, induction of degranulation) which result from activation of PKC by this lysophospholipid (Oishi *et al.*, 1988). In addition to pro-inflammatory activity LPC, and to a lesser extent arachidonate, as well as saturated fatty acids, are microbicidal for Gram-positive bacteria (Kondo & Kanai, 1985; Steel *et al.*, 1999), suggesting a role for both cytosolic and secretory PLA_2 in neutrophil antimicrobial function (Weinrauch *et al.*, 1996).

While LPC is converted to biologically "inert" glycerophosphocholine and saturated fatty acid (usually palmitate) by phospholipase A_1, arachidonic acid in neutrophils is converted by cyclooxygenase 1/cyclooxygenase 2 to prostaglandin E_2 (PGE_2) and by 5'-lipoxygenase to leukotriene B_4 (Alonso *et al.*, 1998). PGE_2 possesses a range of pro-inflammatory activities (increase of blood flux, potentiation of oedema, haemorrhagic necrosis, activation of transcription and initiation of the acute phase response - Alonso *et al.*, 1998). In addition, PGE_2 acting via adenylyl cyclase-linked EP_2 receptors and adenosine 3',5'-cyclic monophosphate (cAMP) prolongs the lifespan of activated neutrophils by inhibiting apoptosis (Rossi *et al.*, 1995). LTB_4

also possesses a range of pro-inflammatory actions (mobilisation of intracellular Ca^{2+}, pro-adhesive, chemotactic – Alonso *et al.*, 1998). While neutrophils possess membrane receptors for LTB_4, they do not synthesise the cysteinyl leukotrienes (LTC_4, LTD_4, LTE_4), neither do they possess membrane receptors for these pro-inflammatory lipids (Lam & Austen, 1992).

Neutrophils also produce PAF by the so-called, PLA_2-dependent, remodelling pathway during which 1-0-alkyl-2 acyl-sn-glycero-3-phosphocholine is converted by PLA_2 to lyso-PAF, which in turn is converted by acetyltransferase to PAF (Zimmerman *et al.*, 1992). PAF is undoubtedly the most potent and versatile of the biologically active lipids produced by neutrophils and other inflammatory cells, and when released extracellularly can amplify inflammatory responses by interacting with G-protein/PLC-coupled PAF receptors on target cells, particularly eosinophils and neutrophils (Zimmerman *et al.*, 1992; Prescott, 1999).

1.6 Calcium Fluxes and Restoration of Calcium Homeostasis in Activated Neutrophils

Transient elevations in cytosolic free Ca^{2+} ($[Ca^{2+}]i$) precede and are a prerequisite for the receptor-mediated activation of many neutrophil functions, including activation of $ß_2$-integrins and adhesion to vascular endothelium, superoxide production, granule enzyme release and activation of pro-inflammatory cytosolic nuclear transcription factors, including NF-*k*B (Lew *et al.*, 1986; Thelen *et al.*, 1993; Pettit & Hallet, 1996; Dolmetsch *et al.*, 1997).

1.6.1 Release of calcium from stores

Intracellular Ca^{2+} in neutrophils is reportedly stored in specialised storage vesicles termed calciosomes (Favre *et al.*, 1996). This may be somewhat of an over-simplification, however, since there are two distinct cellular locations for Ca^{2+} stores in neutrophils which may have differential involvement in activation of pro-inflammatory functions and which may utilise different molecular/biochemical mechanisms of Ca^{2+} mobilisation (Pettit & Hallet, 1996). One site is located

peripherally under the plasma membrane and appears to be involved in activation of $ß_2$-integrins, while the other (probably calciosomes) is localised in the juxtanuclear space and is mobilised by the chemoattractant, FMLP (Pettit & Hallet, 1996).

Because differences between the molecular/biochemical mechanisms of mobilisation of Ca^{2+} from these two different storage sites have not yet been established, I will focus on those utilised by FMLP. Occupation of neutrophil membrane receptors for this chemotactic tripeptide results in receptor-G-protein coupling with consequent activation of PLC and generation of inositol triphosphate by hydrolysis of phosphatidylinositol 4,5-biphosphate (Favre *et al.*, 1996; Alonso *et al.*, 1998). Inositol triphosphate then interacts with Ca^{2+}-mobilising receptors on calciosomes, resulting in discharge of the cation into the cytosol. These events are extremely rapid, occurring within less than 1 second after the ligand-receptor interaction (Favre *et al.*, 1996; Anderson & Goolam Mahomed, 1997). In the case of neutrophils, the abrupt increase in cytosolic Ca^{2+} following exposure to FMLP, results exclusively from release of the cation from intracellular stores with little or no contribution at this early stage (within the first 30-60 seconds) from extracellular Ca^{2+} and results in an increase in the basal $[Ca^{2+}]i$ from around 100 nM to ± 1 µM (Favre *et al.*, 1996; Anderson & Goolam Mahomed, 1997; Geiszt *et al.*, 1997). Influx of extracellular Ca^{2+} is delayed, being detectable at around 1 minute after the addition of FMLP and terminating at around 5 minutes. This type of influx is characteristic of a store-operated Ca^{2+} influx (ie primarily involved in re-filling of stores as opposed to activation of neutrophils) and is operative in a large variety of cell types, including neutrophils.

1.6.2 Restoration of calcium homeostasis

Peak cytosolic $[Ca^{2+}]i$ is achieved abruptly in FMLP-activated neutrophils, peaking at around 10-20 seconds, and subsiding rapidly thereafter to close to base-line values within 3-5 minutes. Restoration of Ca^{2+} homeostasis in activated neutrophils is essential to prevent Ca^{2+} overload and hyperactivity of these cells. This is achieved by rapid clearance of Ca^{2+} from the cytosol of the cells in the setting of carefully regulated influx of extracellular cation, which is efficiently diverted into stores. Clearance of cytosolic Ca^{2+} is accomplished through the action of the plasma

membrane and endo-membrane Ca^{2+}-ATPases operating in unison, while extracellular cation is excluded from the cells through the membrane depolarising activity of NADPH oxidase.

The plasma membrane Ca^{2+}-ATPase of neutrophils is modulated by calmodulin which shifts the pump to a higher affinity state for Ca^{2+}, resulting in enhanced maximal velocity (Lagast *et al.*, 1984). A dramatic and transient (over a 30 second time course) increase in efflux of Ca^{2+}, coincident with the release of the cation from stores, is observed in FMLP-activated neutrophils which results in the extrusion of about 50% of cell-associated cation (Anderson & Goolam Mahomed, 1997). Activation of neutrophils with the chemoattractant (and several other G-protein/PLC-coupled receptor-mediated stimuli) also results in immediate activation of adenylate cyclase (Snyderman & Uhing, 1992), which appears, at least in part, to be linked to activation of cAMP-dependent protein kinase (PKA) and up-regulation of the sarcoplasmic reticulum (calciosome), endo-membrane Ca^{2+}-ATPase (Tao *et al.*, 1992; Villagrasa *et al.*, 1996; Anderson *et al.*, 1998). The endo-membrane Ca^{2+}-ATPase contributes to Ca^{2+} clearance from the cytosol of activated neutrophils by promoting re-sequestration of the cation. Operating in harmony, these two Ca^{2+} pumps (plasma membrane and endo-membrane) are the major, if not the sole contributors to clearance of cytosolic Ca^{2+} in activated neutrophils, since the Na^{+}/Ca^{2+} exchanger does not appear to mediate Ca^{2+} efflux in these cells (Simchowitz & Cragoe, 1988).

