THE ROLE OF THE NEUTROPHIL IN RHEUMATOID ARTHRITIS

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy by Fiona Stewart White.

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I declare that this thesis entitled:

'The role of the neutrophil in rheumatoid arthritis'

is entirely my own work, except where collaboration is indicated in the text.

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ABSTRACT

The neutrophil is the first line of defence against invading pathogens. However, it can also cause damage to the host in inflammatory diseases such as Rheumatoid Arthritis (RA). This damage is thought to occur primarily by release of preformed granule enzymes and reactive oxidants from activated neutrophils. The aim of this thesis was to investigate the role of protein synthesis by neutrophils in the pathogenesis of RA.

Initially, the overall pattern of proteins synthesised by neutrophils in response to synovial fluid (SF) and immune complexes (IC) was studied. Neutrophils were stimulated with these agents and the proteins synthesised were radiolabelled and analysed by SDS-PAGE. It was found that both SF and IC stimulated an increased rate of protein synthesis in neutrophils and that soluble IC was the most effective agent. A similar effect was seen when SF-derived IC were used. These results suggest that neutrophils within the rheumatoid joint are synthesising and secreting proteins which may be of pathological relevance.

IL-1β and TNF-α , 2 cytokines thought to be of particular importance in joint destruction in RA were studied in more detail. Radiolabelled cell associated and secreted IL-1β and TNF-α were immunoprecipitated and analysed by SDS-PAGE. SF and IC were able to stimulate the synthesis of cell associated IL-1β and TNF-α but only SF induced secretion of IL-1β and no secretion of TNF-α could be detected. Neutrophils isolated from RA patients' blood and SF were also seen to synthesise IL-1β and TNF-α . These results indicate that neutrophils in the rheumatoid joint may be synthesising these cytokines, and contributing to disease pathology.

The ability of IL-1β and TNF-α to prime neutrophils was studied by assaying the activity of the NADPH oxidase and changes in intracellular Ca^{2+}. Both IL-1β and TNF-α were able to prime the NADPH oxidase of neutrophils although TNF-α was a more effective priming agent. A similar result was obtained when intracellular Ca^{2+} changes were assayed. Hence, IL-1β and TNF-α synthesised by neutrophils within the rheumatoid joint may be able to prime fresh neutrophils entering the joint.

Finally, neutrophil apoptosis was examined following exposure to rheumatoid SF. Neutrophils exposed to SF underwent more rapid apoptosis, however, neutrophils incubated in the presence of ROI scavengers showed delayed apoptosis in the presence of low concentrations of SF but not high concentrations.

These results suggest that neutrophils may be able to direct the immune response in RA through the production of cytokines, and possibly still to be identified pro-inflammatory proteins. This activation of neutrophils in the joint may then quickly exhaust the cell's capacity to respond leading to rapid apoptosis.

ABBREVIATIONS

ActD	Actinomycin D
APS	Ammonium persulphate
BSA	Bovine serum albumin
C5a	Complement fragment 5a
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
CGD	Chronic Granulomatous Disease
CHAPS	(3-[3-Cholamidopropyl)dimethylammonio]-1-propanesulphonate
CHX	Cycloheximide
cpm	counts per minute
CR	Complement receptor
EDTA	Ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-*bis*-(β-aminoethyl)-N,N,N',N'-tetraacetic acid
ENA-78	Epithelial Neutrophil Activating peptide 78
FcR	Fc receptor
FMLP	Formyl-methionyl-leucyl-phenylalanine
G-CSF	Granulocyte Colony Stimulating Factor
GM-CSF	Granulocyte Macrophage-Colonly Stimulating Factor
H_2O_2	Hydrogen peroxide
HEPES	N-2-Hydroxyethylpiperazine-N'2-ethanesuphonic acid
HSA	Human serum albumin
HOCl	Hypochlorous acid
IC	Immune complex
ICAM	Intercellular adhesion molecule
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist
LPS	Lipopolysaccharide
LTB_4	Leukotriene B_4
LUMINOL	5-amino-2,3-dihydro-1,4-phthalazinedione
MAPK	Mitogen activated protein kinase
MHC	Major histocompatibility complex
MIP-1α,β	Macrophage inhibitory protein-1α,β

mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
O_2^-	Superoxide
OH^-	Hydroxyl radical
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDA	Piperazine diacrylamide
PMA	Phorbol-12 Myristate Acetate
PMN	Polymorphonuclear leukocyte
PMSF	Phenylmethylsulphonylfluoride
RA	Rheumatoid arthritis
RNI	Reactive nitrogen intermediate
ROI	Reactive oxygen intermediate
rpm	Revolutions per minute
SDS	Sodium dodecylsulphate
SF	Synovial fluid
SOD	Superoxide dismutase
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGFβ	Transforming growth factor β
TNF-α	Tumour necrosis factor-α
Tris	Tris(hydroxymethyl)methylamine

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CHAPTER 1

INTRODUCTION

1.1 GENERAL

The neutrophil is the first line of defence of the immune system when the host is attacked by invading pathogens. It is the first cell to arrive at the site of an infection and immediately begins phagocytosing bacteria and fungi, killing them intracellularly using a variety of anti-bacterial and -fungal agents. However, the neutrophil has another, more sinister side to its activity. Under certain circumstances, the toxic agents used to such good effect against invading pathogens can be released into the surrounding tissue causing a great deal of damage. This tissue destruction mediated by neutrophils is a feature of the chronic inflammatory disease Rheumatoid arthritis (RA).

1.2 NEUTROPHIL PHYSIOLOGY

The neutrophil is one of 3 major types of white blood cell known as polymorphonuclear leukocytes (PMN). It is the most abundant white blood cell, accounting for 40-65% of the total cell numbers and its abundance can increase dramatically during an inflammatory reaction or infection. The other 2 types of PMN are eosinophils and basophils which are much less abundant accounting for 1-7% and <1% respectively of the total leukocyte pool. Examination by electron microscopy shows that the neutrophil has a polymorphic nucleus and many granules (Baggiolini *et al*, 1984) but little or no endoplasmic reticulum or Golgi apparatus (Bainton *et al*, 1971). These morphological observations have led to the erroneous suggestion that the neutrophil is a terminally differentiated cell with little or no need or capacity for protein synthesis (Cline, 1966).

It is also difficult to locate any mitochondrial structures within the cytoplasm and, in fact, neutrophils derive most of their energy requirements from the O_2-independent process of glycolysis. This feature allows the neutrophil to perform efficiently in the relatively low oxygen concentrations found at inflammatory sites (Edwards, 1994).

The life span of the neutrophil is only around 8-20 h in the circulation, but this can be increased to several days if the cell becomes activated. These cells are therefore subject to a massive daily turnover with 10^{11} cells being produced by the bone marrow each day. Thus, the control of neutrophil generation must be a very tightly regulated process.

1.3 NEUTROPHIL GRANULES

The abundant granules observed in the cytoplasm are of 3 types:

1 Primary or Azurophil granules (contain peroxidase) (Bainton *et al*, 1971),

2 Secondary or Specific granules (contain no peroxidase) (Bainton *et al*, 1971),

3 Tertiary or Gelatinase containing granules (Morel *et al*, 1994).

In addition to these granules, there are also recently identified secretory vesicles (Borregaard *et al*, 1993). The cytoplasmic granules are important in the intracellular killing of pathogens as they contain the neutrophil's repertoire of anti-bacterial and anti-fungal proteins. Some of the anti-pathogen proteins present within the granules include: cathepsins, defensins, elastase and myeloperoxidase in azurophil granules; collagenase, gelatinase, lactoferrin and plasminogen activator in specific granules; gelatinase and acetyltransferase in gelatinase-containing granules (Borregaard *et al*, 1993). The proteins found in secretory granules include CD14 and CD16 (Detmers *et al*, 1995).

A second important function of the granules is the transport of proteins to the cell surface. Many important membrane proteins are stored on the granule membranes and when fusion of the granule with the plasma membrane occurs these proteins become integrated into the plasma membrane of the cell. Proteins mobilised in this way include CR1, CR3, CD14, CD16 (FcγRIIIb), the FMLP-receptor and cytochrome b_{558} (Sengeløv *et al*, 1995; Detmers *et al*, 1995).

1.4 THE ROLE OF THE NEUTROPHIL IN INFLAMMATION

1.4.1 Neutrophil Exit From the Bloodstream

Under normal circumstances, around 50% of the blood neutrophil population will be marginated on the blood vessel walls. The circulating population can be increased very rapidly in response to inflammation and also to exercise. In an inflammatory situation, circulating neutrophils are induced to enter the tissues by chemotactic factors such as IL-8, bacterial products (eg N-formylated peptides) and the complement fragment C5a. These chemoattractants also stimulate up-regulated expression of adhesion molecules on both neutrophils and endothelial cells. Initially, a loose adhesion is formed between the first set of adhesion molecules expressed, the Selectins (L-selectin on neutrophils and E-selectin on endothelial cells). This loose adhesion allows the neutrophil to "roll" along the capillary. There then follows a much stronger adhesion between later expressed integrins (CD11a/CD18 or LFA-1 and CD11b/CD18 or Mac-1 on neutrophils and ICAM-1 and -2 on endotheluim). The neutrophil now enters the tissue by migrating between the endothelial cells, a process termed diapedesis (Simon *et al*, 1995; Crockett-Torabi *et al*, 1995).

1.4.2 Phagocytosis and Killing

Upon arrival at the inflammatory site neutrophils begin phagocytosing and killing the bacteria. Opsonised bacteria coated with antibody and complement bind to the Fc and complement receptors on the neutrophil surface (FcγRI,II and IIIb and CR1 and CR3). The bound bacterium is then engulfed by the plasma membrane and internalised in a phagosome. Once inside the cell, the phagosome fuses with granules to form a phagolysosome and the granule contents are released into it killing the bacteria (reviewed by Smith, 1995).

1.4.3 Respiratory Burst

The respiratory burst of the neutrophil is also activated during phagocytosis (Smith, 1995). The many components required for the NADPH oxidase enzyme complex translocate to and assemble on the phagolysosome and reactive oxygen intermediates (ROI) are formed.

The first oxidant species generated during the respiratory burst is superoxide (O_2^-), which is formed by the reduction of O_2 by a single electron. This is then converted to H_2O_2 by the enzyme, superoxide dismutase. The hydroxyl radical (OH•) may also be formed from H_2O_2 and O_2^- in the presence of an Fe or Cu salt, although evidence for the formation of this reactive oxidant by phagocytosing neutrophils is controversial. The granule enzyme myeloperoxidase catalyses the conversion of H_2O_2 to hypohalous acid. It is generally thought that hypochlorous acid is predominantly produced by neutrophils because chloride is the most abundant physiologic halide ion present (Edwards, 1994). All of these ROI are toxic to both prokaryotic and eukaryotic cells with HOCl being most toxic followed by OH^-, H_2O_2 and then O_2^-. These short lived free radicals are generated in the phagolysosome and contribute to the killing of the pathogen. The

importance of the respiratory burst in intracellular killing is illustrated by the human condition Chronic Granulomatous Disease (CGD) in which the NADPH oxidase is impaired or absent. Patients with CGD suffer persistent, chronic and life-threatening bacterial and fungal infections which their neutrophils are unable to control because of inadequate generation of ROI (reviewed by Miller & Britigan, 1995).

1.4.4 Reactive Nitrogen Intermediates

Neutrophils have also been shown to synthesise Nitric oxide (NO). This molecule has been shown to be produced at high levels by mouse macrophages and is extremely toxic to intracellular pathogens such as Leishmania major (Liew *et al*, 1990). As yet it is unknown if NO contributes to neutrophil killing of pathogens (reviewed by Miller & Britigan, 1995). However, ROI and RNI are able to interact to form other reactive species for example, superoxide reacts with NO to form peroxynitrite (ONOO-) a molecule which is also toxic to microbial pathogens and may mediate several of the cytotoxic effects of NO (Darley-Usmar *et al*, 1995).

1.5 TISSUE DAMAGE CAUSED BY NEUTROPHILS

Under certain inflammatory conditions, such as those found in certain autoimmune diseases, the pathogenic killing mechanism can be turned against the body and result in tissue damage. In some diseases such as glomerulonephritis, antibodies and immune complexes are deposited onto tissues. The neutrophil, unable to phagocytose a large area of tissue coated with opsonin will undergo a process termed frustrated phagocytosis. Instead of being released into the phagolysosome, the granule contents will be released into the environment and the toxic enzymes and ROI so effective against foreign pathogens, will destroy cells and structures in the surrounding tissues (reviewed by Smith, 1995; Weiss, 1989; Fujishima & Aikawa, 1995).

1.6 NEUTROPHIL PRIMING

It is now widely accepted that the neutrophil can exist in at least 3 functional states - quiescent, primed and activated. Priming is defined as a state of enhanced responsiveness induced after the addition of agonists at a sub-stimulatory concentration or by exposure to certain cytokines. A primed neutrophil will therefore give a greater and more rapid response to further stimulation. Priming may thus constitiute an important and highly controlled mechanism for regulating neutrophil activation during inflammation. Thus, full activation may require 2 separate stimuli (a priming stimulus and an activating stimulus). This 2 step activation process may decrease the possibility of non-specific activation (Edwards, 1994).

1.6.1 Priming Agents and Receptors

Many neutrophil agonists have been identified as priming agents when used at concentrations much lower than those required to fully activate the cell. The bacterial formyl peptide FMLP has been shown to prime neutrophils at a concentration 20 fold lower than that required to activate the respiratory burst (McPhail *et al*, 1984; Hughes *et al*, 1987). In addition to FMLP it has also been shown that certain cytokines are able to prime neutrophils. For example, IFNγ (Weisbart *et al*, 1985), TNF-α (Berkow *et al*, 1987; Klebanoff *et al*, 1986), IL-8 (Yuo *et al*, 1991), IL-6 (Biffl *et al*, 1994; Borish *et al*, 1989) GM-CSF and G-CSF (Weisbart *et al*, 1985; Yuo *et al*, 1990) have all been demonstrated to prime the NADPH oxidase, phagocytosis, chemotaxis, intracellular killing and protein biosynthesis in neutrophils (Perussia *et al*, 1987; Edwards *et al*, 1990; Humphreys *et al*, 1991; Bober *et al*, 1995).

It has also been shown that cross-linking adhesion molecules can result in priming of neutrophils. Cross-linking of CD62L (L-Selectin) up-regulates CD18 expression and adhesion (Simon *et al*, 1995) while cross-linking of CD18 by antibodies, mimicking

adhesion to the extracellular matrix, can prime the neutrophil respiratory burst (Lilies *et al*, 1995). Ligation of CD18 has also been shown to be important in the mechanism of TNF-α priming (Dapino *et al*, 1993).

1.6.2 Short Term Effects Of Priming

The short term effects of priming are seen within 15-60 min of addition of the primer and result in a greatly enhanced respiratory burst when neutrophils are exposed to a further stimulus, such as immune complexes, FMLP, C5a and LTB_4 (Edwards *et al*, 1989; Lilies *et al*, 1995). This process is associated with a change in cell shape from the spherical shape seen in suspension and in whole blood, to the more ameboid shape seen in adherent neutrophils and alterations in the cytoskeleton have been noted (Edwards, 1994).

Cell surface expression of some receptors is increased during priming, such as the FMLP-receptor, CR1 and CR3. This rapid increase in expression of these receptors is unaffected by incubation with the protein synthesis inhibitor, cycloheximide or the mRNA synthesis inhibitor, actinomycin D indicating that these receptors are mobilised from preformed intracellular stores which are located on the membranes of specific and gelatinase granules, and the secretory vesicles (Edwards *et al*, 1990). The expression and functional activity of the CD11b receptor has been shown to be up-regulated while another adhesion molecule, CD62L is shed from the cell surface (Condliffe *et al*, 1996). CR3 activity is also rapidly up-regulated during priming (Edwards, 1995)

The ability to both phagocytose and kill bacteria are enhanced after priming, both of which may be predicted following increased oxidant production and greater receptor expression on the cell surface (Bober *et al*, 1995; Perussia *et al*, 1987). There has also been a report of increased intracellular production of Platelet Activating Factor (PAF) following priming with LPS (Worthen *et al*, 1988).

1.6.3 Long Term Effects Of Priming

Longer term effects of priming are detectable many hours and even days after exposure to the priming agent. The survival time *in vitro* of primed neutrophils is increased from 8-20 h characteristic of circulating neutrophils to several days. This much longer survival time would seem to indicate a necessity for *de novo* protein synthesis. Indeed, addition of cycloheximide decreases oxidant production in primed cells even after 2 h. This decrease is greater in cells where the second stimulus is FMLP (which is a receptor mediated response) compared to PMA (which is not receptor mediated). However, this PMA response is also decreased after prolonged incubation with cycloheximide (Edwards *et al*, 1990). The decrease in function was accompanied by decreased expression of receptors from the cell surface, although there may also be decreased expression of oxidase components during cycloheximide treatment. These observations indicate that protein synthesis is needed to maintain the cell in a primed state for a prolonged length of time (Edwards *et al*, 1990).

Neutrophil protein synthesis has also been studied. It has been found that although an overall increase of 3-5 fold in the amount of ^{35}S-methionine incorporated into proteins is seen, certain proteins only show a 1-2 fold increase in labelling while others show an increase of 10-20 fold (Hughes *et al*, 1987; Humphreys *et al*, 1989; Edwards *et al*, 1990). Hence, there is a rapid and selective up-regulation of protein synthesis seen when neutrophils are primed.

1.6.4 Signal Transduction

There are 3 types of receptors that trigger priming in neutrophils. These are: the 7 transmembrane domain, GTP-binding protein (G protein) linked receptors, such as the FMLP-receptor; single transmembrane domain receptors that require cross-linking for

activation, such as integrins; single transmembrane domain receptors for growth regulating cytokines, such as the GM-CSF-receptor. Although all 3 of these receptor types can prime neutrophils, only the first 2 can also activate the cell. When a neutrophil is activated, there is a 10 fold increase in cytosolic free Ca^{2+} which correlates with oxidase activation and inhibition of this Ca^{2+} increase also inhibits the activation of the oxidase (Al-Mohanna & Hallett, 1988; Hallett *et al*, 1990). Hence, the Ca^{2+} signal is a key signalling event in activation. However, when neutrophils are primed with GM-CSF or substance P, there is no Ca^{2+} rise and priming has no effect on the Ca^{2+} changes seen after further stimulation (Lloyds & Hallett, 1993). Therefore, Ca^{2+} signals appear to have no direct role in the signal transduction mechanisms responsible for priming (reviewed by Hallett & Lloyds, 1995).

It is thought that another signalling event could be important in priming, namely tyrosine phosphorylation (Gomez-Cambronero *et al*, 1989; McColl *et al*, 1991). There are several lines of evidence to support this theory:

1. Tyrosine phosphorylation is triggered in neutrophils at concentrations of agonists too low to activate the cells.
2. The timing of phosphorylation is consistent with a causative role in priming.
3. Inhibition of tyrosine kinases also inhibits priming (reviewed by Hallett & Lloyds, 1995).

Lilies *et al* (1995) have also shown tyrosine phosphorylation to be important in the priming of neutrophils by cross-linking CD18. Several other groups have demonstrated the association of tyrosine phosphorylation with priming. This phenomenon is thought to be important in priming by TNF-α and G-CSF (Akimaru *et al*, 1992), substance P (Lloyds *et al*, 1995) and priming by GM-CSF in conjunction with PLA_2 (Roberts *et al*, 1996). Tyrosine phosphorylation may be important in transducing signals that control cell activation following ligation of cytokine receptors.

In neutrophils cross-linked with anti-CD18 antibodies and then stimulated with TNF-α, tyrosine phosphorylation of 3 major proteins was observed. One of these proteins, pp40 could be a member of the mitogen activated protein kinase family (MAPK) which is thought to be involved in the signalling of priming (Ohta *et al*, 1992; Thompson *et al*, 1993). MAPK also appears to be involved in LPS priming via the CD14 molecule (Nick *et al*, 1996). Hence, adhesion dependent tyrosine phosphorylation may be necessary to couple the TNF-α receptor to activation of the oxidase.

1.7 GENE EXPRESSION

The neutrophil was originally thought to be a terminally differentiated cell with only a passive role in the efferent arm of the immune system, using its preformed granule enzymes for bactericidal activity (Cline, 1966). However, it is now recognised that the neutrophil is capable of *de novo* protein synthesis and that they do make a range of proteins that are important in the inflammatory response. Such proteins include some receptors and cytokines (Granelli-Piperno *et al*, 1979; Jack & Fearon, 1988; Cassatella, 1995). Such cytokine production by neutrophils can make a contribution to the afferent limb of the immune system and hence play an important role in the regulation of the inflammatory response.

1.7.1 Gene Expression Products

There are 3 broad classes of gene products expressed by neutrophils. The first group of genes are expressed constitutively in the absence of any activating agent. These are "housekeeping genes" such as actin and phosphoglycerokinase and their expression is not altered following activation of the cell. Secondly, there are genes encoding proteins with a high turnover rate including some of the oxidase components and certain receptors. When the cell is activated, the expression of these genes increases by 2-3

fold. Finally, there are genes not expressed in resting bloodstream neutrophils eg. those encoding cytokines which undergo a large increase in expression following activation.

1.7.2 Receptor Gene Expression

There are several plasma membrane receptors on the neutrophil which have a rapid turnover rate due to either shedding or internalisation of the receptor. These include FcγRIIIb, CR1, CR3 (CD11b only) and MHC class I (Edwards, 1994; Lloyd & Oppenheim, 1992). These receptors are constantly being shed or internalised in resting cells, but are replaced by *de novo* protein synthesis and by mobilisation from internal stores. If the cell is activated, the synthesis of these receptors increases by 2-5 fold. For example, FcγRIIIb, CR1 and CR3 expression is increased by exposure of neutrophils to GM-CSF (Bober *et al*, 1995).

Other receptors synthesised by the neutrophil include the MHC class II antigen (DR only) which is not detectable on resting cells but is induced by both GM-CSF and IL-3 acting in synergy, and by IFNγ or GM-CSF alone (Smith, 1995). The purpose of this expression is unclear as neutrophils do not possess the co-stimulatory molecule B7 required for antigen presentation and have so far not been shown capable of processing and presenting an antigen to T cells (Smith *et al*, 1995). Another receptor not expressed on resting cells but up-regulated on activated cells is FcγRI, the receptor with a high affinity for monomeric IgG. The expression of this receptor is stimulated by IFNγ (Perussia *et al*, 1987) and its expression *in vivo* has also been reported to correlate with the amount of G-CSF present in the plasma (Gericke *et al*, 1995). Another receptor induced in activated cells is the decoy IL-1 receptor type II. This receptor binds IL-1 on

the cell surface but does not have any signalling capacity. Synthesis of this receptor in neutrophils is induced by IL-13, an anti-inflammatory cytokine (Colotta *et al*, 1994).

1.7.3 NADPH Oxidase Components

Neutrophils incubated with cycloheximide lose their ability to produce oxidants over time in culture, indicating that constitutive expression of at least one of the oxidase components or a protein involved in oxidase activation is needed for prolonged neutrophil function. In unstimulated cells, mRNA can be detected for gp91-*phox* and p22-*phox*, the subunits of cytochrome b and for p47-*phox* and p67-*phox*, the cytosolic components. IFNγ treatment for 2-4 h increases the ability of neutrophils to generate oxidants in response to FMLP and PMA but this increased ability is lost if the cells are also incubated with cycloheximide. Hence, synthesis of oxidant components is necessary for the neutrophil to retain function in culture. Analysis of the expression of the oxidase genes shows that while p22-*phox* expression is unchanged by IFNγ treatment, gp91-*phox* expression increases but p47-*phox* is down-regulated (Edwards, 1994). It has also been shown that phagocytosis of opsonised or non-opsonised yeast particles results in a down-regulation of the steady state levels of gp91-*phox* and gp47-*phox* mRNA (Cassatella *et al*, 1993).

1.7.4 Cytokines

The neutrophil can synthesise a range of cytokines including IL-1β (Tiku *et al*, 1986), TNF-α (Djeu *et al*, 1990), IL-8 (Fujishima *et al*, 1993), IL-12 (Cassatella *et al*, 1995) IL-1ra (McColl *et al*, 1992), G-CSF, M-CSF, TGFβ (Grotendorst *et al*, 1989) and IFNα (reviewed by Cassatella, 1995; Lloyd & Oppenheim, 1992). The common

feature of neutrophil synthesis of cytokines distinct from that of monocytes, is the rapid and transient nature of the expression. In general, mRNA can be detected within 1 h after stimulation, peaking at 1-2 h and quickly returning to baseline levels. This is in contrast to the pattern of cytokine expression by monocytes, in which expression is slower and can be continued for periods of up to and beyond 24 h. It should also be noted that neutrophils synthesise much less (usually 5-20 fold less) cytokine protein (on a per cell basis) than monocytes. However, at an inflammatory site, the neutrophil is the first cell to arrive and often in very large numbers. Hence, relatively low levels of production of cytokines by large numbers of neutrophils can generate significant quantities during acute inflammation.

1.7.4.1 Interleukin-1β

Interleukin-1β (IL-1β) is a pleiotropic cytokine secreted by many cells, including monocytes, neutrophils, endothelial cells and synoviocytes. Included in the many effects of IL-1β are pyrogenicity, T and B cell activation, and the stimulation of the synthesis of many other cytokines (reviewed by Dinarello, 1991; Brazel *et al*, 1992).

Neutrophil synthesis of IL-1β has been reported by many authors in response to a variety of stimuli. LPS has been shown to be a potent stimultor of *de novo* synthesis and secretion of IL-1β (Lord *et al*, 1991) as has TNF-α and IL-1β itself (Marucha *et al*, 1991). It has also been observed that GM-CSF treatment can induce IL-1β expression (Lindemann *et al*, 1988; Quayle *et al*, 1994), although this has been disputed by Malyak *et al*, (1994). These later authors were unable to detect any IL-1β secretion following GM-CSF or TNF-α treatment, although these cytokines did enhance mRNA levels transiently. It has also been demonstrated that LPS-stimulated IL-1β production can be

inhibited by the anti-inflammatory cytokine, IL-10 (Cassatella *et al*, 1994; Jenkins *et al*, 1994). Further details of neutrophil-dependent synthesis of IL-1β are described in Chapter 4.

1.7.4.2 Interleukin-1 Receptor Antagonist

The Interleukin-1 receptor antagonist (IL-1ra) is an anti-inflammatory cytokine secreted by many cells and specifically antagonises the action of IL-1 by binding competitively to the IL-1 receptor (Reviewed by Dinarello, 1991). This receptor binding does not lead to cell activation.

Expression of the IL-1ra gene and secretion of the mature protein has been observed in neutrophils in response to a number of mediators including GM-CSF, G-CSF, LPS, IL-4 and TNF-α but not IL-1 itself, IL-10, IFNγ, TGFβ FMLP, C5a or IL-8 (Re *et al*, 1993; Jenkins *et al*, 1994; Muzio *et al*, 1994). The enhanced expression of IL-1ra induced by TNF-α is augmented by IL-4 and IL-10 (Marie *et al*, 1996). IL-10 also up-regulates IL-1ra expression stimulated by LPS (Cassatella *et al*, 1994). McColl *et al* (1992) showed *de novo* synthesis and secretion of IL-1ra by neutrophils stimulated with GM-CSF or TNF-α. The IL-1ra produced represented the major *de novo* protein of the neutrophils and was in an excess of 100 fold over IL-1β production. This level of IL-1ra production was found to be sufficient to inhibit 50% of the activity of recombinant IL-1β. However, other authors have shown a much lower production of IL-1ra by neutrophils of only a 3-4 fold excess of IL-1β which would not be sufficient to inhibit its activity (Malyak *et al*, 1994).

1.7.4.3 Tumour Necrosis Factor-α

TNF-α is another pro-inflammatory cytokine sharing many effects with IL-1β (Duff, 1993, Dinarello, 1991). It is also known to increase adherence, phagocytosis and degranulation in neutrophils and can prime the respiratory burst. Synthesis and secretion of TNF-α has been reported after stimulation with LPS, an effect that is abrogated by incubation with cycloheximide, indicating that *de novo* biosynthesis occurs (Palma *et al*, 1992). This synthesis stimulated by LPS is thought to act via CD14 through the binding of LPS to an LPS-binding protein (Haziot *et al*, 1993). TNF-α mRNA has also been detected in neutrophils following stimulation with GM-CSF (Quayle *et al*, 1994).

Treatment of neutrophils with IL-2, which can act via the constitutively expressed and functional IL-2Rβ, results in TNF-α expression (Wei *et al*, 1993). It has also been observed that following phagocytosis of yeast particles opsonised with IgG, neutrophils can synthesise and secrete a higher level of TNF-α than that seen with LPS (Bazzoni *et al*, 1991). Synthesis of TNF-α is also seen when neutrophils phagocytose non-opsonised yeast particles (Djeu *et al*, 1990). This is an important result which links phagocytosis to cytokine production. Phagocytosis of a pathogen could then allow the neutrophil to direct the subsequent immune response through the secretion of immunomodulatory cytokines.

1.7.4.4 Interleukin-6

IL-6 is a third pro-inflammatory cytokine which also shares some effects with IL-1β and TNF-α (Duff, 1993; Dinarello, 1991). It is important in the terminal differentiation

of B cells. In addition to these properties and also acts in synergy with IL-1 and IL-3 on haematopoietic progenitors.

There are conflicting reports regarding neutrophil synthesis of IL-6 with some authors suggesting that the expression seen is the result of monocyte contamination of cultures (Cassatella, 1995). GM-CSF and TNF-α have both been shown to induce IL-6 production in neutrophils although GM-CSF is much more effective (Cicco *et al*, 1990). Using a very sensitive PCR assay, Melani *et al* (1993) demonstrated constitutive transcription of IL-6 which was up-regulated by GM-CSF. IL-6 secretion has also been shown to be induced by LPS (Palma *et al*, 1992).

1.7.4.5 Interleukin-8

IL-8 is a member of the chemokine family of chemotactic cytokines. It is chemotactic mainly for neutrophils but also for a subset of T cells. Neutrophils do not constitutively express IL-8, but, when adherent to plastic *in vitro*, *de novo* synthesis of IL-8 occurs (Streiter *et al*, 1992). Synthesis of IL-8 is also seen in response to LPS (Fujishima *et al*, 1993) and this production is increased when C5a, FMLP or LTB_4 are also present, although these agents alone do not stimulate the synthesis of IL-8 (Strieter *et al*, 1992).

This transient production of IL-8 is also seen when cells are stimulated with IL-1β and TNF-α, with mRNA levels and protein secretion peaking at around 2 h (Strieter *et al*, 1992; Fujishima *et al*, 1993; Kasama *et al*, 1994). IFNγ stimultes IL-8 synthesis but this is thought to occur as a result of the secondary production and autocrine effects of TNF-α (Kasama *et al*, 1995). One group have shown that phagocytosis of yeast particles also induces the synthesis and secretion of IL-8 (Cassatella *et al*, 1993). In contrast to the results reported by Strieter and co-workers, Cassatella *et al* (1992)

showed IL-8 synthesis and secretion following stimulation with FMLP. It was found that concentrations sufficient to stimulate chemotaxis, but not the respiratory burst, were needed to induce this production. Hence, neutrophils once chemotactically stimulated could enhance the attraction of further neutrophils to an inflammatory site by the production of IL-8.