Efficient Ca^{2+} clearance by these systems is greatly facilitated by the membrane depolarising actions of NADPH oxidase which limit influx of extracellular Ca^{2+}. The dramatic decrease in membrane potential which accompanies activation of the oxidase following exposure of FMLP, and which is coincident with release of Ca^{2+} from granulocyte intracellular stores and activation of superoxide production, has been attributed to the electrogenic activity of the oxidase (Henderson *et al.*, 1988; Schrenzel *et al.*, 1998; Jankowski & Grinstein, 1999), as well as to the action of a rapidly activated H^{+} conductance with resultant influx of H^{+} (Bánfi *et al.*, 1999). This type of abruptly-occurring depolarisation, which accompanies activation of various types of inflammatory cells, including basophils, mast cells and neutrophils, has been shown to limit the influx of extracellular Ca^{2+} (Di Virgilio *et al.*, 1987; Mohr & Fewtrell,

1987; Penner *et al.*, 1988; Geiszt *et al.*, 1997). It has been proposed that when the cells are depolarised the driving force for entry of Ca^{2+} is abolished because the electrical component of the electrochemical gradient for Ca^{2+} is markedly reduced.

Recovery of membrane potential in FMLP-activated neutrophils becomes evident at around 1 minute after exposure to the chemoattractant and proceeds gradually over a 5-10 minute time course. Interestingly, the kinetics of influx of extracellular Ca^{2+} (for store-refilling) are virtually superimposable on those of membrane repolarisation (Anderson & Goolam Mahomed, 1997; Geiszt *et al.*, 1997; Tintinger *et al.*, 2001). Repolarisation appears to be achieved through the action of the Na^+/Ca^{2+} exchanger operating in reverse mode i.e. Na^+ efflux/Ca^{2+} influx (Simchowitz & Cragoe, 1988), and by a slowly activatable H^+ conductance which allows only H^+ extrusion and repolarisation (Schrenzel *et al.*, 1998). This H^+ conductance appears to be a function of $gp91^{phox}$ (Bánfi *et al.*, 2000). Carefully regulated influx of Ca^{2+} probably ensures efficient diversion by the endo-membrane Ca^{2+}-ATPase of incoming cation into stores, thereby preventing flooding of the cytosol with Ca^{2+} and possible hyperactivation of the cells.

1.7 Anti-Inflammatory Actions of cAMP

The broad-spectrum anti-inflammatory potential of physiologic and pharmacologic cAMP-elevating agents, which spans many different types of immune and inflammatory cells, including neutrophils, has been recognised for more than two decades (Moore & Willoughby, 1995). These agents suppress the pro-inflammatory activities of human neutrophils, eosinophils, monocytes and lymphocytes by PKA-dependent mechanisms which are both dependent and independent of gene transcription (Moore & Willoughby, 1995; Barnes & Adcock, 1997).

1.7.1 Cyclic AMP and neutrophils

Receptor-mediated activation of human neutrophils with Ca^{2+} mobilising stimuli (chemoattractants, opsonised particles) is associated with an immediate, transient

(peaks within 30 seconds in the case of chemoattractants) increase in intracellular cAMP (Snyderman & Uhing, 1992; Goolam Mahomed & Anderson, 2000). This transient increase in cAMP appears to be due to the release of adenosine, an endogenous anti-inflammatory agent (Iannone *et al.*, 1989; Cronstein, 1994), by activated neutrophils. Adenosine in turn causes autocrine activation of G-protein/adenylate cyclase-linked subtype A_{2A} adenosine receptors (which are reviewed in a later section, 1.9.3, page 43) on neutrophils, resulting in transiently elevated levels of cAMP. Cyclic AMP in turn may fulfil an anti-inflammatory function by restoring Ca^{2+} homeostasis in activated neutrophils, resulting in down-regulation of the pro-inflammatory activities of these cells (Anderson *et al.*, 1998).

1.8 Neutrophil-Directed, Anti-Inflammatory Chemotherapeutic Strategies

Anti-inflammatory chemotherapeutic agents can be classified into two groups, those which have selective targets in immune and inflammatory cells and those with broad-spectrum activity affecting several different molecular targets. There are many selective anti-inflammatory chemotherapeutic agents, some of which are shown in Table 1.5 (page 30). With the possible exception of the leukotriene receptor antagonists in the treatment of mild-to-moderate bronchial asthma, monotherapy with these agents is either ineffective or of limited value (and in some cases impractical) in the treatment of patients with acute and chronic inflammatory conditions. On the other hand, the elite group of broad-spectrum anti-inflammatory agents currently consists of only one member, the corticosteroids. These agents are the mainstay of anti-inflammatory chemotherapy in bronchial asthma. The value of inhaled corticosteroids in the treatment of this chronic inflammatory disorder is captured in the following quotation:

> "If ever there was a magic potion that should resolve the symptoms of an affliction, it is the use of corticosteroids in asthma" (McFadden, 1998).

Table 1.5: Anti-inflammatory agents which are currently used or are under investigation for the treatment of asthma

Selective	Broad-spectrum
Cyclooxygenase (Cox 1/Cox 2) inhibitors	Corticosteroids
5'-Lipoxygenase inhibitors	
Leukotriene receptor antagonists	
PAF receptor antagonists/degrading enzymes	
Cytokine receptor antagonists	
Chemokine receptor antagonists	
Anti-oxidants	
Anti-proteases	
Adhesion molecule antagonists	

1.8.1 Corticosteroids

These agents have a unique triple mechanism of anti-inflammatory action resulting from their interactions with cytosolic glucocorticoid (GC) receptors (GR). GR are members of the nuclear receptor superfamily that includes other steroids (oestrogen, progesterone), receptors for vitamins (vitamins A and D) and thyroid hormone (Barnes & Adcock, 1998). GR are expressed in most types of cells in an inactive form bound to a protein complex that includes two molecules of a 90 kDa heat shock protein (hsp90) and an immunophilin, which act as nuclear chaperones, protecting the nuclear localisation site. GC bind to GR in the cytoplasm, resulting in the dissociation of the inactive complex and rapid translocation to the nucleus of the GC/GR complex (reviewed in detail by Barnes & Adcock, 1998).

Interestingly, GC/GR interactions result in at least three different types of anti-inflammatory actions (Barnes & Adcock, 1998). These are:

- activation of genes encoding anti-inflammatory polypeptides
- activation of histone deacetylase activity, preventing the interaction of transcription factors with binding regions on target genes, leading to gene repression

- direct protein-protein interactions between the activated GR and activated, pro-inflammatory cytosolic-nuclear transcription factors.

With respect to the first of these, activated GR form homodimers to interact with GC response elements (GRE) in the promoter regions of target genes, resulting in increased gene transcription. This type of interaction results in the increased transcription of several anti-inflammatory polypeptides, including lipocortin-1 (antagonist of PLA_2), serum leukoprotease inhibitor, the IL-1 receptor antagonist (Barnes & Adcock, 1998), and possibly I*k*B-α, the antagonist of NF-*k*B (Scheinman *et al.*, 1995). In addition, this type of interaction also results in the increased transcription of $ß_2$-adrenoreceptors (Mak *et al.*, 1995), which although of primary importance in maintaining the sensitivity of the airways to $ß_2$-receptor agonists, may also contribute to anti-inflammatory activity since inflammatory cells, including neutrophils, contain adenylate cyclase-linked $ß_2$-adrenoreceptors (Mueller *et al.*, 1988).

Interestingly, however, relatively few genes possess GRE, indicating that an alternative mechanism, or mechanisms, must account for the major anti-inflammatory actions of corticosteroids. It is now accepted that the major anti-inflammatory effects of corticosteroids are achieved via repression of immune and inflammatory genes and, although it was thought that these were likely to be mediated through negative GRE, resulting in gene repression, none of the immune and inflammatory genes that are switched off by steroids appears to have nGRE in their promoter sequences (Barnes & Adcock, 1998).