1.7.4.6 Other Cytokines

There are several other cytokines reported to be synthesised by neutrophils. These are TGF-β (Grotendorst *et al*, 1989), the potent monocyte chemoattractants MIP-1α and β (Kasama *et al*, 1994), IL-12 (Cassatella *et al*, 1995), IFNα (Cassatella, 1995), G-CSF and M-CSF (Lindemann *et al*, 1989). The production of IL-8, MIP-1α and β may allow neutrophils to control the infiltration of different types of leukocytes in either acute or chronic inflammation. This would allow the neutrophil to elicit a greater control over the immune response than has previously been thought possible.

1.7.5 Other Proteins

There are many other proteins that are synthesised by activated neutrophils which affect the immune response. The mitogens, Concanavalin A and PMA (only at low concentrations) stimulate the production of plasminogen activator (Lloyd & Oppenheim, 1992), whilst GM-CSF has been shown to increase *de novo* synthesis of the enzyme 5'-lipoxygenase (Pouliot *et al*, 1994). Neutrophils isolated from synovial fluid secrete fibronectin which promotes fibroblast adhesion to cartilage-collagen matrices and so may promote cartilage destruction. Under conditions of increased temperature, neutrophils will produce a range of heat shock proteins (Maridonneau-Parini *et al*, 1988).

1.8 APOPTOSIS

Two kinds of cell death exist that are morphologically and biochemically distinct from each other. The first and most common form of death is necrosis. In necrotic cell death, the cell dies as a result of sudden and severe trauma such as metabolic poisons, ischemia, complement attack and direct cell trauma (reviewed by Ueda & Shah, 1994). Necrosis is characterised by swelling of the cytoplasm and organelles, leading to eventual rupture of the plasma membrane and leakage of the cell contents.

The second type of cell death, apoptosis (derived from the Greek "to fall off" like leaves or petals) is characterised by condensation of the chromatin in the nucleus, shrinkage of the cell, fragmentation of the nucleus into membrane bound segments and budding of the plasma membrane to result in the formation of apoptotic bodies (Kerr *et al*, 1972). The chromatin is cleaved by an endogenous endonuclease into DNA fragments of 200 base pairs. This feature may be used as a biochemical marker for apoptosis as chromatin fragmentation can be detected as a ladder pattern on an agarose gel. Apoptosis is important in a range of biological processes including differentiation, development, cell maturation and also cell injury. Apoptotic cells are phagocytosed and so cell elimination occurs without damaging adjacent cells (reviewed by Stewart, 1994). It is also an important process in the shaping of the repertoire of B and T cells during development (reviewed by Williams, 1994).

1.8.1 Apoptosis Genes

Much attention has recently been focussed on elucidating the genetic control of apoptosis and many "death genes" have been identified in different systems. The very varied results suggest that the control of apoptosis differs between cell types. An important family of genes is the *bcl-2* family. It includes both apoptosis-promoting

and -inhibiting genes. Those that inhibit apoptosis are *bcl-2* (Stewart, 1994), *A1* (Lin *et al*, 1996), *mcl-1*, *bcl-x* and *bax*. *Bcl-x* has 3 alternatively spliced isoforms, $bcl\text{-}x_L$ and $bcl\text{-}x_{\Delta TM}$ which protect against apoptosis and $bcl\text{-}x_S$ which promotes apoptosis (Fang *et al*, 1995). The tumour suppressor gene *p53* is also involved in apoptosis. It is thought to induce apoptosis in cells with irrepairable DNA damage (reviewed by Payne *et al*, 1995). Other genes shown to have a role in apoptosis are *c-myc* (reviewed by Ueda & Shah, 1994) and *fas* (Iwai *et* al, 1994; reviewed by Lilies & Klebanoff, 1995; Clément & Stamenkovic, 1996), both of which promote apoptosis.

1.8.2 Intracellular Signalling in Regulation of Apoptosis

Little is known about the intracellular signalling pathways involved in the apoptosis pathway especially those involved in the regulation of DNA cleavage. Once again, the results obtained vary depending on the cell studied suggesting that there is no unique pathway which controls apoptosis. Ca^{2+} changes are thought to be involved, particularly in the activation of the endonuclease (McConkey *et* al, 1990; Kluck *et al*, 1994). Protein phosphorylation also seems to be important (reviewed by Gjertsen & Døkesland, 1995; Chen & Faller, 1996) as does the level of cAMP (McConkey *et al*, 1990; Rossi *et al*, 1995).

1.8.3 Neutrophil Apoptosis

The neutrophil has the shortest lifespan of all the leukocytes and undergoes apoptosis constitutively *in vitro* and *in vivo* unless rescued by exposure to inflammatory mediators. Neutrophils undergoing apoptosis lose expression of surface CD16 (FcγRIIIb), CD15, CD43 and CD62-ligand (Dransfield *et al*, 1994; Dransfield *et al*, 1995). Expression of the β_2-integrins is maintained but these receptors appear to be non-functional possibly due to dysregulation of their intracellular signalling pathways

(Dransfield *et al*, 1995). Many other neutrophil functions are down-regulated as the cells undergo apoptosis. These include degranulation, respiratory burst and chemotactic responses (Whyte *et al*, 1993). These changes have led to the hypothesis that the biochemical events associated with apoptosis may functionally inactivate the cell prior to its removal by scavenger phagocytes (Lilies & Klebanoff, 1995).

1.8.3.1 Genetic Control of Neutrophil Apoptosis

A number of studies have indicated that apoptosis in neutrophils is regulated differently to most other cell types. This may be because the neutrophil constitutively undergoes apoptosis while other cells must be stimulated to undergo apoptosis. Mature neutrophils do not express *bcl-2* hence it is not thought to influence apoptosis in these cells (Iwai *et al*, 1994). A more important gene is the *fas* gene. Human neutrophils are highly susceptible to *fas* mediated cell death (Lilies *et al*, 1994). The Fas Ligand (FasL) is expressed on the neutrophil cell surface but to a lesser extent on monocytes or eosinophils supporting the view that Fas is important in neutrophil apoptosis (Iwai *et al*, 1994). It is possible that constitutive co-expression of Fas and its ligand may commit neutrophils to rapid cell death and early studies suggest that this process may be modulated by G-CSF, glucocorticoids and tyrosine kinase inhibitors (Lilies *et al*, 1995). Another gene which may be important is the *ice* gene encoding the IL-1β converting enzyme. This gene is homologous to *ced-3*, one of the apoptosis genes from the nematode *C. elegans* (Yuan *et al*, 1993). As neutrophils are known to express *ice*, it may also play a role in neutrophil apoptosis (Ayala *et al*, 1994).

1.8.3.2 Intracellular Signalling in Neutrophil Apoptosis

Once again, a number of studies suggest that the signals which control apoptosis in other cells have different effects in neutrophils. Increases in intracellular Ca^{2+} which have been shown to promote apoptosis in many cells may actually inhibit neutrophil

apoptosis (Cousin *et al*, 1995; Whyte *et al*, 1993). An increase in cAMP has also been shown to inhibit neutrophil apoptosis, again in contrast to results for other cell types (Rossi *et al*, 1995). Protein synthesis, while important for the progression of apoptosis in many cells appears to be necessary for the inhibition of apoptosis in neutrophils (Takeda et al, 1992; Stringer *et al*, 1996).

1.8.3.3 Uptake of Apoptotic Neutrophils by Macrophages

Apoptotic neutrophils are recognised and phagocytosed by macrophages, a process thought to regulate the resolution of inflammation as neutrophils are removed from the site without leakage of their toxic contents (Savill *et* al, 1989; reviewed by Haslett, 1992). Recognition of neutrophils by macrophages appears to involve a negatively charged molecule on the neutrophil surface as it is inhibited by cationic molecules and decreased pH. Interestingly, a number of neutrophil products are cationic (eg. elastase) and the pH at sites of chronic inflammation is very low. Hence, the local microenvironment of a chronically inflamed site could inhibit the phagocytosis of apoptotic neutrophils (reviewed by Haslett, 1992).

The molecules on macrophages thought to be responsible for the recognition of apoptotic neutrophils are the integrin $\alpha_v\beta_3$, the vitronectin receptor and CD36 (a thrombospondin receptor) (Savill *et al*, 1990). It is thought that these receptors link via the bridging molecule thrombospondin to an as yet uncharacterised anionic molecule on neutrophils (Haslett *et al*, 1994). The neutrophils are then phagocytosed by the macrophage without leakage of their toxic contents. The usual response of the macrophage to phagocytosis is to release a large quantity of inflammatory mediators. However, the ingestion of apoptotic neutrophils fails to stimulate this response, even when the maximal number of neutrophils are ingested. If apoptotic neutrophils are cultured beyond apoptosis to a point where they fail to exclued Trypan blue, their subsequent ingestion by macrophages does produce a massive release of pro-

inflammatory mediators. This response is a function of the special mechanism by which these neutrophils are phagocytosed (reviewed by Haslett *et al*, 1995).

1.8.3.4 Factors Which Delay Neutrophil Apoptosis

Much research has centred on agents which are capable of delaying apoptosis in the neutrophil. This research has shown that many inflammatory mediators are able to delay the onset of apoptosis. IL-6 has been shown to delay apoptosis at concentrations that would be found at inflammatory sites (Biffl *et al*, 1996). Other cytokines able to delay apoptosis are G-CSF (Lilies *et al*, 1995), GM-CSF (Brach *et al*, 1992; Colotta *et al*, 1992; Yamamoto *et al*, 1993), IFNγ (Klebanoff *et al*, 1992), IL-2 (Pericle *et al*, 1994), and TNF-α (Colotta *et al*, 1992). However, one group has found that TNF-α actually increased the rate of apoptosis in human neutrophils in as short a time as 3 hours and the action of TNF-α remains controversial (Takeda *et al*, 1993).

There are other factors which are reported to improve neutrophil survival *in vitro* including LPS (Yamamoto *et al*, 1993), C5a, FMLP (Lee *et al*, 1993), an elevation in cAMP levels (Rossi *et al*, 1995) and glucocorticoids which have been found to accelerate apoptosis in thymocytes (Lilies *et al*, 1995). Interestingly, another factor which improves neutrophil survival is hypoxia (Hannah *et al*, 1995). This is of considerable relevance because the concentration of oxygen *in vivo* at inflammatory sites is very low.

1.9 RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a chronic inflammatory disease which affects the peripheral synovial joints and can additionally be associated with a multitude of extra-articular features eg. vasculitis, respiratory and ocular problems (Grennan, 1984).

Worldwide, approximately 1% of the population is afflicted with RA. Although disease onset can occur at any time from the teens through to old age, the peak onset is from 35-55 years in women and 40-60 years in men (Starkebaum, 1993; Grennan, 1984). RA is more common in women with a female preponderance over men of 3:1 although this is seen mostly in younger sufferers and the incidence becomes roughly equal between the sexes as age increases (Akil & Amos, 1995a).

1.9.1 Aetiology

Currently, the aetiology of RA remains unknown although there are several theories in existence. Genetic predisposition is a widely accepted theory and correlations have been found between the major histocompatability complex (MHC) class II gene haplotypes DR1 and DR4β and susceptibility to RA. The amino acid sequences of the peptide binding grooves are virtually identical (Starkebaum, 1993; McCulloch *et al*, 1993) and may be presenting similar arthritogenic peptides to T cells. The shared MHC epitope can also be found in 2 microbial proteins, Epstein Barr virus gp110 and dnaj, a heat shock protein of *Escherichia coli*. Exposure to the proteins of these ubiquitous organisms in an individual with the susceptible MHC molecule could lead to an autoimmune reaction through molecular mimicry (reviewed by Starkebaum, 1993; Klareskog *et al*, 1995).

An alternative theory suggests that RA is caused by a slow bacterial infection. Many clinical similarities have been noted between RA and chronic mycobacterial infections including autoantibody production, synovitis and systemic illness. *Mycobacterium tuberculosis*, while still continuing slow replication, can exhibit the characteristics of a stationary phase bacteria but will still be expressing 30-50 new proteins especially heat shock proteins and superantigens. Hence, it is thought that these bacteria, undetectable by standard histological techniques, could be the driving force behind the chronic inflammation of RA (McCulloch *et al*, 1993; reviewed by Bodman & Roitt, 1994).

None of these explanations can account for all cases of RA as only 30% have the gene haplotye associated with increased susceptibility. It is generally thought that a combination of genetic and environmental factors lead to the onset of disease.

1.9.2 Pathology

The initial stimulus for the onset of RA remains a mystery. Tissue samples from early RA are rare but those studied show no features to distinguish them from other forms of acute synovitis (Zvaifler, 1995). Initially, memory T cells are thought to enter the joint, and in response to an unknown antigen, secrete cytokines (IFNγ, IL-2, IL-4 and IL-6) stimulating lymphocyte proliferation, macrophage activation, and IgG and IgM secretion by B cells. Rheumatoid Factors which recognise other immunoglobulins form immune complexes (IC) leading to complement activation, which results in the release of anaphylatoxins (C3a) and chemotactic factors (C5a) which can activate neutrophils (Ehrengruber *et al*, 1994).

Vascular permeability increases allowing greater exudation of leukocytes and fibrinogen which leads to fibrin depostition and the activation of the fibrinolytic system. This, in turn, releases more pro-inflammatory mediators and destructive enzymes. The destructive tissue known as pannus is formed from the synovium and includes macrophage-like MHC class II^+ cells (Allard *et al*, 1990), which advances over the bone and cartilage. Enzyme release from pannus cells and neutrophils causes bone and cartilage destruction, and osteoclast activating factor from macrophages increases bone demineralization. Hence, bone and cartilage are gradually eroded and the joints become swollen, painful, deformed and non functional.

1.9.3 Effect of Cytokines in Rheumatoid Arthritis

The synovial fluid of the rheumatoid joint contains a range of inflammatory cytokines including IL-1β, TNF-α, IL-6, IL-8 and GM-CSF. These cytokines have varied effects on the cells present within the joint and contribute to the pathology (reviewed by Rafter, 1988; Koch *et al*, 1995b; Arend & Dayer, 1995). IL-1β and TNF-α stimulate chondrocyte GM-CSF production which in turn increases the proliferation of pannus cells (Alsalameh *et al*, 1994). Other effects of IL-1β and TNF-α include stimulation of the resorption of bone *in vitro* and a correlation with bone destruction (Panagakos *et al*, 1994; Neidel *et al*, 1995), stimulation of IL-8 synthesis by synovial fibroblasts and chondrocytes (Rathanaswami *et al*, 1993; Recklies & Gold, 1992), up-regulation of neutrophil adhesion (reviewed by Koch *et al*, 1995b) and the induction of synthesis of several other cytokines, including IL-1β itself (Seid *et al*, 1993).

In addition to the effects of IL-1 and TNF, IL-6 is present in the joint in sufficient quantities to activate the acute phase response (McNiff *et al*, 1995). Studies have shown that the amounts of IL-1, IL-6 and IL-8 present in the joint are very high and are all correlated (Bertazzolo *et al*, 1994). There are also anti-inflammatory cytokines present in the joint, including TGF-β which has been shown in animal models to reduce the effects of IL-1β (Rédini *et al*, 1993; van Beuningen *et al*, 1993). Also present is IL-10 which is found to be elevated in RA serum and synovial fluid (Cush *et al*, 1995) and when neutralized, results in an increase in the levels of IL-1β and TNF-α (Katsikis *et al*, 1994). IL-1ra is also found to be elevated in 80% of SF of RA patients (Malyak *et al*, 1993). Although many monokines are found to be present in rheumatoid SF, the

levels of T cell derived cytokines (such as IL-2, IL-3, IL-4, IFNγ) are found to be very low or undetectable (Firestein *et al*, 1988; Chen *et al*, 1993).

1.9.4 Treatment

There are many drug treatments available to RA patients which can alleviate symptoms, although they generally become ineffective with long term use and all have multiple side effects. Drugs used include non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, corticosteroids, antimalarials and gold compounds. When joint destruction becomes advanced, surgery to replace worn out joints can be performed (Akil & Amos, 1995b). There are now new therapies being tested including neutralizing the cytokine TNF-α (using anti-TNF-α antibodies) which is thought to be one of the major cytokines involved in disease pathology (Maini *et al*, 1995) and the use of recombinant IL-1RI (Drevlow *et al*, 1996).