There has recently been increasing evidence that corticosteroids may affect the chromatin structure of DNA, thereby preventing the interaction of pro-inflammatory cytosolic nuclear transcription factors with their target genes (Barnes & Adcock, 1998). The association of transcription factors with their target genes is facilitated by interaction with large co-activator molecules such as cyclic AMP response element binding factor (CREB) binding protein (CBP) and p300, which bind to the basal transcription factor apparatus. These co-activator molecules have an intrinsic histone acetyltransferase (HAT) activity, resulting in acetylation of histone proteins

around which DNA is wound in the chromosome (Ogryzko *et al.*, 1996; Wolffe, 1997). This leads to unwinding of DNA and facilitates binding of transcription factors, resulting in increased gene transcription. Activated GR bind to a steroid receptor co-activator that is bound to CBP, interfering with the activation of CBP and leading to deacetylation of histone residues. This results in tighter coiling of DNA, excluding transcription factors such as NF-*k*B and AP-1 from binding to DNA (Kamei *et al.*, 1996). The consequence is interference with the synthesis of a range of pro-inflammatory polypeptides including cytokines, chemokines, COX2, iNOS, adhesion molecules and immunoreceptors (Barnes, 1997; Lane *et al.*, 1998).

The third, and perhaps somewhat more controversial mechanism of anti-inflammatory action of corticosteroids, is by direct protein-protein interactions between activated GR and several different types of pro-inflammatory cytosolic nuclear transcription factors, resulting in functional inactivation of the latter. Activated GR have been reported to bind to activated NF-*k*B, AP-1 and signal transduction-activated transcription factors (STATs) and prevent their binding to promoter regions on target genes (Barnes, 1997; Barnes & Adcock, 1998).

1.8.2 Limitations of corticosteroids

In spite of their clinical efficacy, enthusiasm for corticosteroids must be tempered by an awareness of their limitations. These include concerns about their long-term safety (including small, but nevertheless significant effects of inhaled corticosteroids on the growth of children), steroid-resistance (especially in asthma), slow onset of action, and the relative insensitivity of neutrophils to these agents (McFadden, 1998). Corticosteroid resistance may be due to decreased affinity and/or numbers of GR in the cytosol of immune and inflammatory cells, or to excessive production of AP-1 by these cells (Lane *et al.*, 1998), while the slow onset of action of these agents may reflect the time required for *de novo* synthesis of anti-inflammatory polypeptides, as well as the time required to suppress biosynthetic processes already initiated by pro-inflammatory, cytosolic nuclear transcription factors.

1.8.3 Neutrophils and corticosteroids

The reported insensitivity of the rapidly-activatable functions of neutrophils to therapeutically-relevant concentrations of corticosteroids *in vitro* is due to the independence of these pro-inflammatory activities on *de novo* protein synthesis. The insensitivity of oxidant production, adhesion, chemotaxis and phagocytosis to corticosteroids has been described by many investigators and these reports are summarised in Table 1.6 and 1.7 (pages 34-36 and 37-38 respectively). Although some authors have described inhibitory effects of corticosteroids on rapidly-activatable neutrophil functions (summarised in Table 1.6), it is possible that these may be due to non-GR-mediated effects such as membrane stabilisation.

Somewhat worryingly, there are also reports that corticosteroids may potentiate the pro-inflammatory activities of neutrophils by delaying apoptosis in these cells (Cox, 1995; Meagher *et al.*, 1996; Daffern *et al.*, 1999). This contrasts with the apoptosis-inducing effects of corticosteroids in other types of immune and inflammatory cells, including eosinophils (Meagher *et al.*, 1996). The differences between neutrophils and other types of inflammatory cells have been attributed not only to the paucity of mitochondria in the former, but also to differences in the structure of these organelles.

The insensitivity of neutrophils to corticosteroids may account for the low therapeutic efficacy of these agents in the treatment of conditions associated with hyperactivity of neutrophils. These include ARDS, chronic obstructive pulmonary disease, cystic fibrosis, bronchiectasis and certain categories of asthmatics. On the other hand, corticosteroids (which would be expected to inhibit slowly-activatable neutrophil pro-inflammatory functions such as cytokine synthesis), may be most effective in controlling neutrophil-mediated inflammation when used in combination with agents which inhibit rapidly-activatable neutrophil functions. This contention is supported by the observation that glucocorticoids inhibit IL-8 production by neutrophils, while superoxide production by these cells was unaffected (Cox, 1995)

Table 1.6: Summary of Reports on the Effects of Corticosteroids on Early-Activatable Neutrophil Functions *In Vitro*

Functions Investigated:					
Adhesion	**Oxidant Production**	**Chemotaxis**	**Phagocytosis**	**Degranulation**	**Author**
0 (stimulated), slight ↓ (spontaneous)		0		0	Schleimer *et al.*, 1989
0		↓			Hirata *et al.*, 1980
		↓			Kurihara *et al.*, 1984b
0 (bovine)		↑ (bovine)			Jayappa & Loken, 1983
		↓ (rabbit)			Ward, 1966
		↓			Rivkin *et al.*, 1976
	0	↓			Freischlag *et al.*, 1992
				↓	Burnett *et al.*, 1989
	↓				Feunfer *et al.*, 1979
	0	↓ (suprapharm. concentr)			Lomas *et al.*, 1991
	0	↓		↓	Llewellyn-Jones *et al.*,1994
	↓				Goldstein *et all.*, 1976
	↓				Jones *et al.*, 1983
	↓				Umeki & Soejima, 1990
				0	Røshol *et al.*, 1995
		0 (rabbit)			Borel 1973
		0			Rhinehart *et al.*, 1974
	0 (bovine)				Phillips *et al.*, 1987
	0/↑ (suprapharm.concentr)				Horan *et al.*, 1982

Table 1.6 (continued)

Adhesion	Oxidant Production	Chemotaxis	Phagocytosis	Degranulation	Author
	0				Van Dyke *et al.*, 1979
0/↓ (canine suprapharm concentration)	0	0	0 (↑ at suprapharm concentration)		Trowald-Wigh *et al.*, 1998
	↓ (high concentr)				Levine *et al.*, 1981
	0 (except Dex)				Niwa *et al.*, 1987
	0		0		Olds *et al.*, 1974
	0 (bovine) /↓ (suprapharm concentr ex Betameth)			0	Hoeben *et al.*, *1998*
	0				Humphreys *et al.*, 1993
				0	Mandell *et al.*, 1970
				↓	Wright & Malawista, 1973
				0	Persellin & Ku, 1974
	0	0		↓	Gadaleta *et al.*, 1994
↓					Pichyangkul *et al.*, 1988
0		0			MacGregor *et al.*, 1974
↓	↓				Bratt & Heimburger, 1999
0 (unstim. PMN /↓ (stim. PMN)				0	Filep *et al.*, 1997
↓					Liu *et al.*, 2000
↓	↓				Heimburger *et al.*, 2000
↓					Zouki *et al.*, 2000
↓					Suzuki *et al.*, 2000

Table 1.6 (continued)

Adhesion	Oxidant Production	Chemotaxis	Phagocytosis	Degranulation	Author
↓	↓				Filep *et al.*, 1999
↓ (bovine)+ human N's					Shimoyama *et al.*, 1997
↓ (rat)					Sakamoto *et al.*, 1997
↓					Wheller & Perretti, 1997
↓ (activated N's)				0	Filep *et al.*, 1997
↓		↓			Yoshida *et al.*, 1997

Table 1.7: Summary of Reports on the Effects of Corticosteroids on Early-Activatable Neutrophil Functions *In Vivo*