1.10 THE ROLE OF THE NEUTROPHIL IN RHEUMATOID ARTHRITIS

The role of neutrophil granule enzymes and ROI in rheumatoid arthritis has been studied extensively. However, as it is now becoming apparent that the neutrophil can synthesise and secrete important inflammatory mediators including cytokines, perhaps more emphasis should be put on this process in the regulation of the afferent response in rheumatoid arthritis.

1.10.1 Immune Cells in the Rheumatoid Joint

The neutrophil is the most abundant cell present in the synovial fluid of rheumatoid arthritis patients and can account for up to 90% of the total cell numbers (Kitsis &

Weissmann, 1991). Neutrophils are attracted into the joint by the chemotactic factors such as the cytokine IL-8 or the complement fragment C5a or LTB_4, and may also be attracted by TGF-β as seen in rat models (Fava *et al*, 1991). It has been shown that neutrophils are primed and activated within the joint (Nurcombe *et al*, 1991a). Neutrophils in the joint have an activated phenotype, ie, low L-selectin, up-regulated CD11b (Humbría *et al*, 1994). Recent evidence suggests that neutrophils may be exposed to priming agents in the blood prior to entering the joint (Quayle *et al*, 1995). T cells, B cells and macrophages present within the joint are generally found in the synovial membrane rather than in the synovial fluid (Bodman & Roitt, 1994).

1.10.2 Neutrophil Mediated Tissue Damage

Neutrophils mediate tissue damage via their toxic arsenal of granule enzymes and reactive oxidants. Immune complexes are considered to be the major neutrophil activating factors present in the rheumatoid joint and neutrophils are thought to bind to and phagocytose immune complexes resulting in prostaglandin and leukotriene release (Crockett-Torabi *et al*, 1992), degranulation (Zhang *et al*, 1995b) and activation of the respiratory burst (De Clerck *et* al, 1991; Robinson *et* al, 1992; Robinson *et al*, 1993). Immune complexes have also been shown regulate selectin and integrin expression on neutrophils (Molad *et al*, 1994) and to induce chemotaxis of these cells (Trevani *et al*, 1995). The complement system is known to be activated in RA SF and correlates with neutrophil activation (Abbink *et al*, 1992). Receptors for immune complexes are up-regulated on synovial fluid neutrophils. These include the C1q receptor which binds C1q bearing immune complexes (Crockard *et al*, 1993) and the complement receptors CR1 and CR3 (Crockard *et al*, 1992).

It is also of note that the high affinity receptor for monomeric IgG is up-regulated in SF neutrophils (Watson *et al*, 1993). Another Fc receptor, FcγRIIIb (CD16) is found in soluble form in RA SF and may be shed from activated neutrophils (Fleit *et al*, 1992).

1.10.2.1 Release of Granule Enzymes

A process known as "regurgitation during feeding" can occur during neutrophil phagocytosis. In this process, the release of granule contents into the phagosome occurs before closure of the phagosome is complete. Hence, the granule contents and oxygen metabolites are released into the SF (reviewed by Pillinger & Abramson, 1995). It has been suggested that this persistent release of granule contents may propagate the formation of pannus.

However, it is unlikely that neutrophil granule contents released into the SF will come into contact with cartilage. There are several factors which can inhibit the granule contents, including anti-proteinases and the buffering capacity of SF proteins which can consume reactive oxygen metabolites before they can reach the cartilage. It has been shown that 2 of the anti-proteinases in SF α-1 antitrypsin and α-1 antichymotrypsin, have been inactivated to varying extents by proteolysis, possibly by neutrophil elastase and myeloperoxidase (Abbink *et al*, 1993). This may allow some activity of neutrophil enzymes to remain in SF, although they must first diffuse through the SF to the cartilage surface. It should also be noted that the granule enzyme, collagenase which is particularly important in the degradation of cartilage is secreted in an inactive form and requires activation by cathepsin G or HOCl. This is unlikely to be achieved in SF where the activators can readily diffuse away from the site of release. ROI can damage tissue directly. Superoxide can break down bovine SF and HOCl is able to inactivate anti-proteinases allowing granule enzymes to diffuse in an active form within the joint space (reviewed by Kitsis & Weissmann, 1991).

1.10.2.2 Adherence to Cartilage

Several groups have now demonstrated that neutrophils can adhere to cartilage *in vitro* via immune complexes which are trapped within the cartilage (Ugai *et al*, 1983; Chatham *et al*, 1993). The neutrophils will then attempt to phagocytose the cartilage and when unable to, will undergo frustrated phagocytosis and release the granule contents directly onto the cartilage surface. The space between the neutrophil and cartilage will be protected by the neutrophil membrane from SF proteinases. However, the limited space formed between the neutrophil and the surface will allow for the build-up of high local concentrations of granule enzymes and ROI. This will activate latent proteases and inactivate anti-proteinases. Elastase and cathepsin G can then degrade proteoglycan allowing collagenase access to its substrate, collagen. The proteases can also diffuse through the cartilage matrix to cause deep damage.

This theoretic sequence of events has been demonstrated in experimental models. Ugai *et al*, (1983) have shown that neutrophils incubated with antigen-induced arthritis cartilage containing trapped IC, adhered to the surface and then invaded the tissue. Electron micrographs showed that the neutrophils had discharged their granule contents. PMA activated neutrophils will degrade bovine cartilage *in vitro* and this activity is associated with released proteolytic enzymes (Moore *et al*, 1993). Others have observed that neutrophils incubated with SF will adhere to human cartilage and degrade both proteoglycan and collagen (Chatham *et al*, 1993). This adherence was found to be facilitated by immunoglobulins bound to the cartilage surface. GM-CSF has been shown to increase neutrophil adherence to, and subsequent destruction of, cartilage *in vitro* (Kowanko *et al*, 1991). The neutrophils may be attracted to the cartilage surface by the chemotactic complement fragment C5a, by IL-8 secreted by chondrocytes, or by the immune complexes themselves.

1.10.2.3 Arachidonic Acid Derivatives

In addition to granule enzymes and oxygen metabolites, neutrophils can contribute to the inflammatory reaction by the production of oxidation products of the arachidonic acid cascade, such as prostaglandins and leukotrienes. Prostaglandins are generally pro-inflammatory although PGE_2 may be anti-inflammatory. LTB_4 has a variety of effects on neutrophils including stimulation of chemotaxis and degranulation. The production of these lipid mediators can be induced by a number of stimuli including immune complexes (Crockett-Torabi *et al*, 1992), C5a, the Fc portion of immunoglobulins and LTB_4 itself (reviewed by Kitsis & Weissmann, 1991).

1.10.3 Neutrophil Derived Cytokines

There has been much recent interest in neutrophil production of cytokines, although little research has been carried out into the production of these cytokines in disease states. Neutrophil synthesis of cytokines may modulate the immune response in the rheumatoid joint. Recent research has demonstrated the synthesis of IL-1β (Quayle *et al*, 1995), Groα (Koch *et al*, 1995a), IL-8 and IL-1ra (Beaulieu & McColl, 1994) by SF neutrophils. It may be predicted that the neutrophil may also produce other cytokines under the inflammatory conditions of the rheumatoid joint, such as TNF-α, and IL-6 but this remains to be shown. The production of cytokines by neutrophils on a per cell basis, represents only around 10% of that seen in monocytes. However, as the numbers of neutrophils in the rheumatoid joint far exceed those of any other cell type, neutrophil derived synthesis of cytokines has the potential to be of pathological importance.

IL-1β is a very important cytokine in RA. It can induce the secretion of several other cytokines, including IL-1β, -6 and -8 and can induce bone and cartilage resorption by chondrocytes and osteoclasts (reviewed by Koch *et al*, 1995b; Duff, 1994). With respect to neutrophils, IL-1β may have autocrine effects including priming of the respiratory burst and stimulation of further IL-1β synthesis. In a murine model of RA, neutralization of IL-1 reduced neutrophil infiltration and cartilage degradation (van Lent *et al*, 1994).

Other cytokines present in the rheumatoid joint which may be produced by neutrophils have a pathogenic effect on the neutrophils themselves and other cells. TNF-α is a very important cytokine in the pathology of RA. It shares many effects with IL-1β including activation of osteoclasts and stimulating the production of other pro-inflammatory cytokines. It also primes and activates neutrophils, increases their ingress into the joint by up-regulating neutrophil adhesion to endothelial cells (Koch *et al*, 1995b) and increases phagocytosis of immune complexes (Fava *et al*, 1993).

There is no direct evidence for an action of IL-6 on neutrophils in RA although IL-6 levels have been shown to correlate strongly with lactoferrin which is released from activated neutrophils. This observation may suggest a link between IL-6 concentration and neutrophil activation (van Leeuwen *et al*, 1995). Also, IL-6 can induce neutrophil degranulation *in vitro* and correlates with levels of elastase-α_1–proteinase-inhibitor complex, a marker of degranulation, *in vivo* (Bank *et al*, 1995). GM-CSF has been shown to augment neutrophil adhesion to cartilage and its subsequent degradation (Kowanko & Ferrante, 1991). An increase in FcγRI expression on neutrophils *in vivo* has been demonstrated following treatment with IFNγ (Goulding *et al*, 1992). This will

increase the neutrophils ability to adhere to antibody coated cartilage. Finally, there are several chemokines which have been reported to be responsible for chemotaxis of neutrophils into the rheumatoid joint. These include IL-8 (Koch *et al*, 1995b), Groα (Koch *et al*, 1995a) and Epithelial Neutrophil Activating Peptide-78 (ENA-78) (Koch *et al*, 1994). IL-8 may can also chemotactically attract a subset of T cells. Both IL-8 and Groα are also synthesised by neutrophils while ENA-78 is synthesised mainly by synovial tissue macrophages.

1.10.4 Neutrophil Apoptosis in RA

Much interest has recently focussed on the role of apoptosis in the control of cell number and function within the immune system. The neutrophil is a cell which constitutively undergoes apoptosis unless rescued by a signal provided by inflammatory mediators. Hence, it may be predicted that neutrophils in the rheumatoid joint would have an enhanced survival rate due to the presence of high concentrations of cytokines, many of which have been shown to inhibit neutrophil apoptosis *in vitro*. A recent report which contradicts this theory has shown that peripheral blood or SF neutrophils incubated with either autologous or heterologous SF show increased rates of apoptosis in a dose dependent manner (Bell *et al*, 1995). It may be that the massive activation of neutrophils in the rheumatoid joint exhausts the cell leading to early apoptosis. There is evidence to suggest that proteolytic enzymes including trypsin, chymotrypsin and elastase may enhance apoptosis in neutrophils and that such a mechanism may be important in the resolution of inflammation (Trevani *et al*, 1996). Neutrophils contain elastase stored in granules and this is externalised in the rheumatoid joint. Hence, the neutrophil could enhance its own apoptosis through degranulation in an inflammatory situation.

The experiments carried out by Bell and co-workers (1995) were done under normoxic conditions and recent reports have demonstrated enhanced neutrophil survival under hypoxic conditions (Hannah *et al*, 1995). The oxygen concentration in the rheumatoid joint has been measured to be approximately 10-20% of that in air (Edwards *et al*, 1984). Hence, it may be that neutrophil survival could be prolonged in the rheumatoid joint because of the combined effects of a low concentration of oxygen and presence of cytokines. It has also been noted that a low pH inhibits the removal of apoptotic neutrophils by phagocytes (Haslett *et al*, 1994). As sites of chronic inflammation have a low pH, this clearance of apoptotic neutrophils may be inhibited in the rheumatoid joint. Hence, the neutrophils may go beyond apoptosis and either spill their toxic contents into the joint or eventually be phagocytosed by macrophages. They may induce a massive release of pro-inflammatory mediators from the macrophage which can cause more joint damage.

1.11 AIMS OF THE THESIS

The aims of this thesis were 4-fold:

1 To investigate the effects of synovial fluid and its components on the protein synthesis of neutrophils.

2 To investigate the synthesis and secretion of the cytokines IL-1β and TNF-α by both normal blood neutrophils and those isolated from the blood and SF of RA patients.

3 To investigate the priming effects of IL-1β and TNF-α upon normal blood neutrophils.

4 To study the effect of synovial fluid on neutrophil apoptosis.

CHAPTER 2

MATERIALS AND METHODS

2.1 PREPARATION OF NEUTROPHILS

2.1.1 Ficoll-Hypaque

Neutrophils were isolated from buffy coats kindly supplied by the Liverpool Blood Transfusion Service as described previously (Edwards & Swan, 1986). The majority of erythrocytes were removed by sedimentation (1g) in dextran T-501 (1% w/v). Remaining erythrocytes were eliminated by hypotonic lysis for 20s in distilled water with tonicity restored by the addition of NaCl to a final concentration of 0.9% (w/v). The cells were then washed once in PBS (150 mM NaCl, 10 mM K_2HPO_4, pH 7.4) and layered onto ficoll-hypaque (Pharmacia Biotech) and the neutrophils pelleted by centrifugation in an MSE Centaur 2 centrifuge at 1500 rpm for 30 min. Neutrophils were then washed once in PBS and centrifuged at 2000 rpm (600g) for 3 min to pellet them. The cells were then resuspended in RPMI 1640 medium (ICN) and counted using a Fuchs-Rosenthal Haemocytometer slide.

2.1.2 Neutrophil Isolation Medium

For smaller volumes of blood, the following method as described by (Gasmi *et al*, 1996) was used. Blood was layered onto Neutrophil Isolation Medium (Cardinal Associates Inc) and centrifuged at 600 g for 15 min. The upper layer of cells obtained on the gradient contained lymphocytes and monocytes and was discarded. The lower layer contained neutrophils and this was removed and washed once with RPMI. Any contaminating red blood cells were removed by hypotonic lysis as described above.

The cells were washed once more in RPMI and counted using a Fuchs-Rosenthal Haemocytometer slide. Cells were 99% neutrophils and >99% viable after purification.

2.2 PROTEIN LABELLING

Neutrophils at a density of $2x10^7$ cells/ml were first preincubated with 60 μCi/ml ^{35}S-methionine (Amersham, ICN) for 15 min at 37°C. Following this, either GM-CSF (final concentration 14 ng/ml, 50 U/ml), cell free synovial fluid (SF), fractions of SF (see 2.5, 5% v/v) or synthetic immune complexes (see 2.4, 10% v/v) were added. Incubation was continued at 37°C with gentle agitation for times of up to 24 h. Control cells contained no additions. All cell suspensions also contained 0.1% w/v BSA.

2.3 PROTEIN BIOSYNTHESIS

^{35}S-methionine incorporation into TCA-precipitable proteins was measured by scintillation counting, during incubation, as described previously (Hughes *et al* 1987). Briefly, at selected time points triplicate samples of $2x10^6$ cells were removed from the cell cultures and added to 2 ml casein hydrolysate (4% w/v casein hydrolysate in 10% w/v TCA) and incubated at 4°C overnight. Precipitated proteins were filtered onto Whatman GF/A filters, washed with 70% (v/v) ethanol followed by 10% w/v TCA and finally again with 70% (v/v) ethanol. The filters were then dried, placed in scintillation vials and 4ml of scintillation fluid added (BDH Scintran - Cocktail T). Radioactivity in the TCA precipitates was measured using a Wallac 1219 RACKBETA Scintillation Counter.

2.4 PREPARATION OF IMMUNE COMPLEXES

2.4.1 Titration of Antibody and Antigen

Anti-human serum albumin (HSA, Dako) was dialysed overnight to remove preservatives in 2-3 l of PBS with one change of buffer then diluted to a concentration of 5 mg/ml total protein with PBS. The HSA protein (Sigma) was diluted to 5 mg/ml with PBS. Immune complexes were then made as described previously (Crockett-Torabi & Fantone, 1990). The antibody was then titrated against the HSA protein in a round bottomed microtitre plate and incubated for 1 h at 37°C with gentle agitation to allow the formation of immune complexes (IC). The absorption of each sample was measured at 450 nm on a Bio-Rad model 3550 Microplate Reader.

2.4.2 Synthesis of Immune Complexes

The ratio of antibody to antigen at equivalence point (the highest absorbance reading on the microplate reader) was used to form insoluble immune complexes. Soluble immune complexes were then formed at a 6 fold antigen excess than required to form insoluble IC. The appropriate amounts of antibody and antigen were added to 15 ml Falcon tubes and incubated for 1 h at 37°C. The insoluble IC were washed 3 times with PBS and the soluble IC were centrifuged at 3000 rpm (1060 g) for 5 min to pellet any contaminating insoluble IC. The soluble IC containing supernatant was retained. Both types of IC were stored at 4°C until use.

2.5 PREPARATION OF SYNOVIAL FLUID

2.5.1 Removal of Cells from Synovial Fluid

Synovial fluid samples from patients with a variety of arthritic diseases were kindly supplied by Dr R C Bucknall, Royal Liverpool University Hospital. The fluids were first filtered through gauze to remove any clumps of cells then centrifuged at 2000rpm for 15 min. The supernatant of cell free synovial fluid was retained and stored in aliquots at -20°C until use.

2.5.2 Fractioning Synovial Fluid

To pellet insoluble IC from cell free synovial fluid, the fluid was spun in a microfuge at 13000 rpm (9450 g) for 10 min. The pellet of insoluble IC was resuspended in the same volume of PBS immediately prior to use to minimalize resolublization of the IC. The supernatant was either untreated to retain soluble IC or depleted entirely of IC by incubation with 150 µl Pansorbin ® (Calbiochem) per 1 ml of fluid and incubating at 4°C for 1 h. The Pansorbin was pelleted by centrifugation in a microfuge at 13000 rpm and the supernatant stored at 4°C until use. The removal of soluble and insoluble IC was assessed by measuring neutrophil chemiluminescence (2.8). These 2 types of IC activate neutrophils with distinct kinetics (Robinson *et al*, 1993).

2.6 IMMUNOPRECIPITATION

IL-1β and TNF-α were immunoprecipitated from cell pellets and culture supernatants as previously described (Perregaux *et al*, 1992). Briefly, cell pellets were initially lysed in lysis buffer (10 mM Tris, pH 7.2, 5 mM EDTA, 0.15 M NaCl, 1% v/v Nonidet P40, 1 mM PMSF, 3 µl/ml protease inhibitor cocktail) then both pellets and culture

supernatants were treated in the same way. Firstly, samples were incubated with 25 µl of Pansorbin® (*Staphylococcus aureus* bacteria coated with protein A, Calbiochem) on ice for 10 min to remove proteins binding non-specifically. They were then centrifuged at 13000 rpm for 3 min to pellet the Pansorbin® which was discarded. The remaining supernatant was incubated at 4°C for 2 h with 1 µl of a 1:10 dilution of sheep anti-IL-1β antibody or sheep anti-TNF-α antibody (NIBSC). Following this 75 µl of Pansorbin® was added and incubation continued on ice for a further 30 min. Samples were centrifuged to pellet the Pansorbin® and the pellet washed 5 times with wash solution (10 mM Tris, pH 8.0, 10 mM EDTA, 0.4% w/v deoxycholic acid, 1% v/v Triton X-100, 0.1% w/v SDS) and once with 50 mM Tris, pH 6.8. The pellet was then resusupended in SDS-PAGE sample buffer, boiled for 3min and the resulting suspension centrifuged to remove the Pansorbin®. The supernatant was loaded onto an SDS-PAGE gel.

2.7 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

2.7.1 One Dimensional SDS-PAGE

Samples of ^{35}S-labelled cells were solublized in SDS sample buffer (12.5 mM Tris, pH 6.8, 8% v/v glycerol, 2.3% w/v SDS, 5% β-mercaptoethanol, trace of bromophenol blue), heated in a boiling water bath for 3-5 min and then centrifuged for 3 min at 13000 rpm to remove any particulate matter. The samples were then loaded on an SDS slab gel (Laemmli, 1970) of either 12% or 15% acrylamide with a stacking gel of 3% acrylamide and electrophoresed at 35 mA per gel in a Bio-Rad Protean II xi gel kit or a Bio-Rad mini Protean II gel kit. Gels were fixed overnight in 40% v/v methanol, 10%

v/v acetic acid with gentle agitation, washed briefly in 10% v/v ethanol for 10 min and submerged in a fluorographic reagent (Amplify, Amersham) for 1 h. Gels were then dried onto filter paper using a Bio-Rad model 443 slab dryer at 60°C and exposed to preflashed Fuji X-ray film at -80°C for 1-4 weeks. Molecular mass markers used were ^{14}C-labelled (Gibco, Amersham).

2.7.2 Two Dimensional SDS-PAGE

Samples of ^{35}S-labelled cells were first solublized in urea sample buffer (9 M urea, 2% v/v Nonidet P40, 2% w/v CHAPS, 5% v/v ampholytes (pH3.5-10), 5% v/v β-mercaptoethanol). 10 µl PMSF (15 mg/ml in ethanol) was added per 100 µl sample buffer. The samples were vortexed and centrifuged at 13000 rpm for 3 min. Isoelectric focussing was carried out as described by O'Farrell (1975). Samples were applied to the top of an IEF tube gel (9 M urea, 3% w/v acrylamide/PDA (ratio of 24:1, w/w), 2% w/v CHAPS, 2% v/v Nonidet P40, 5% v/v ampholytes (pH 5-8 and pH 3.5-10), 5% w/v APS, 0.2% v/v TEMED). Anolyte (0.01M H_3PO_4) was placed in the bottom of the tank and catolyte (0.1 M NaOH) in the upper buffer chamber and electrophoresis was carried out at 400 V for 4 h and 1000 V for 10 min. The gels were then extruded from the capillary tubes and equilibrated in SDS-PAGE sample buffer for 15min before being laid along the top of a 13% slab gel and electrophoresed as for 1D SDS-PAGE. The gels were then treated in the same way as 1D gels and exposed to film.

2.8 MEASUREMENT OF CHEMILUMIINESCENCE

Freshly isolated cells at a concentration of $1x10^7$/ml were incubated with GM-CSF (14 ng/ml), IL-1β (5,10 and 25 ng/ml) and TNF-α (10, 25 and 50 ng/ml) for periods of up to 1 h to prime the cells. After priming, the oxidase activity in $5x10^5$ cells was assayed. Luminol was added to the cells (final concentration of 10 µM) followed by 10% v/v

soluble or insoluble immune complexes (as described in 2.4.2). The final volume assayed was 1 ml. Chemiluminescence was measured on an LKB Wallac 1251 Luminometer and the results plotted in graph form.

2.9 MEASUREMENT OF INTRACELLULAR Ca^{2+}

2.9.1 Preparation of Neutrophils

Before use, neutrophils were loaded with the Ca^{2+} indicator dye Fluo-3 acetomethoxy ester. The freshly isolated cells were resuspended at a concentration of $1x10^7$ cells/ml in Ca^{2+} free Hepes buffer (145mM NaCl, 5mM Kcl, 1mM Na_2HPO_4, 0.5mM $MgSO_4.7H_2O$, 20mM HEPES) and incubated with gentle agitation at 37°C with 2 μM Fluo-3 for 30 min. The cells were then washed twice in Ca^{2+} free Hepes buffer and resuspended in either Ca^{2+} free buffer (for experiments designed to measure changes in Ca^{2+} in the absence of extracellular Ca^{2+}) or in Hepes buffer containing 1 mM Ca^{2+} (for experiments to measure changes in the presence of extracellular Ca^{2+}).

2.9.3 Measurement of Ca^{2+} Changes

The measurement of the fluorescence of Fluo-3 was carried out as described by Merrit *et al*, 1990 using a Perkin Elmer 3000 Fluorescence Spectrometer. Briefly, the fluorimeter was set to read excitation at 505 nm and emission at 530 nm and was blanked using 3 ml of Hepes buffer. $2x10^6$ cells in a final volume of 3 ml were added to the cuvette and the resting fluorescence trace of the intracellular Ca^{2+} level measured. Following this, the stimulus was added and the change in Ca^{2+} levels was measured. Stimuli used were fMLP (final concentration 100 nM) and soluble immune complexes (6.67% v/v). The final volume was made up with either Ca^{2+} free Hepes buffer or buffer containing 1 mM Ca^{2+}. Calibration of fluorescence changes with Ca^{2+} levels was performed as described by Merrit *et al*, (1990).

2.10 NEUTROPHIL INCUBATIONS FOR APOPTOSIS ASSAY

Neutrophils were isolated as described in 2.1.2 and resuspended at a final concentration of $5x10^6$ cells/ml in RPMI 1640. The neutrophils were then incubated in the presence or absence of synovial fluid (5-75% v/v), superoxide dismutase (250, 500 μg/ml) and catalase (200, 400 μg/ml) for 18 h at 37°C with gentle agitation. All samples also contained foetal calf serum (5% v/v) and L-Glutamine (2 mM).

2.11 CYTOSPINS

Following incubation as described in 2.10, $1.5x10^5$ cells were spun onto slides using a Shandon cytospin centrifuge. The slides were allowed to dry in air at room temperature and then stained using May-Grünwald and Giemsa stains for 10 and 5 min respectively. The slides were washed briefly in distilled water and allowed to dry at room temperature. Finally, coverslips were affixed to the slides using DPX mountant. The number of apoptotic and non apoptotic cells were counted in random fields on a Zeiss microscope at a magnification of x25. Apoptotic cells were defined as smaller round cells with a condensed, darkly staining nucleus and non apoptotic cells were defined as larger cells with the characteristic multi-lobed nucleus and lighter staining. A total number of 500 cells were counted on each slide.

2.12 CHEMILUMINESCENCE AS A MARKER OF APOPTOSIS

Following incubation as described in 2.10, the NADPH oxidase activity of the neutrophils was measured using an LKB Wallac 1251 Luminometer. The chemiluminescence was measured in a final volume of 1 ml containing $5x10^5$ cells and a final concentration of luminol of 10 μM. To stimulate oxidase activity either PMA (0.1

μg/ml) or fMLP (1 μM) were added and the chemiluminescence generated by the reaction of oxidants with luminol was measured.

CHAPTER 3

NEUTROPHIL PROTEIN SYNTHESIS

3.1 INTRODUCTION

The neutrophil has long been described as a terminally differentiated cell with little or no capacity for mRNA or protein synthesis (Cline, 1966). However, recent work has shown the neutrophil can, in fact, synthesise a range of proteins both constitutively, i.e. housekeeping genes and in response to inflammatory stimuli (Granelli-Piperno *et al*, 1979; Jack & Fearon, 1988). It has also been demonstrated that this protein synthesis is selective. For example, the labelling of certain proteins by ^{35}S-methionine can increase 1-2 fold following stimulus, while the labelling of others increases by 10-20 fold (Hughes *et al*, 1987; Edwards *et al*, 1990). This indicates differential control of gene expression during priming and activation of neutrophils.

Many authors have reported biosynthesis of many different proteins by neutrophils. Among those proteins now known to be synthesised by neutrophils are cytokines, components of the NADPH oxidase and several housekeeping proteins such as actin (reviewed by Edwards, 1994). In response to inflammatory stimuli, neutrophils increase synthesis and secrete several cytokines. Amongst the secreted cytokines are IL-1β (Tiku *et al*, 1986), TNF-α (Djeu *et al*, 1990), IL-8 (Fujishima *et al*, 1993), G-CSF, M-CSF, TGF-β (Grotendorst *et al*, 1989) and IFNα (Shirafuji *et al*, 1990). These cytokines are potentially important in RA because the synthesis of oxidase components could lead to a more sustained respiratory burst thereby causing more oxidative damage within joints. Each of the cytokines mentioned are known to be

present in the rheumatoid joint, although their presence is attributed to macrophages or synoviocytes. Neutrophil protein synthesis has not been extensively studied in rheumatoid arthritis. There are many potential neutrophil stimulating agents present in the synovial fluid, which include cytokines, such as those mentioned above (reviewed by Rafter, 1988), complement fragments such as C5a (Ehrengruber *et al*, 1994), and immune complexes.

Immune complexes are thought to be the most potent neutrophil activating agents present in the SF of RA patients (Robinson *et al*, 1993; Crockett-Torabi *et al*, 1992). There are 2 types of IC, present within these fluids, namely soluble and insoluble complexes. The soluble IC are probably dimers and trimers, whilst insoluble IC are much larger and are easily removed from solution by centrifugation. The effects of these 2 types of IC on the respiratory burst of the neutrophil have been studied (Robinson *et al*, 1992a). Insoluble IC are able to activate the respiratory burst in resting neutrophils (Robinson *et* al, 1992b) while soluble IC are only able to activate the oxidase in neutrophils which have been previously primed (Robinson *et al*, 1992a; Robinson *et al*, 1993). This finding suggests that the 2 types of complexes act via different receptor signalling pathways to achieve their effects. Soluble IC act via a G protein dependent, protein kinase C independent mechanism while insoluble IC act largely via a G protein independent, PKC dependent pathway (Robinson *et al*, 1994).

These IgG-containing IC will activate neutrophils following occupancy of Fcγ receptors (FcγR). FcγRII is thought to play a major role during phagocytosis of large particles, such as opsonised bacteria or fungi. Conversely, it is generally believed, FcγRIIIb ligation does not initiate phagocytosis or the respiratory burst. FcγRIIIb is a GPI-linked protein and does not have an intracellular domain, which has cast some doubt on whether the receptor can transduce signals into the cell, although it has been shown that

occupation of the receptor does induce intracellular Ca^{2+} transients (reviewed by Edwards, 1994). Other groups have suggested that the function of FcγRIIIb is to bind complexes and present them to FcγRII, which will then send a signal into the cell leading to activation (Brunkhorst *et al*, 1992; Moser *et al*, 1995; reviewed by Indik *et al*, 1995).

In the experiments presented in this Chapter, I have studied the effects of SF from RA patients on the activation of protein biosynthesis by control, blood neutrophils. The experiments are designed to mimic the events that occur when blood neutrophils are recruited into joints and are exposed to the complex mixture of activating factors present in SF. In addition to this, I have studied the effects of the immune complexes on neutrophil protein synthesis. Initially, this was done using synthetic IC because it is difficult to isolate soluble IC from SF in a pure form. I have also studied the effects of fractioned synovial fluid in which both soluble and insoluble immune complexes were depeleted from the SF of RA patients, and used separately to activate neutrophil protein synthesis.

The neutrophil is an important cell in RA. It is the most abundant cell in SF, accounting for up to 90% of the total cell numbers (Kitsis & Weissmann, 1991). There is also a constant influx of fresh neutrophils into the joint. This is an unusual occurence in a chronic inflammatory condition where the initial influx of neutrophils in the acute stage is replaced by macrophage recruitment as the inflammation becomes chronic. Thus, there must be some factors which continue to attract neutrophils into the joint and this factor may be a neutrophil product.

The rate of cytokine synthesis by neutrophils on a per cell basis is approximately 10% of that observed by macrophages. However, there are very few macrophages present in the synovial fluid and an enormous population of neutrophils. Hence, any synthesis

of pro-inflammatory proteins by neutrophils will be significant because of their large numbers within joints. If the proteins produced in the neutrophil in response to infiltration into the rheumatoid joint have pro-inflammatory properties, (eg cytokines, receptors and oxidase components), this may have an important effect on the progress of the immune response in RA. If changes in protein synthesis are observed, then this would indicate that the neutrophil could play a central role in directing the progress of the inflammatory events in this disease.