		Functions Investigated:			
Adhesion	**Oxidant Production**	**Chemotaxis**	**Phagocytosis**	**Degranulation**	**Author**
↓ (suprapharm concentr)		0			Clark *et al.*, 1979
↓ (suprapharm concentr)		↓			MacGregor *et al.*, 1974
↓		↓			Ackerman *et al.*, 1982
	↓				Dandona *et al.*, 1999
	↑				Kuzniar *et al.*, 1991
	0	↓			Lomas *et al.*, 1991
	0	↓			Fleming *et al.*, 1991
	↓	↓		↓	Adams *et al.*, 1989
	↓				Røshol *et al.*, 1995
	↑ (bovine)				Phillips *et al.*, 1987
	↓ (bovine)				Roth & Kaeberle, 1981
	0	0		0	Llewellyn-Jones *et al.* 1996
↓ (canine)	↑	↑	↑		Trowald-Wigh *et al.*, 1998
		↑ (canine, pharm. conc)			Guelfi *et al.*, 1985
		↑ (equine, pharm. conc)	↑		Morris *et al.*, 1988
				0	Persellin & Ku, 1974
		↑ (unstim) ↓ (stim.ster.sens asthma)			Zak-Nejmark *et al.*, 1996
		↑ (stim.ster.resist.asthma)			

Table 1.7 (continued)

Adhesion	Oxidant Production	Chemotaxis	Phagocytosis	Degranulation	Author
0					Oda & Katori, 1992
		↓			Davis *et al.*, 1991
↓ (methyl-pred) 0 (Dex)					Bassaris *et al.*, 1987
↓	↑			↑	Hetherington & Quie, 1985
	↓				Macconi *et al.*, 1993
↓					Nakagawa *et al.*, 1999
↓		↓			Crockard *et al.*, 1998
	0		0	0	Dale *et al.*, 1998
0				0	Tailor *et al.*, 1997
↓					Van Overveld *et al.*, 2000
↓		0			Lim *et al.*, 1998
↓ (rat)		↓			Davenpeck *et al.*, 1998
↓					Youssef *et al.*, 1996
↓					Perretti *et al.*, 1996
↓ (bovine)					Burton & Kehrli, 1995
↑ ($ß_2$-integrin)	↑	↓			Barton *et al.*, 2000
↓ (L-selectin)					
↓	↓	↓	↓		Goulding *et al.*, 1998
↓					Hill *et al.*, 1994

Second generation type 4 phosphodiesterase inhibitors (Torphy, 1998; Wang *et al.*, 1999) and adenosine receptor agonists operative at the level of the subtype A_{2A} receptor (Cronstein, 1994; Hannon *et al.*, 1998; Underwood *et al.*, 1998) are promising pharmacologic inhibitors of early activatable, pro-inflammatory functions of neutrophils.

1.9 Adenosine and Neutrophils

Adenosine is a ubiquitous autocoid with a broad-spectrum of biological activities, including modulation of leucocyte function. It is a physiologic anti-inflammatory agent released by many cell types and is a normal constituent of all body fluids in which its levels are raised by hypoxia and ischaemia. Adenosine is released by endothelial cells and neutrophils in response to physiologic stimuli (Fredholm, 1997).

Adenosine was first administered to humans on an experimental basis in 1930 and was found to induce transient sinus bradycardia and AV nodal block. Its clinical usefulness was only recognised in the 1980s and it is now one of the most commonly used agents in the diagnosis and treatment of supraventricular arrhythmias, as well as in the diagnosis of narrow complex tachycardias, while its ability to produce coronary vasodilation is utilised in combination with myocardial perfusion scintigraphy to assess whether perfusion defects occur as a consequence of coronary artery stenosis. A potential therapeutic use is to provide protection to the heart during ischaemia or infarction, a process known as preconditioning. This approach may be applied to unstable patients about to undergo complex angioplasty or coronary bypass surgery (Olah & Stiles, 1995). Reperfusion injury may be reduced by adenosine-mediated inhibition of neutrophil activation (Jordan *et al.*, 1997).

1.9.1 Adenosine effects on neutrophil function

Adenosine, of both exogenous and endogenous (neutrophil-derived) origin, is a potent modulator of several neutrophil functions including superoxide production,

adhesion to vascular endothelium, migration and phagocytosis (Iannone *et al.*, 1989; Cronstein *et al.*, 1993).

Superoxide production

In 1983 Cronstein *et al.* reported that adenosine inhibited neutrophil O_2^- generation stimulated by the chemoattractants (FMLP) and C5a, as well as the Ca^{2+} ionophore A23187, but not by phorbol myristate acetate (a direct activator of protein kinase C). Adenosine appeared to mediate these effects extracellularly since blockade of purine uptake did not reverse the effect of added adenosine on neutrophil function. Adenosine inhibited O_2^- generation via occupancy of specific adenosine receptors (AR). It was also found that removal of endogenously released adenosine (by addition of adenosine deaminase) resulted in enhanced O_2^- generation by stimulated neutrophils. This observation led to the hypothesis that adenosine is an endogenously released anti-inflammatory agent. Confirmation of this was subsequently derived from both *in vitro* and *in vivo* studies (Church &Hughes, 1985; Cronstein *et al.*, 1986; Gunther & Herring, 1991).

After exposure to agents such as endotoxin, phorbol myristate acetate, platelet-activating factor and TNF-α, neutrophils become primed to generate greater quantities of H_2O_2 and O_2^- after stimulation with chemoattractants. Adenosine has been reported to inhibit TNF-α-mediated priming of adherent neutrophils, but not neutrophils in suspension, although it did inhibit PAF-mediated priming of neutrophils in suspension (Steward & Harris, 1993). The biochemical basis of priming and the mechanism by which adenosine inhibits priming remain to be clarified.

Chemotaxis

Rose *et al* (1988) found that adenosine and its analogues augmented neutrophil chemotaxis to FMLP and C5a at concentrations two to three orders of magnitude below those which inhibited O_2^- generation. This was ascribed to occupancy of A_1 receptors. However, when primed, neutrophils demonstrated a reduced chemotactic response to chemoattractants. The effect of TNF-α on neutrophil chemotaxis was reversed by adenosine (Takenawa *et al.*, 1986).

Degranulation

In 1980 Marone *et al* reported that adenosine had no effect on degranulation by stimulated neutrophils. These results were widely accepted and for several years no further research was performed in relation to the effect of adenosine on neutrophil degranulation. In 1988 Cronstein *et al* confirmed that adenosine at best poorly inhibited granule release from stimulated neutrophils, while McGarrity *et al.*, (1989) also reported only modest effects of adenosine on secretion of granule proteins from neutrophils.

In contrast to these findings, Bouma *et al.*, (1997) demonstrated that adenosine dose-dependently inhibited human neutrophil degranulation in whole blood activated with either LPS or TNF-α, and that it attenuated the release of three different azurophil granule proteins, neutrophil elastase, bactericidal/permeability-increasing protein and defensin, to approximately the same extent. The inhibitory effect was also of comparable magnitude when the neutrophils were stimulated with agents which activate different signal transduction pathways, i.e. FMLP and STZ (serum-treated zymosan). The reported lack of adenosine effects on degranulation demonstrated in previous *in vitro* studies (Cronstein *et al.*, 1988; McGarrity *et al.*, 1989), had been attributed to the use of cytochalasin B to enhance neutrophil secretion in suspension. It had also been suggested that neutrophil adherence to biological surfaces might be required for mediation of the inhibitory effects of adenosine on the respiratory burst, as well as degranulation. However, Bouma *et al.*, (1997) in their study observed no major difference in the magnitude of the inhibitory effects of adenosine on degranulation in either whole blood or isolated, cytochalasin-B-pretreated neutrophils in suspension, using LPS or TNF-α as activators, both of which are very potent and selective inducers of azurophilic granule release.

Aggregation

Cronstein *et al.* (1983) reported that adenosine did not inhibit leucocyte aggregation (neutrophil-neutrophil adhesion).