3.2 RESULTS

3.2.1 *Incorporation of radiolabel into newly synthesised neutrophil proteins.*

Rheumatoid SF contains many cytokines and other proinflammatory agents which are capable of activating the neutrophil oxidase and stimulating degranulation. Hence, it is possible that these agents may also stimulate protein synthesis in neutrophils. Measurement of radiolabel (^{35}S-methionine) incorporation into proteins gives a quantifiable method to assess the degree of protein synthesis occuring. Neutrophils were therefore incubated with ^{35}S-methionine for 24 h in the absence (control) and presence of GM-CSF (positive control) or cell free synovial fluid. Each sample was measured in triplicate and errors are shown. Figure 3.1 shows a histogram of the incorporation of ^{35}S-methionine into TCA precipitable proteins of neutrophils incubated with GM-CSF (14 ng/ml) or with cell free SF (5% v/v) from 12 different patients (patients 1-7 had RA, patient 8 had RA and ankylosing spondylitis, patients 9-12 had other inflammatory arthritis conditions). In each case, control levels were taken to be 100% synthesis and the synthesis of protein induced by SF and GM-CSF calculated as a percentage of the control value. In all samples of SF tested, the amount of protein synthesis as measured by radiolabel incorporation was greater than the control level although there were wide variations in the potency of different SFs. GM-CSF was used as a positive control and the amount of radiolabel incorporated into GM-CSF stimulated cells is comparable with that of SF stimulated cells.

3.2.2 *Analysis of proteins synthesised by neutrophils incubated with SF*

3.2.2.1 *2-dimensional SDS-PAGE*

In order to study more closely the proteins being synthesised by neutrophils and to obtain some information on their molecular properties, 2D gels of radiolabelled proteins

were analysed. This technique gives better separation of proteins than 1D gels as it is able to separate proteins on the basis of both their size and their isoelectric point. These experiments were repeated 4 times, with reproducible results. Figure 3.2 shows fluorographs of 2-dimensional SDS-PAGE gels of radiolabelled proteins from experiments in which neutrophils were incubated with GM-CSF or SF, as in 3.2.1 above. These experiments indicate 2 important features. First, protein synthesis is elevated above control values in neutrophils incubated with SF and GM-CSF. Second, it can be seen that the pattern of protein biosynthesis seen with both SF and GM-CSF is extremely similar. It can also be noted that certain proteins, indicated with arrows, have increased markedly in labelling compared to others which have only increased in labelling by a small amount, or not at all, following stimulation. There are several proteins which were undetectable in control cells and may not be synthesised at all by unstimulated blood cells.

It was then necessary to determine which of these newly-labelled proteins were cell associated, (and hence likely to be internal in neutrophil function) and those which are secreted and hence likely to play extracellular roles. Figure 3.3 shows fluorographs of the 2D gels on which radiolabelled proteins present in culture supernatants were analysed. Once again, there are more proteins secreted by cells incubated with SF or GM-CSF compared to control. The cells incubated with SF have all secreted 2 particular proteins marked by the arrows which have low molecular weights of similar value (approximately 20-25 kDa) and are not seen secreted by control cells or by cells incubated with GM-CSF. These proteins may thus represent neutrophil products that are secreted into diseased rheumatoid joints.

3.2.3 *Effect of synthetic immune complexes on neutrophil protein synthesis and secretion.*

Immune complexes are thought to be the major neutrophil stimulating agent present in synovial fluid which activate the respiratory burst and degranulation (DeClerck *et al*, 1991; Zhang *et al*, 1995). Synthetic soluble and insoluble IC (made from HSA and anti-HSA antibodies, as described in 2.4) have been shown to activate neutrophils by analogous processes to those by which SF IC act (Robinson *et al*, 1993, 1994). Therefore, the ability of synthetic IC to stimulate neutrophil biosynthesis was investigated.

3.2.3.1 *Incorporation of radiolabel into newly synthesised proteins.*

Neutrophils were incubated for 4 h and 24 h in the absence (control) and presence of soluble or insoluble IC in medium containing ^{35}S-methionine. Incorporation of radiolabelled TCA-precipitable proteins was measured by scintillation counting and compared with that obtained following incubation with GM-CSF. Each sample was measured in triplicate and error bars are shown. Figure 3.4 shows a graph depicting the amounts of radiolabel incorporated into proteins synthesised by neutrophils in these experiments. At 4 h, the amount of ^{35}S-methionine incorporated into newly synthesised proteins was only slightly higher after incubation with soluble or insoluble IC compared with control. However, at 24 h, cells stimulated with soluble IC had much higher levels of protein biosynthesis than in both controls and insoluble IC stimulated suspensions. This figure is representative of 5 experiments. The soluble IC always stimulated protein synthesis to levels at least 2-fold greater than in control cells and in some cases as much as 12x. Insoluble IC did not differ significantly from control cells, maximally stimulating only around 1.3x the amount of protein synthesis of controls. The level of protein synthesis seen in GM-CSF stimulated cells was generally greater

than that seen with insoluble IC stimulated cells, usually varying between 1.5-fold and 2-fold greater than control.

3.2.3.2 *1-Dimensional SDS-PAGE*

Whilst soluble and insoluble IC exhibited marked quantitative differences in their ability to stimulate protein biosynthesis, it was necessary to determine if there were any qualitative differences in biosynthetic profiles. Figure 3.5 shows fluorographs of 1D gels on which newly-synthesised proteins both cell associated and secreted by neutrophils after 24 h incubation have been analysed. Three identical samples of each stimulus from the same experiment have been analysed on this gel and the degree of biological variation can clearly be seen between samples. They closely parallel the experiments measuring radiolabel incorporation. Soluble IC induced a much greater degree of protein synthesis than either control or insoluble IC stimulated cells. Of particular interest is the high level of secreted proteins following activation by soluble IC. Cells incubated with insoluble IC show a greater degree of protein synthesis than control cells, but far less than was observed with soluble IC.

3.2.3.3 *2-Dimensional SDS-PAGE.*

Figure 3.6 shows fluorographs of 2D gels on which cell associated proteins from neutrophils incubated with GM-CSF or IC were separated. The fluorographs show the proteins synthesised at 4 h and 24 h. Even at 4 h, it can be seen that soluble IC induced a greater amount of protein synthesis than was seen in control suspensions and during incubation with insoluble IC. However, at this time point, protein biosynthesis stimulated by soluble IC was comparable with that induced by GM-CSF. At 24 h, the amount of protein synthesis seen with soluble IC stimulated cells increased dramatically and was greater even than in suspensions treated with GM-CSF. Insoluble

IC stimulated cells also showed an increased level of protein synthesis that was approximately equal to that observed following treatment with GM-CSF.

Figure 3.7 shows fluorographs of radiolabelled, secreted proteins, following incubation of suspensions for 4 h and 24 h. Even by 4 h incubation, the amount of radiolabelled proteins secreted by soluble IC stimulated neutrophils was much greater than that in conrol suspensions, or after incubation with GM-CSF or insoluble IC. Again, this difference was more marked at 24 h. The above experiments have been repeated 4 times with reproducible results.

3.2.4 *Comparison of synthetic immune complexes and those isolated from synovial fluid.*

Having established that synthetic IC (particularly soluble IC) could stimulate neutrophil biosynthesis, it was then necessary to compare these effects with those of complexes present in rheumatoid SF. This would add strength to the idea that activated neutrophil biosynthesis is important *in vivo*. It was impossible to remove soluble IC from SF and to determine their effects *in vitro* because once removed from the fluid by affinity chromatography, the antigen:antibody ratio is disturbed and the properties of the complexes change (Robinson, 1994). Hence, I have sequentially removed insoluble and soluble IC from SF and determined the effects of the depleted SF on protein biosynthesis. I thus isolated insoluble IC from SF (by centrifugation) then used the remaining SF containing both soluble IC and the other components of SF (eg cytokines) in labelling experiments. I then depleted SF of all IC (by adsorption with Pansorbin, 2.5.2) incubated neutrophils with this fraction. SF depleted of all IC may stimulate biosynthesis via the cytokines (and perhaps other undefined factors) contained within it. These experiments have been repeated 3 times with reproducible results.

3.2.4.1 *Incorporation of radiolabel into newly synthesised proteins.*

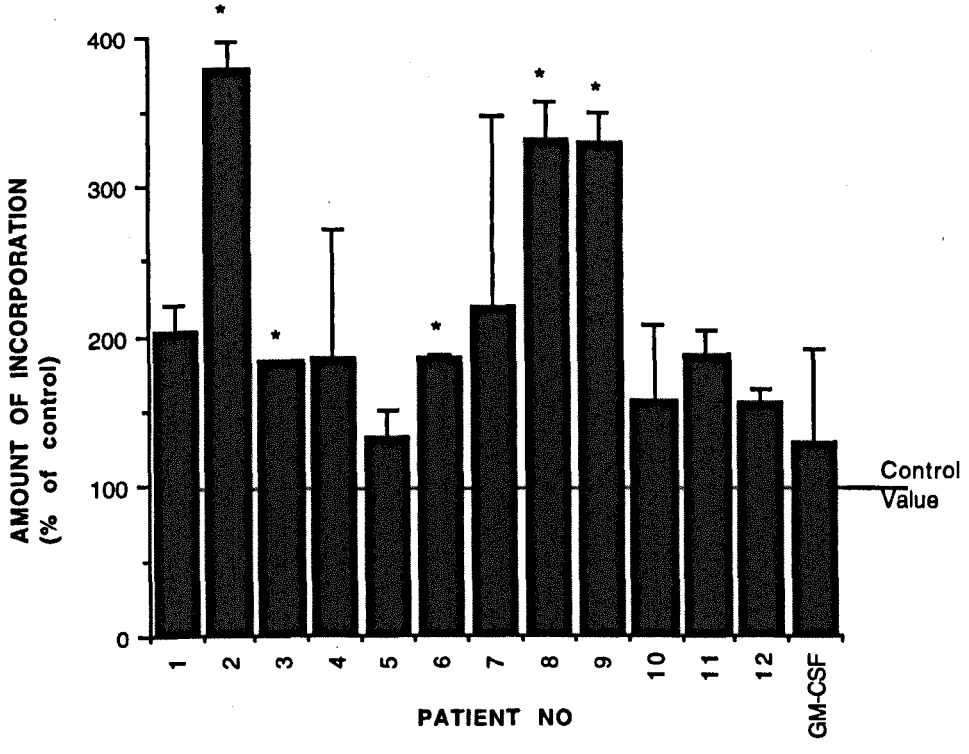
Figure 3.8 shows a graph of the amount of ^{35}S-methionine incorporated into newly-synthesised proteins by neutrophils incubated with different fractions of synovial fluid and synthetic IC. These results suggest that immune complexes within SF activate neutrophils in similar ways as synthetic IC. The amount of radiolabel incorporated into neutrophil proteins stimulated by soluble IC was very similar to that by cells incubated with SF containing only soluble IC (ie SF depleted of insoluble IC). When soluble IC are also removed from SF, then the level of incorporation decreased slightly to 67.5% (± 7.8%) of the level observed in cells incubated with SF containing soluble IC. Insoluble IC from SF and synthetic insoluble complexes induce similar, low levels of protein synthesis as observed before. Whole, unfractioned SF, induced a greater level of protein synthesis than control cells but this was slightly less than that stimulated by soluble IC, both synthetic and from SF.

3.4.2.2 *1-Dimensional SDS-PAGE.*

Figure 3.9 shows fluorographs of SDS-PAGE gels on which newly-radiolabelled proteins both cell associated and secreted by neutrophils after 4 h and 24 h incubation have been analysed. These results parallel measurements of radiolabel incorporation shown in Figure 3.8. After 4 h incubation, protein synthesis levels in all cells was similar. However, at 24 h, the cells incubated with synthetic soluble IC and SF containing only soluble IC (ie depleted of insoluble IC) have synthesised and secreted greater amounts of proteins. It can be noted that the pattern of synthesis was also very similar. Neutrophils incubated with whole SF also showed a similar pattern of protein synthesis to those incubated with soluble IC, both synthetic and SF-derived. Both types of insoluble IC induce only low levels of protein synthesis. SF depleted of all IC showed a similar level of protein synthesis as that seen with whole SF. Hence,

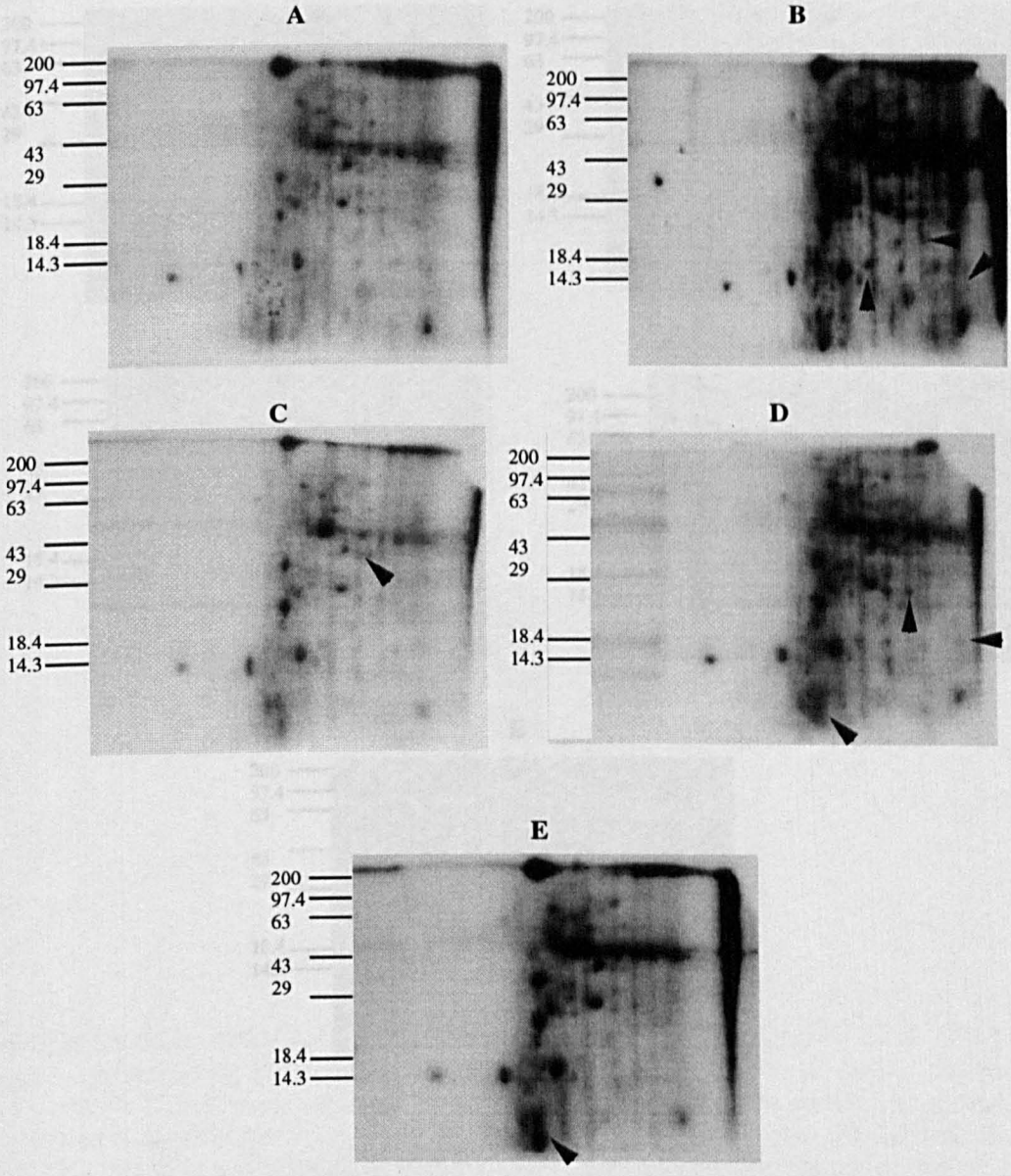
synovial fluid cytokines may play a more important role in the induction of neutrophil biosynthesis and IC may play only a minor role.