Adherence and phagocytosis

The stable adenosine analogue 2-chloroadenosine has been reported to inhibit adhesion of stimulated neutrophils to vascular endothelial cells (Cronstein *et al.*, 1986), whereas low concentrations of compounds specific for the adenosine A_1-receptor promote adhesion of stimulated neutrophils to cultured endothelial cells and other surfaces. Higher concentrations of adenosine and adenosine A_2-receptor specific agonists inhibited the adherence of activated neutrophils to endothelial cells (Cronstein *et al.*, 1992).

Low concentrations of adenosine A_1-receptor agonists promote phagocytosis of immunoglobulin-coated red blood cells, while higher concentrations of adenosine A_2-receptor agonists have been reported to inhibit phagocytosis of Ig-coated red blood cells (Salmon & Cronstein, 1990).

Leukotriene production

Adenosine accumulates in leucocyte suspensions as a consequence of the extracellular breakdown of ATP. This accumulation of adenosine results in attainment of levels of this agent that exert suppressive effects on neutrophil functions, including LTB_4 synthesis. Thus, endogenous adenosine is an efficient modulator of LTB_4 biosynthesis by neutrophils and in inflammatory exudates rich in neutrophils, accumulation of adenosine may play an important role in modulating the local generation of important lipid mediators of inflammation (Krump *et al.*, 1997).

Calcium

Adenosine does not interrupt the early PLC-mediated signals (the early wave of diacylglycerol and inositol 1,4,5-triphoshate production, as well as Ca^{2+} mobilisation observed 10-20 seconds after chemoattractant receptor occupancy), but decreases by 50% the sustained increase in diacylglycerol synthesis in these cells(Walker *et al.*, 1990; Cronstein & Haines, 1992), as well as the late increment in $[Ca^{2+}]i$, presumably by attenuating the influx of extracellular Ca^{2+} (Cronstein, 1994).

1.9.2 Adenosine and polypeptide mediators of inflammation

Adenosine has been reported to inhibit the synthesis of pro-inflammatory cytokines, such as TNF-α by LPS-treated monocytes and macrophages (Parmely *et al.*, 1993), as well as the secretion of complement C2 by stimulated monocytes (Lappin & Whaley, 1984). In lymphocytes, adenosine inhibits the synthesis of immunoglobulins (Moroz & Stevens, 1980), as well as lymphocyte-mediated cytolysis (Wolberg *et al.*, 1975).

1.9.3 Adenosine receptors (ARs)

When acting on cell surface receptors, adenosine elicits a large number of responses throughout several organ systems. Activation of adenosine receptors can occur in response to endogenous adenosine or upon administration of adenosine (exogenous) or its more stable pharmacologic analogues. The anti-inflammatory and immunosuppressive potential of adenosine is, however, restricted by several factors *viz* the short half-life (2 seconds) in the circulation, receptor promiscuity, and potent cardiovascular effects (hypotension and bradycardia). These limitations have been overcome by the development of pharmacologic adenosine receptor agonists which are resistant to adenosine deaminase and which have receptor specificity (Cronstein, 1994).

Four different types of adenosine receptors have been identified, A_1, A_{2A}, A_{2B} and A_3. There is indirect evidence for the presence of A_1 and A_3 receptors on granulocytes (Fredholm *et al.*, 1996; Bouma *et al.*, 1997; Walker *et al.*, 1997) and fairly compelling evidence for the presence of A_{2A} receptors on neutrophils (Varani *et al.*, 1998). However, the exact involvement of these receptors in mediating the anti-inflammatory effects of adenosine remains to be conclusively established (Bouma *et al.*, 1997; Fredholm *et al.*, 1996), as does the role of cAMP (Cronstein, 1994; Varani *et al.*, 1998).

A_1R

This receptor is a single polypeptide, consisting of 326 amino acids with a molecular weight of ~ 36 kDa and is expressed in brain, spinal cord, fat, testis, heart and kidney. The A_1R has been classically associated with the inhibition of adenylyl cyclase (i.e. decreased production of cAMP) and is linked to G_i signal transduction proteins. The chemoattractant receptors of neutrophils are similarly linked to these pertussis toxin-sensitive signal transduction systems and amplification of G_i-stimulated signals by A_1 agonists may account for their enhancement of chemotaxis and phagocytosis. Alternatively, occupancy of A_1 receptors may promote more efficient recycling of chemoattractant and Fc receptors to effect more rapid chemotaxis and phagocytosis (Cronstein, 1994).

$A_{2A}R$

This is also a single polypeptide of 410-412 amino acids and molecular weight 45 kDa. A_{2A} receptors are present in human brain, heart, lung, kidney, liver and platelets. Occupation of $A_{2A}R$ is associated with activation of adenylyl cyclase via G_s-protein and increased intracellular cAMP. Resultant generation of cAMP is responsible for $A_{2A}R$-mediated inhibition of platelet aggregation, modulation of neutrophil function as described below and possibly vasodilation of certain vascular beds. The receptor demonstrates high affinity binding of several pharmacologic adenosine mimics such as CGS 21680, PAPA-APEC and NECA.

Interestingly, sustained elevations in circulating adenosine concentrations, leading to uncontrolled interaction with A_{2A} receptors on T- and B-lymphocytes is thought to underpin the immunologic hyporesponsiveness of these cells in patients with the adenosine deaminase deficiency variant of severe combined immunodeficiency disease.

Adenosine, via occupancy of $A_{2A}R$ on neutrophils, inhibits their adherence to endothelial cells, as well as generation of superoxide and phagocytosis (Cronstein, 1994). A_{2A} Receptor activation exerts cardioprotection, primarily during reperfusion, by inhibiting neutrophil function (superoxide radical production) and reducing the adherence of these cells to vascular endothelium (Jordan *et al.*, 1999; Harada *et al.*, 2000). A_{2A} Receptor activation has also been reported to protect against

inflammation-mediated renal injury by preventing neutrophil adhesion (Okusa *et al.*, 2000). The mechanism by which adenosine inhibits neutrophil function is not fully understood. However, it is evident that the $A_{2A}R$ is involved and that engagement of this receptor results in elevated intracellular cAMP, which has long been recognised as a cellular event leading to inhibition of the functional responses of neutrophils (Iannone *et al.*, 1989). Some studies do, however, cast doubt on the hypothesis that cAMP is the intracellular messenger for inhibition of O_2^- generation via activation of $A_{2A}R$. It has been reported for example that treatment of neutrophils with cell-soluble analogues of cAMP, inhibits O_2^- generation which is completely reversed by inhibitors of cAMP-dependent protein kinase (protein kinase A) (Cronstein *et al.*, 1992). Supporting a role for cAMP is the finding of synergistic effects of A_{2A} AR agonists and type IV phosphodiesterase inhibitors (Sullivan *et al.*, 1995). The inhibitory effects of $A_{2A}R$ occupancy on O_2^- generation are, however, reported to be unaffected, or only partially counteracted, by protein kinase A inhibitors, indicating that adenosine does not utilise the cAMP-protein kinase A system to inhibit O_2^- generation (Cronstein *et al.*, 1992). It is possible, however, that other neutrophil functions are affected as a result of $A_{2A}R$-mediated increases in intracellular cAMP concentrations (Cronstein, 1994). Nevertheless, the possible involvement of cAMP in $A_{2A}R$-mediated inhibition of superoxide production by activated neutrophils requires additional investigation.