FIGURE 3.1: INCORPORATION OF ^{35}S-METHIONINE INTO PROTEINS SYNTHESISED BY NEUTROPHILS INCUBATED WITH SYNOVIAL FLUID



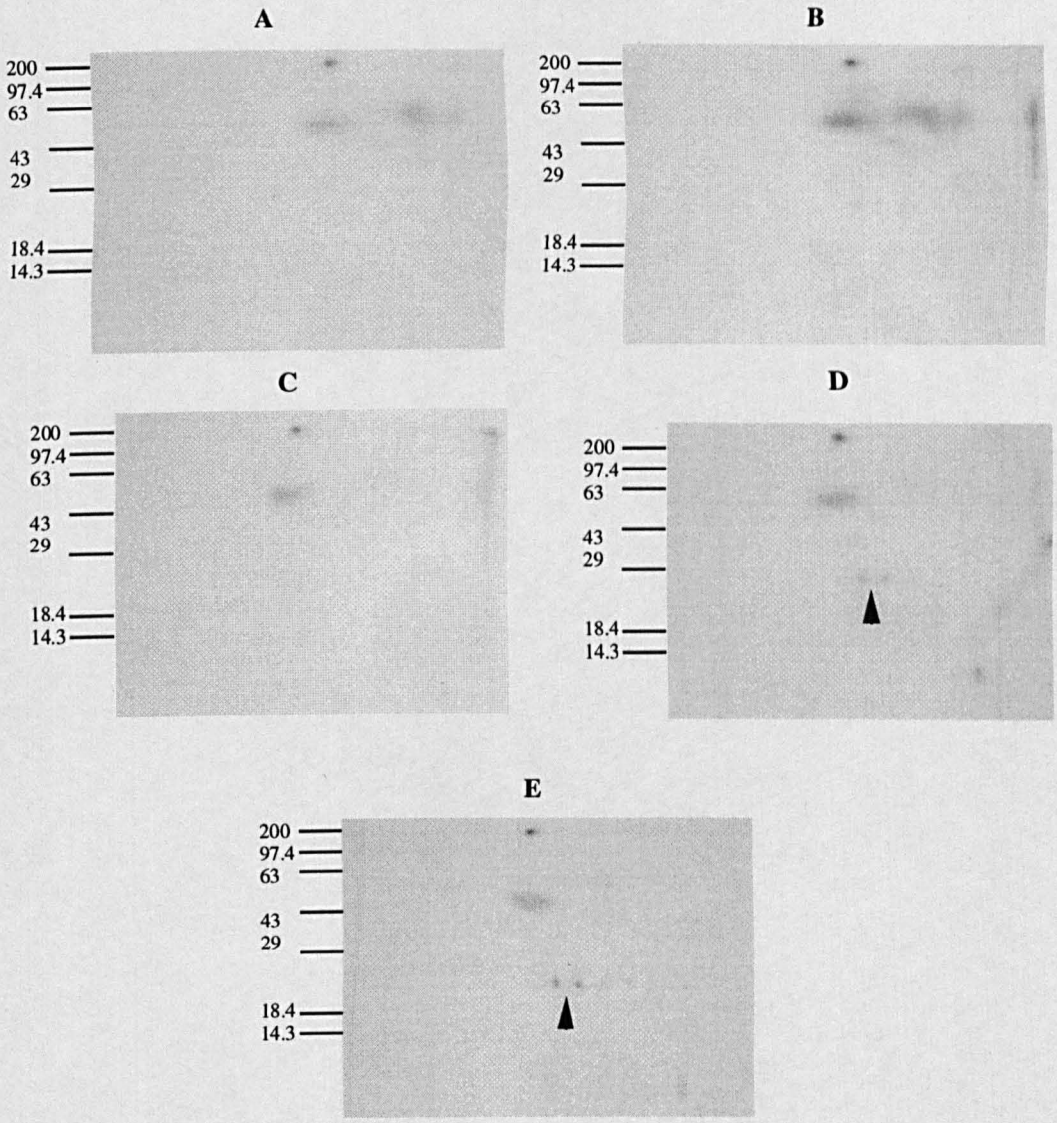
Neutrophils were incubated in the presence of 60 μCi/ml ^{35}S-methionine and in the presence and absence of cell free synovial fluid obtained from the knee joints of patients with inflammatory joint disease. Following incubation for 24 h at 37°C, triplicate samples of 2 x 10^6 cells were removed and the amount of radiolabel incorporated into TCA precipitable proteins was measured using a scintillation counter, as described in 2.3. The amount of radiolabel incorporated into control cells was taken to be 100% and that incorporated into stimulated cells was calculated as a percentage of those control values. Samples marked with * are significantly different from the control value ($p<0.03$).

FIGURE 3.2: CELL ASSOCIATED PROTEIN SYNTHESISED BY NEUTROPHILS INCUBATED WITH SYNOVIAL FLUID



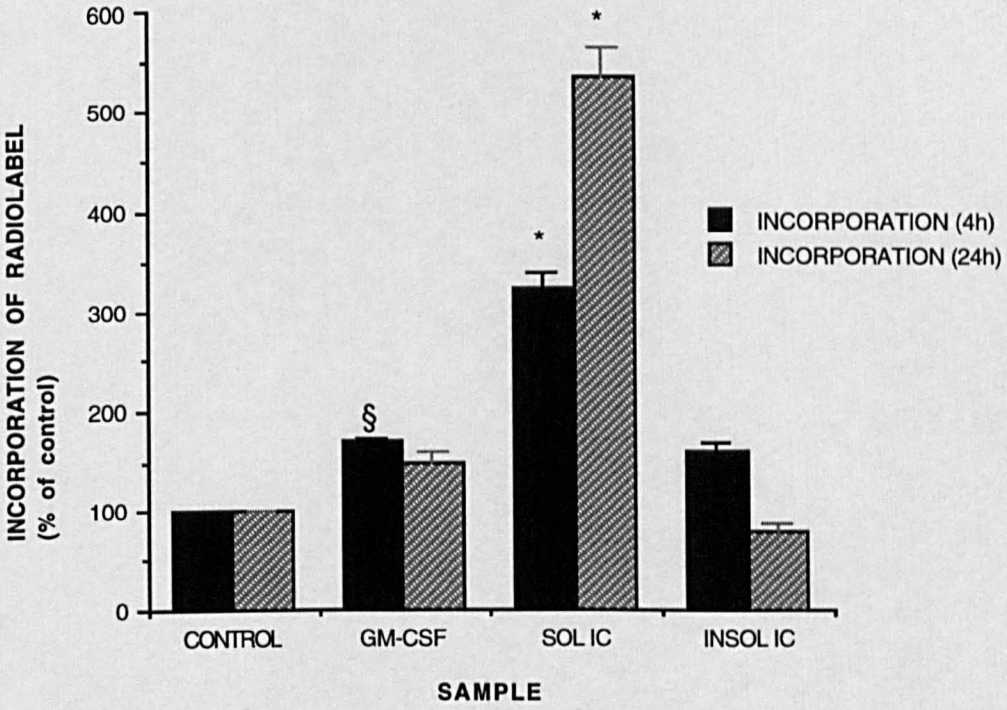
Neutrophils were incubated in the absence (control) and presence of GM-CSF (14 ng/ml) and cell free synovial fluid from 3 different RA patients (5% v/v) for 24 h in the presence of 35S-methionine. The newly-labelled, cell associated proteins were then analysed by 2D SDS-PAGE and fluorography. The fluorographs shown above are of proteins detected in control cells (A), GM-CSF stimulated cells (B) and SF stimulated cells (C-E). The arrows indicate *de novo* synthesis of proteins undetectable in control cells. The numbers indicate the mobilities of molecular mass markers (kDa).

FIGURE 3.3: PROTEINS SECRETED BY NEUTROPHILS INCUBATED WITH SYNOVIAL FLUID



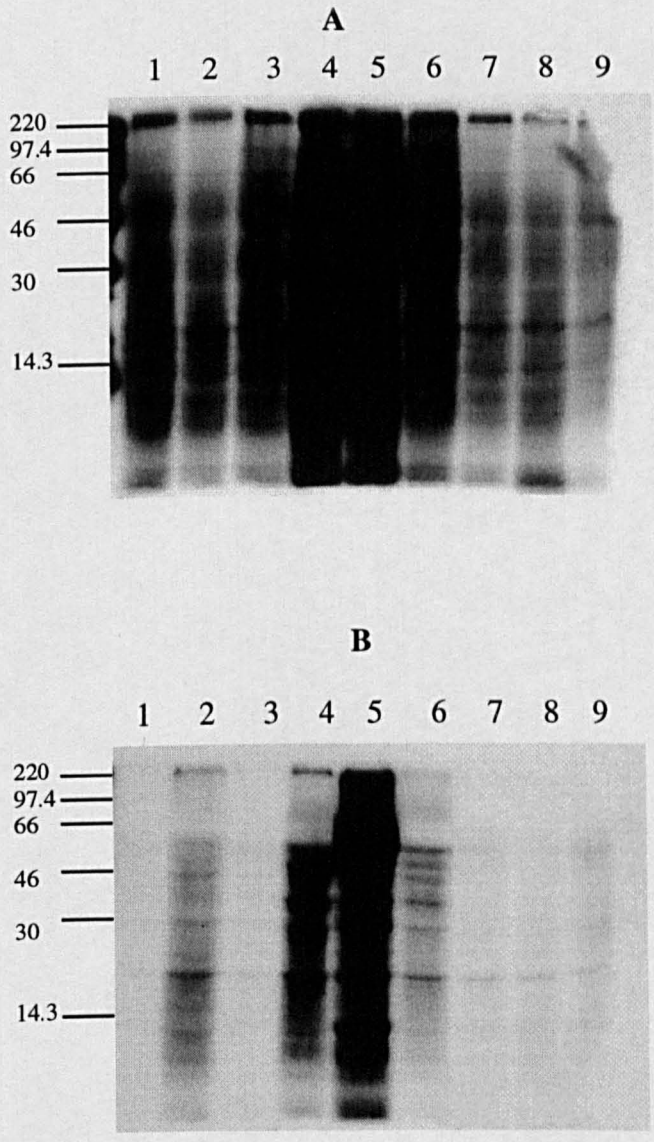
Neutrophils were incubated as described in Fig 3.2 in the absence (control) and presence of GM-CSF and cell free SF of 3 RA patients. The radio-labelled, acetone-precipitable proteins secreted into the culture medium by neutrophils were analysed by 2D SDS-PAGE and fluorography. Shown above are proteins isolated from control suspensions (A), GM-CSF stimulated suspensions (B) and SF stimulated suspensions (C-E). The arrows indicate 2 low molecular mass proteins secreted only by 2 of the SF stimulated suspensions (D & E).

FIGURE 3.4: INCORPORATION OF ^{35}S-METHIONINE INTO PROTEINS SYNTHESISED BY NEUTROPHILS INCUBATED WITH IMMUNE COMPLEXES



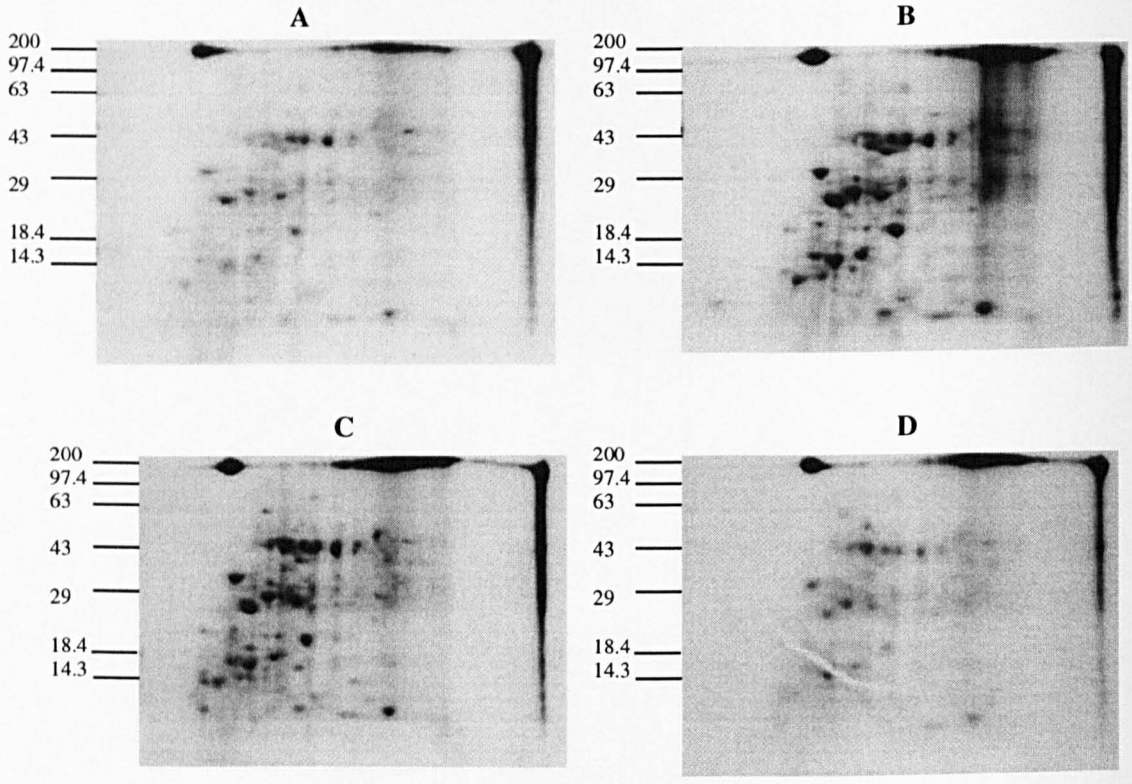
Neutrophils were incubated in the presence of 60 μCi/ml ^{35}S-methionine and in the presence or absence of GM-CSF (14ng/ml), soluble or insoluble IC (10% v/v) for 4 h or 24 h at 37°C. At these time points triplicate samples were removed and the amount of radiolabel incorporated was measured using a scintillation counter as described in 2.3. This graph is representative of 5 separate experiments, the results of which could not be statistically compared due to biological variation. Samples marked with * and § are significantly greater than control values ($p<0.03$ and $p<0.04$ respectively).