As mentioned above, coupling of the $A_{2A}R$ to adenylyl cyclase results in enhanced cAMP levels in neutrophils. Other agents that cause elevation of intracellular cAMP e.g. PGE_2, the β-adrenergic agonist isoproterenol and the type 4 phosphodiesterase inhibitor rolipram all inhibit LT biosynthesis and/or AA release in activated neutrophils (Ham *et al.*, 1983; Fonteh *et al.*, 1993). Indirect, but compelling evidence indicates that elevation of intracellular cAMP levels results in profound inhibition of AA release in activated neutrophils and that cAMP is an intracellular mediator of the inhibitory effect of $A_{2A}R$ agonists on LTB_4 biosynthesis in human neutrophils.

Flamant *et al.*, (2000) have reported that CGS 21680, an analogue of adenosine expressing high selectivity for the $A_{2A}R$, potently inhibits LTB_4 biosynthesis, both in heparinised whole blood stimulated with FMLP and in isolated, agonist-stimulated

neutrophils. Studies with adenosine analogues selective for the A_1 and A_{2A} receptors, as well as with selective A_{2A} receptor antagonists, clearly established that the inhibitory effect of adenosine on LT biosynthesis involves occupancy of the $A_{2A}R$ on neutrophils (Krump *et al.*, 1996). The rapid rise in the release of AA by neutrophils primed with LPS and TNF-α and stimulated with PAF was almost completely abolished by the $A_{2A}R$ agonist CGS 21680. CGS 21680 was also shown to completely inhibit AA release in thapsigargin-activated neutrophils (Krump *et al.*, 1996; Flamant *et al.*, 2000).

As the release of AA in activated neutrophils is a Ca^{2+}-dependent process, a mechanism to account for the inhibition of this release by CGS 21680 was ascribed to the inhibitory effect of adenosine on agonist-induced Ca^{2+} influx in neutrophils. However, neutrophils activated by PAF in the absence of extracellular Ca^{2+} consistently produced 5-lipoxygenase products which were inhibitable by CGS 21680. The activation of $cPLA_2$, a cytosolic enzyme in resting neutrophils, involves two events: a Ca^{2+}-dependent translocation to nuclear structures on cell activation and phosphorylation of Ser-505 which results in increased catalytic activity (Leslie, 1997). Neither of these events was affected by CGS 21680 (Flamant *et al.*, 2000). Thus, engagement of the $A_{2A}R$ causes inhibition of AA release in activated neutrophils by an as yet unknown mechanism.

$A_{2B}R$

These receptors mediate a theophylline-sensitive, NECA-induced stimulation of adenylyl cyclase. $A_{2B}R$ transcripts are expressed in caecum, large intestine, bladder, brain, spinal cord and lung. Examples of coupling of the $A_{2B}R$ to phospholipase C stimulation are limited and receptor-mediated increases in inositol phosphate have not been reported. $A_{2B}R$-mediated stimulation of calcium channel activity has been described and this receptor subtype may mediate the secretory action of adenosine on mast cells.

A_3R

This is a polypeptide of 320 amino acids, which has been isolated in testis, kidney, heart, lung and brain. Stimulation of the receptor results in inhibition of adenylyl

cyclase activity. The abundant levels of A_3R mRNA in the lung may be significant in mediating allergic responses in the airways. The A_3 receptor exhibits large differences in structure, tissue distribution and pharmacologic properties between species, hampering the extrapolation of properties of this receptor between species (Linden, 1994). The relevance of non-human models to human A_3 function should, however, be reviewed with caution. Whereas A_3R activation inhibits degranulation of human neutrophils, it facilitates degranulation of rat mast cells (Fozard *et al.*, 1996). The mechanism involved in A_3R-mediated responses in the rat include activation of phospholipase C with elevation of intracellular inositol 1,4,5-triphosphate and Ca^{2+} levels, generation of nitric oxide and subsequent elevation of cGMP (Ramkumar *et al.*, 1993). In contrast, in human macrophages, A_3R-mediated inhibition of TNF-α expression has been shown to be independent of phospholipase C activation (Sajjadi *et al.*, 1996). The underlying mechanisms involved in A_3R-mediated inhibition of neutrophil degranulation remain to be defined.

Much work has focused on the ability of A_3Rs to promote preconditioning, and recently the involvement of this receptor in myocardial ischaemia and reperfusion injury has been investigated. It was demonstrated that pre-treatment with the selective A_3R agonist, IB-MECA, protected isolated rabbit hearts from injury induced by 30 min of regional ischaemia and crystalloid reperfusion (Jordan *et al.*, 1999). Cardioprotection has been shown to be elicited through stimulation of myocardial K_{ATP} channels. The mechanisms involved were investigated by Jordan *et al.*, (1999) who found that IB-MECA, at concentrations of 0.1-100nM, did not regulate superoxide production or the release of myeloperoxidase (degranulation) from isolated canine PMNs. This is in contrast to what was suggested by Bouma *et al.*, (1997) who demonstrated that >1μM IB-MECA inhibited release of bacterial permeability-increasing protein, elastase and defensins from human PMNs in whole blood in response to cytokine and endotoxin stimulation. It is possible that the higher concentrations used by Bouma *et al.*, (1997) may have resulted in inhibition of degranulation by influencing other adenosine receptor subtypes, notably the A_{2A} receptor. Alternatively, the disparate observations may be ascribed to species differences, measurement of different granule proteins, or differences in the stimulants used to activate the PMNs.

It was speculated that the inhibitory effect of A_3R agonists on neutrophil adherence may be mediated by regulation of surface expression of adhesion molecules. Bouma *et al.*, (1997), demonstrated that adenosine reduced the surface expression of E-selectin and VCAM-1 in human endothelial cell cultures stimulated with TNF-α. Preservation of endothelial function was observed when adherence of neutrophils was blocked with antibodies to endothelial adhesion molecules.

1.10 Hypothesis

The hypothesis to be tested is that adenosine receptor agonists possess anti-inflammatory properties which, unlike those of corticosteroids, enable these agents to suppress the harmful, pro-inflammatory, pro-oxidative and proteolytic activities of activated human neutrophils and that these are mediated predominantly through $A_{2A}R$ and increases in intracellular cAMP.

1.11 Objectives

The primary objectives of my study were as follows:

- to investigate the anti-oxidative and anti-inflammatory interactions of adenosine receptor agonists operative at the level of A_1, A_{2A} and A_3 receptors with human neutrophils *in vitro*.
- to identify which receptor subtype(s) are predominantly involved in mediating anti-inflammatory activity.
- to investigate the relationship, if any, between the anti-inflammatory effects of occupation of A_1, A_{2A} and A_3 receptors on neutrophils and alterations in intracellular cAMP.

- to investigate the effects of AR occupancy on Ca^{2+} fluxes and restoration of Ca^{2+} homeostasis in activated neutrophils as possible mechanisms of anti-inflammatory activity.
- to investigate and compare the effects of dexamethasone with those of an $A_{2A}R$ agonist (CGS 21680) on the early-activatable (superoxide production, elastase release) and late-activatable (interleukin-8 production) pro-inflammatory functions of human neutrophils *in vitro*.

Chapter 2

Effects of Dexamethasone on the Early- and Late-Activatable Pro-Inflammatory Functions of Human Neutrophils

2.1 Introduction

Glucocorticoids are the most well-known and potent anti-inflammatory agents in use today to suppress inflammatory and immunologic responses in conditions such as bronchial asthma, cerebral oedema, inflammatory bowel disease and collagen vascular disease. Their mechanism of action has been a topic of intense study and speculation and it has become evident that a vast array of cell types are targets of their action. Neutrophils play a prominent role in the inflammatory response and therefore the effects of glucocorticoids on neutrophil function are of considerable importance.

Rapidly-activatable pro-inflammatory neutrophil functions include adhesion to vascular endothelium, oxidant production, chemotaxis, phagocytosis and degranulation (Witko-Sarsat *et al.*, 2000). Neutrophils were previously thought to be devoid of transcriptional activity with little or no capacity for protein synthesis. However, convincing molecular evidence has now revealed that the neutrophil is not only a target, but also a source of cytokines, chemokines and growth factors (Cassatella, 1999). Interestingly, IL-8 is the most abundantly secreted cytokine by neutrophils and neutrophils are also the primary cellular target of IL-8, which is the most powerful chemoattractant for these cells (Gainet *et al.*, 1998).

Glucocorticoids require considerable time to mediate their anti-inflammatory actions both *in vivo* and *in vitro* and receptor-mediated effects occur at submicromolar concentrations. Most glucocorticoids used experimentally saturate the glucocorticoid receptor at concentrations of 1 μM or less (Ballard *et al.*, 1975). The use of higher concentrations yields results which are probably not relevant to glucocorticoid receptor-mediated effects. Dexamethasone is a commonly used glucocorticoid and is known to be approximately 25 times as potent as hydrocortisone.

The direct modulatory effects of corticosteroids on early-activatable neutrophil functions such as adhesion to vascular endothelium, superoxide production, granule enzyme release and phagocytosis are controversial as already outlined in section 1.8.3 (page 33), while there is some evidence to suggest that these agents may

suppress the later-onset production of pro-inflammatory cytokines such as IL-8 by these cells (Cox, 1995).

The experiments described in this chapter were designed to investigate the effects of a potent glucocorticoid, dexamethasone, on the rapidly-activatable (superoxide production and elastase release), as well as the late-activatable pro-inflammatory functions (IL-8 production) of human neutrophils *in vitro*.

2.2 Materials and Methods

2.2.1 Chemicals and reagents

Unless indicated these were purchased from the Sigma Chemical Co, St Louis, MO, USA.

2.2.2 Neutrophils

Purified neutrophils were prepared from heparinised (5 units of preservative-free heparin/ml) venous blood of healthy adult human volunteers and separated from mononuclear leucocytes by centrifugation on Histopaque®-1077 (Sigma Diagnostics) cushions at 400 *g* for 25 min at room temperature. The resultant pellet was suspended in phosphate-buffered saline (PBS, 0.15 M, pH 7.4) and sedimented with 3% gelatine to remove most of the erythrocytes. Residual erythrocytes were removed by selective lysis with 0.84% ammonium chloride at 4°C for 10 min. The neutrophils, which were routinely of high purity (> 90%) and viability (>95%), were resuspended to 1 x 10^7/ml in PBS and held on ice until used. Purity of isolated neutrophils was assessed microscopically and assessment of viability was done by dye-exclusion using 0.1% methylene blue.

2.2.3 Oxidant generation

This was measured using a lucigenin (bis-N-methylacridinium nitrate)-enhanced chemiluminescence (LECL) method (Minkenberg & Ferber, 1984). Neutrophils (1 x

10^6/ml final) were preincubated for 15 min in 900 µl indicator-free Hanks' balanced salt solution (HBSS, pH 7.4, Highveld Biological, Johannesburg, South Africa) containing 0.2 mM lucigenin in the presence and absence of the glucocorticoid, dexamethasone (1.25, 2.5, 5 and 10 µM, kindly provided by Dr M Johnson, Glaxo Smith Kline, Stockley Park, London, UK), prior to activation with the synthetic chemotactic tripeptide N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 1 µM final). Spontaneous and FMLP (1 µM)-activated LECL responses were then recorded using a LKB Wallac 1251 chemiluminometer after the addition of the stimulant (100 µl). LECL readings were integrated for 5 sec intervals and recorded as mV x seconds $^{-1}$ (mV.sec^{-1}). FMLP was used as stimulant because it causes activation of measurable Ca^{2+}-dependent pro-inflammatory responses. Additional experiments were performed in the same manner to investigate the effect of dexamethasone (10 µM) on the LECL responses of neutrophils activated with PMA (phorbol 12-myristate 13-acetate 25 ng/ml, 100 µl) and the superoxide-scavenging properties of dexamethasone (10 µM) using a cell-free hypoxanthine (1 mM)-xanthine oxidase (17 milliunits/ml) superoxide-generating system.

2.2.4 Elastase release

Neutrophil degranulation was measured according to the extent of release of the primary granule-derived protease, elastase. Neutrophils suspended in HBSS in the presence or absence of dexamethasone (1.25, 2.5, 5 and 10 µM) were incubated for 10 min at 37°C. The stimulant FMLP (0.1 µM) in combination with CB (cytochalasin B, 1 µM final) was then added to the cells which were incubated for 10 min at 37°C. The final neutrophil concentration in each tube was 2 x 10^6 in a volume of 1 ml HBSS. The tubes were then transferred to an ice-bath, followed by centrifugation at 400 *g* for 5 min to pellet the cells. The neutrophil-free supernatants were then decanted and assayed for elastase activity using a micro-modification of a standard spectrophotometric procedure (Beatty *et al.*, 1982). Briefly, 125 µl of supernatant was added to 125 µl of the elastase substrate, N-succinyl-L-alanyl-L-alanyl-L-alanine-*p*-nitroanilide, 3 mM in 0.3% dimethyl sulphoxide in 0.05 M Tris-HCl (pH 8.0). Elastase activity was assayed at a wavelength of 405 nm and the results for dexamethasone-treated systems expressed as the mean percentage of the amount of enzyme released by the corresponding FMLP/CB-activated, drug-free control

systems. Absolute concentrations of the protease were determined from a standard curve constructed using varying concentrations (0.015 – 15.5 units/ml of porcine pancreatic elastase type III).

2.2.5 Interleukin-8 production by neutrophils

Neutrophils (2×10^6) were preincubated for 10 min with and without dexamethasone (10 µM) in HBSS supplemented with bovine serum albumin (1 mg/ml) followed by the addition of FMLP to activate synthesis of IL-8 (Witko-Sarsat *et al.*, 2000). The final volume in each tube was 2 ml. The remaining tubes were then incubated for 0 and 6 hours at 37°C (a predetermined incubation time) after which total and extracellular IL-8 were measured using an antigen capture ELISA procedure (Roche Diagnostics Corp, Indianapolis, USA). Extracellular cytokine was measured in cell-free supernatants following removal of the neutrophils by centrifugation, while total IL-8 was measured in the lysates of neutrophils which had been treated with the detergent, lysophosphatidylcholine (0.01%), following removal of cellular debris by centrifugation. Quantitatively the lower limit of sensitivity for the IL-8 assay is 12.3 pg/ml. Systems (unstimulated and FMLP-activated) containing cycloheximide (10 µg/ml) were also included to distinguish between pre-existing and newly-synthesised cytokine. The results of these investigations are expressed as picograms IL-8/10^6 neutrophils.

2.2.6 Statistical analysis

The results of each series of experiments are expressed as the mean values ± SEM. Levels of statistical significance were calculated by paired Student's *t*-test when comparing two groups, or by analysis of variance (ANOVA) with subsequent Tukey-Kramer multiple comparisons test for multiple groups. A computer-based software system (Instat II®) was used for analysis.

2.3 Results

2.3.1 Effects of dexamethasone on superoxide production by neutrophils

The results of these experiments are shown in Tables 2.1 and 2.2 (page 55) for cells activated with FMLP and PMA respectively. In the case of FMLP-activated neutrophils, superoxide production was modestly, but significantly increased by dexamethasone at concentrations of 2.5 and 5 μM and unaffected by this agent at concentrations of 1.25 and 10 μM. In the case of PMA-activated cells dexamethasone, at the highest concentration tested (10 μM) caused slight inhibition of superoxide production by neutrophils.

Dexamethasone at the highest concentration tested (10 μM) did not possess superoxide-scavenging properties. These results are shown in Table 2.3 (page 56).

2.3.2 Effects of dexamethasone on the release of elastase by neutrophils

These results are shown in Table 2.4 (page 56). Dexamethasone at all concentrations tested did not significantly inhibit the release of elastase from FMLP/CB-activated neutrophils. At a concentration of 10 μM it significantly increased elastase release from FMLP/CB-activated neutrophils.

2.3.3 Effects of dexamethasone on the release of IL-8 from unstimulated and FMLP-activated neutrophils

These results are shown in Table 2.5 (page 57). Relative to the time 0 values, the concentrations of IL-8, both extracellular and total, increased after 6 hours for the system containing unstimulated (no FMLP) cells and were not significantly affected by dexamethasone. Activation of the cells with FMLP resulted in a slight increase in the concentration of extracellular IL-8, while the total concentration was unaffected.

Inclusion of dexamethasone in FMLP-activated systems was not accompanied by significant alterations in either the total or extracellular IL-8 concentrations. The

increase in both extracellular and total IL-8 during the 6 hour incubation period for both resting and FMLP-activated systems was attenuated by inclusion of cycloheximide (2.5 μM), confirming that the observed increases in IL-8 following 6 hours of incubation at 37°C are due to increased synthesis of the cytokine.

Table 2.1: The effects of dexamethasone 1.25, 2.5, 5 and 10 μM on superoxide generation by FMLP-activated neutrophils.

System	Superoxide Production	
	Absolute values ($mV.sec^{-1}$)	Percentage of control
Background	190 ± 13	
FMLP control	968 ± 100	
FMLP + Dex 1.25 μM	964 ± 99	96 ± 3
FMLP + Dex 2.5 μM	1055 ± 105	112 ± 5 *
FMLP + Dex 5 μM	1133 ± 97	116 ± 6 *
FMLP + Dex 10 μM	1056 ± 116	110 ± 4

The results of 14 experiments are presented as the mean percentages ± SEMs of the corresponding control systems for which the absolute peak values, observed at 10 min after the addition of FMLP, were expressed as $mV.sec^{-1}$. *$P<0.05$ for comparison with the control.

Table 2.2: The effects of dexamethasone 10 μM on superoxide production by PMA-activated neutrophils.

System	Superoxide production ($mV.sec^{-1}$)	Percentage of control
FMLP + Control	6503 ± 370	
FMLP + Dex 10 μM	6523 ± 319	86.8 ± 3

The results of 10 experiments are presented as the mean values ± SEM of the Corresponding control system for which the absolute peak values observed at 10 min after the addition of PMA were expressed as $mV.sec^{-1}$.

Table 2.3: Superoxide production by the hypoxanthine (1 mM)-xanthine oxidase (18 milliunits/ml) system in the presence and absence of dexamethasone.

System	Superoxide production ($mV.sec^{-1}$)
Control	1519 ± 193
Dex 10 µM	1476 ± 153

The results of 12 experiments expressed as the mean values ± SEMs in $mV\ sec^{-1}$.

Table 2.4: The effects of dexamethasone on release of elastase from FMLP/CB-activated neutrophils.

System	Elastase release	
	absolute values (milliunits enzyme/10^7 cells)	Percentage of control
Control	80 ± 15	
Dex 1.25 µM	77 ± 14	98 ± 3
Dex 2.5 µM	79 ± 16	95 ± 4
Dex 5 µM	83 ± 16	106 ± 5
Dex 10 µM	90 ± 17	113 ± 5 *

The results of 20 experiments are presented as the mean percentages ± SEMs of the corresponding control systems for which the absolute values were expressed as millinunits enzyme/10^7 cells. *$P<0.05$ for comparison with the control.

2.4 Discussion

The results presented in this chapter are those of experiments which were designed with the primary objective of identifying the effects, if any, of dexamethasone, considered to be a potent anti-inflammatory corticosteroid operating at the level of gene transcription, on several pro-inflammatory functions of human neutrophils *in vitro*. Even at extremely high concentrations dexamethasone did not interfere with

(but rather slightly potentiated) the production of superoxide by, as well as the release of elastase from, FMLP-activated neutrophils, underscoring the independence of these rapidly-occurring events (maximal within 1 minute) on *de novo* protein synthesis (Snydermann & Uhing, 1992). These observations also appear, at least in the case of dexamethasone, to exclude the existence of non-classical/non-conventional mechanisms (eg. membrane-stabilising actions) of anti-inflammatory activity which may be evident at high, therapeutically improbable concentrations of this agent. These observations are in agreement with the findings of Cox (1995) who reported that coincubation of neutrophils with glucocorticoids for up to 24 hours did not interfere with, but rather moderately increased chemoattractant-activated production of superoxide by these cells. This effect after prolonged exposure of neutrophils and glucocorticoids was attributed to the inhibitory effects of these agents on spontaneous apoptosis. Although not included, I have found similar results with fluticasone to those observed with dexamethasone i.e. no inhibitory effects on the production of superoxide by and release of elastase from activated human neutrophils.

Table 2.5: The effect of dexamethasone on the spontaneous and FMLP-activated synthesis of IL-8.

	IL-8 concentrations (pg/10^6 cells)	
System	**Extracellular**	**Total**
Unstimulated time 0	0	100 ± 18
*Unstimulated control	434 ± 79	793 ± 9
Unstimulated control + dexamethasone (10 µM)	232 ± 26	653 ± 87
FMLP-activated control	557 ± 78	629 ± 303
FMLP-activated + dexamethasone (10 µM)	469 ± 36	1083 ± 33
Unstimulated + cycloheximide (2.5 µM)	32 ± 2	213 ± 7

The results of 4 experiments with triplicate determinations for each are presented as the mean values ± SEMs.

*With the exception of the time 0 value, all the other values shown are for systems incubated for 6 hours at 37°C.

Likewise the production of IL-8 by unstimulated and FMLP-activated neutrophils following 6 hours of incubation at 37°C was unaffected by dexamethasone. This observation differs from the findings of Cox (1995) who reported that dexamethasone inhibited the production of IL-8 by unstimulated neutrophils (activated systems were not included). There are, however, some notable differences between my study and that of Cox (1995). Most importantly, Cox (1995) used an incubation period of 24 hours in comparison with the 6 hour incubation used in the current study. I did not go beyond 6 hours because of the onset of spontaneous apoptosis (observations made by my colleague Ms R Cockeran which are a component of her PhD thesis) in neutrophils cultured *in vitro*, which complicates interpretation of results. I do concede, however, that extended exposure of neutrophils may have resulted in decreased synthesis of IL-8 by these cells. Interestingly, dexamethasone appeared to inhibit release of IL-8 in resting neutrophils without affecting synthesis of the chemokine.

In support of my contention that neutrophils are insensitive (at least over the first 6 hours of exposure) to the inhibitory effects of corticosteroids on the biosynthesis of the pro-inflammatory cytokine IL-8, which appears to be the most abundant of the neutrophil-derived cytokines (Witko-Sarsat *et al.*, 2000), it has recently been reported that these cells have relatively low levels of the α-isoform of the glucocorticoid receptor (Strickland *et al.*, 2001). This is the biologically active form of the receptor. Instead, neutrophils have high levels of the GRß isoform which lacks the steroid binding domain and antagonises GRα through the formation of CRα/GRß heterodimers.

Indirect modulatory effects of corticosteroids on neutrophils achieved through inhibition of the production of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-8, as well as bioactive phospholipids such as PAF are not excluded by the current study and their potential importance is acknowledged. However, the apparent insensitivity of the early-activatable, and possibly also the late activatable pro-inflammatory functions of human neutrophils to the direct, inhibitory actions of corticosteroids does underscore the requirement for novel, neutrophil-directed anti-

inflammatory chemotherapeutic strategies and is the topic of the detailed studies presented in the remainder of this thesis.