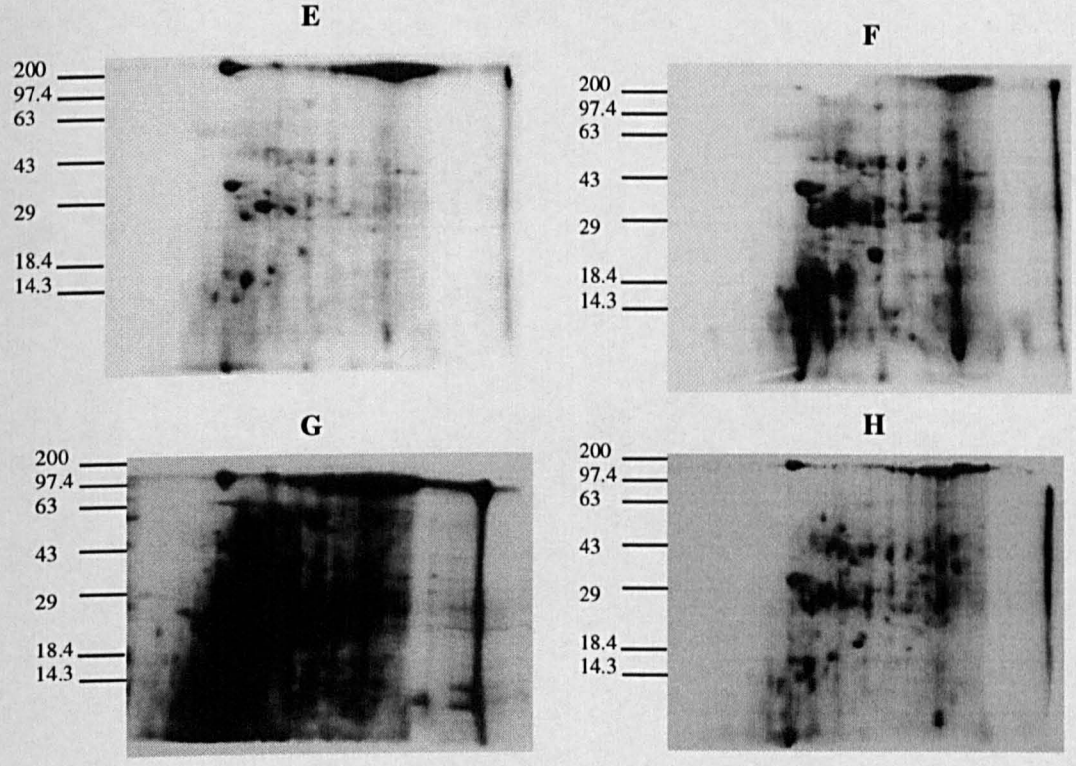
FIGURE 3.5: 1D SDS-PAGE ANALYSIS OF PROTEINS SYNTHESISED BY NEUTROPHILS INCUBATED WITH IMMUNE COMPLEXES



Neutrophils were incubated in the presence of 35S-methionine and in the absence (control) and presence of soluble or insoluble IC (10% v/v). Cell associated and acetone-precipitable secreted proteins synthesised after 24 h incubation were analysed by 1D SDS-PAGE and fluorography. Cell associated proteins are shown on fluorograph A and secreted on fluorograph B. On each fluorograph, lanes 1-3 are control suspensions, lanes 4-6 are soluble IC stimulated suspensions and lanes 7-9 are insoluble IC stimulated suspensions.

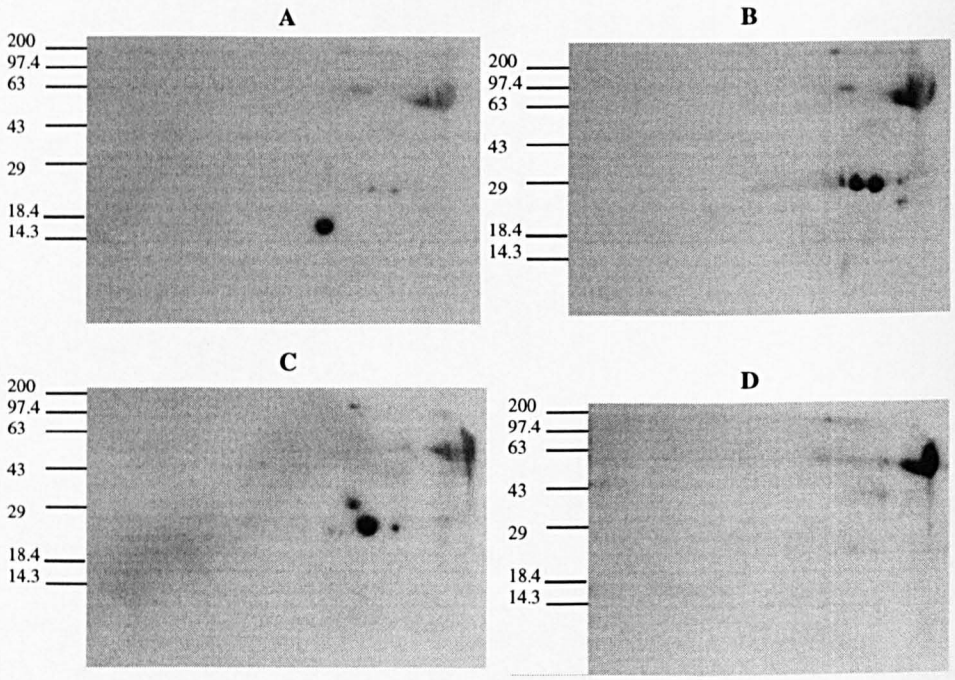
FIGURE 3.6: 2D SDS-PAGE ANALYSIS OF CELL ASSOCIATED PROTEINS SYNTHESISED BY NEUTROPHILS INCUBATED WITH IMMUNE COMPLEXES

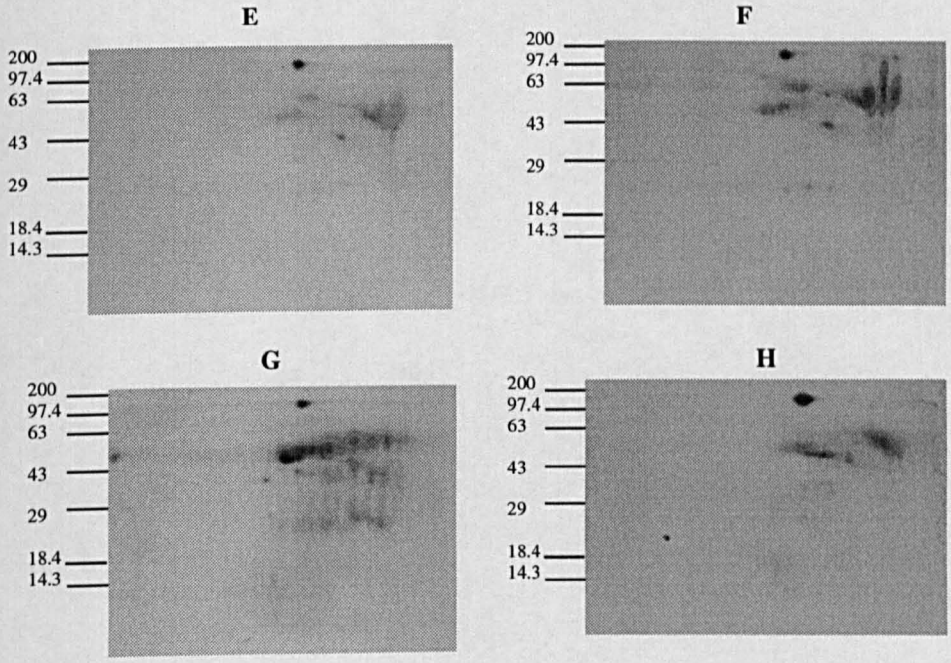




Neutrophils were incubated in the absence (control) and presence of GM-CSF (14 ng/ml), soluble IC and insoluble IC (10% v/v) in medium containing 35S-methionine for 4 h and 24 h. Cell associated proteins were then analysed by 2D SDS-PAGE and fluorography. A & E show proteins synthesised by control suspensions at 4 h and 24 h respectively, B & F, GM-CSF stimulated suspensions at 4 h and 24 h, C & G, soluble IC stimulated suspensions at 4 h and 24 h, D & H, insoluble IC stimulated suspensions at 4 h and 24 h.

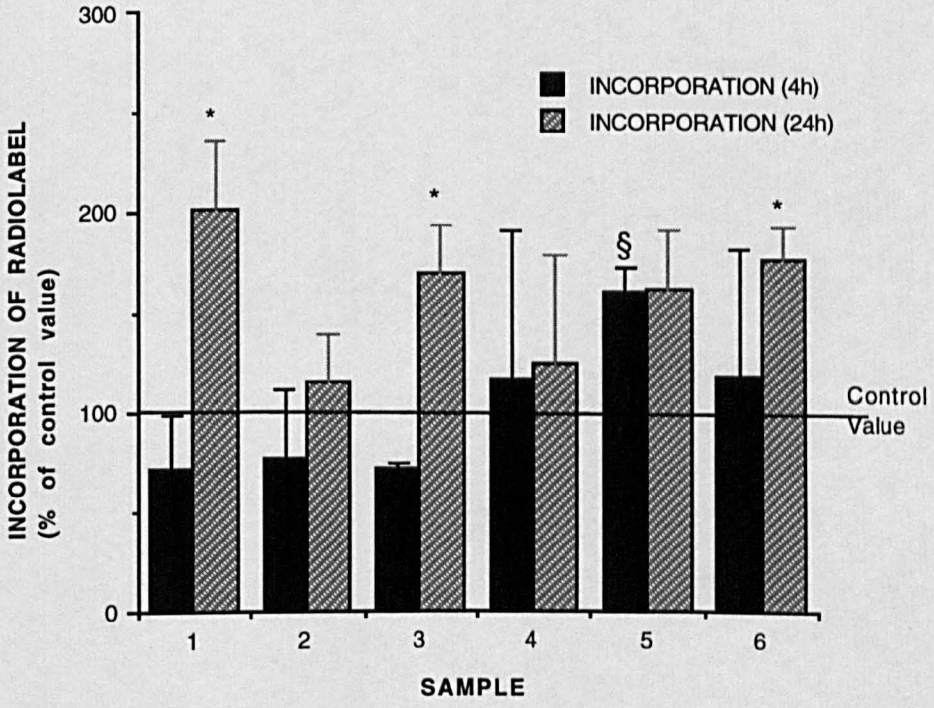
FIGURE 3.7: 2D SDS-PAGE ANALYSIS OF PROTEINS SECRETED BY NEUTROPHILS INCUBATED WITH IMMUNE COMPLEXES





Neutrophils were incubated as described in Fig 3.6. The newly-labelled acetone-precipitable proteins secreted by neutrophils into the culture medium at 4 h and 24 h were then analysed by 2D SDS-PAGE and fluorography. The secreted proteins detected were isolated from control suspensions at 4 h and 24 h (A & E respectively), GM-CSF stimulated suspension (B & F), soluble IC stimulated suspensions (C & G) and insoluble IC stimulated suspensions (D & H).

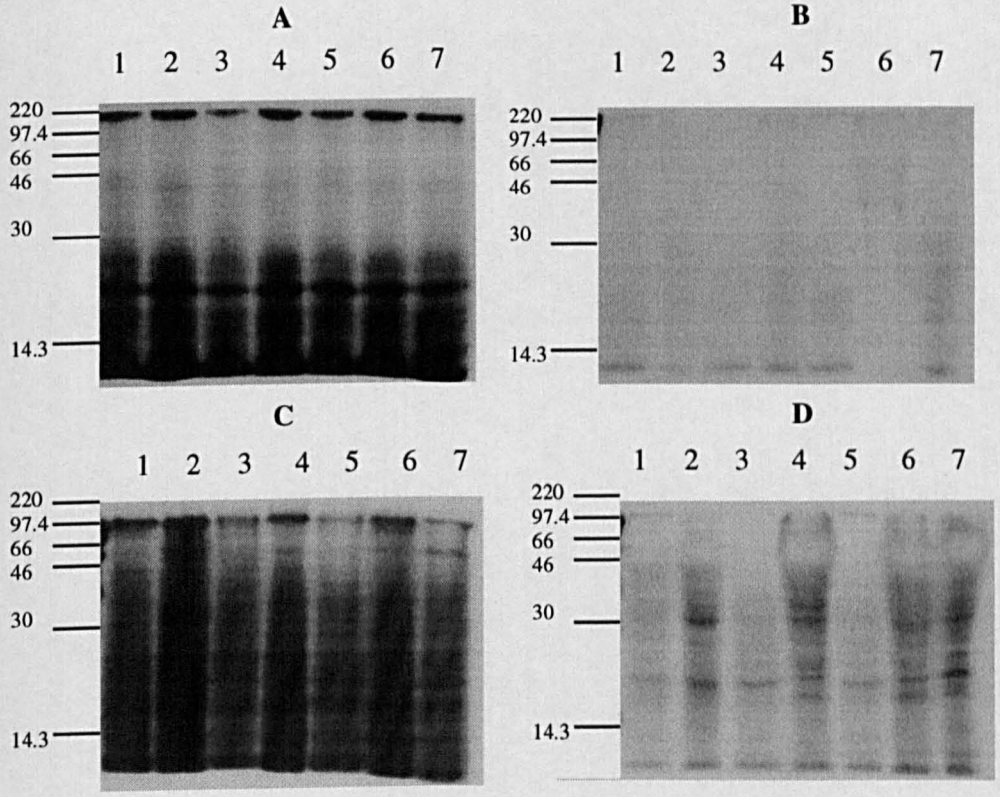
FIGURE 3.8: COMPARISON OF SYNTHETIC IMMUNE COMPLEXES AND IMMUNE COMPLEXES ISOLATED FROM SYNOVIAL FLUID: INCORPORATION OF ^{35}S-METHIONINE



Neutrophils were incubated in the presence of ^{35}S-methionine and with different fractions of SF and synthetic immune complexes for time periods of 4 h and 24 h. This graph is representative of 3 separate experiments. Those samples marked by * and § are significantly greater than control levels ($p<0.04$ and $p<0.02$ respectively). The incubation conditions are as follows:

1 synthetic soluble IC
2 synthetic insoluble IC
3 whole SF
4 insoluble IC isolated from SF
5 insoluble IC depleted SF
6 SF depleted of all IC.

FIGURE 3.9: COMPARISON OF THE EFFECTS OF SYNTHETIC AND SYNOVIAL FLUID DERIVED IMMUNE COMPLEXES ON NEUTROPHIL PROTEIN SYNTHESIS



Neutrophils were incubated in the presence of 35S-methionine and in the absence (control) and presence of synthetic IC and fractions of SF. After 4 h and 24 h incubation, cell associated and acetone-precipitable secreted proteins were analysed by 1D SDS-PAGE and fluorography. (A) and (C) show cell associated proteins at 4 h and 24 h respectively. (B) and (D) show secreted proteins at 4 h and 24 h. On each fluorograph, the lanes correspond to neutrophils stimulated by: (1) control, (2) synthetic soluble IC, (3) synthetic insoluble IC, (4) whole, unfractioned SF, (5) insoluble IC isolated from SF, (6) SF depleted of insoluble IC (ie containing only soluble IC), and (7) SF depleted of all IC.

3.3 DISCUSSION

The results presented in this Chapter confirm the ability of neutrophils to synthesise a wide range of proteins, both cell associated and secreted in response to GM-CSF, a known neutrophil priming agent. This increase in protein synthesis can be up to 2 fold in total, although some proteins are increased by a greater amount than others. This can be seen most clearly on the 2D gels (Figs 3.2 & 3.7). Certain proteins that are seen in primed cells are not present or are below the level of detection in control samples. Among this set of up-regulated cytokine-induced proteins are those which are up-regulated by up to 10-20 fold. There are other proteins which are present in control cells but are up-regulated only slightly by GM-CSF.

A similar pattern of protein labelling was seen when synovial fluid was used to stimulate neutrophils. Total protein synthesis was increased by 1-4 fold in different experiments. Again, several proteins are synthesised *de novo* and are not detectable in control cells. The pattern of proteins synthesised was very similar to that observed after incubation with GM-CSF perhaps suggesting that GM-CSF is an important stimulus in RA synovial fluid. Also, similarly to GM-CSF, the expression of certain proteins is increased by 10-20 fold by SF while expression of others was only increased by 1-2 fold. This indicates a differential regulation of protein synthesis in neutrophils within the rheumatoid joint. When secreted proteins are studied, there were only a few proteins secreted by control neutrophils but this number was increased by stimulation with GM-CSF or by SF. In particular, 2 proteins of similar molecular mass (approximately 26 kDa) appear to be of interest because they are reproducibly seen in SF stimulated neutrophil culture medium. Their similar relative molecular masses but altered PI's indicate that these 2 species may represent protein with altered phosphorylation patterns. These 2 proteins may be of particular significance in RA, and further work is clearly required to identify them.

The most potent neutrophil activating agents present in synovial fluid (in terms of activating the respiratory burst and degranulation) are generally thought to be immune complexes. Hence, I have used synthetic immune complexes made from HSA and anti-HSA antibodies to stimulate protein synthesis in neutrophils. The results obtained show that smaller, soluble IC are much more potent activators of neutrophil protein synthesis than large insoluble IC. The soluble IC can induce up to a 12 fold increase in protein synthesis in control blood neutrophils. In contrast, insoluble IC do not stimulate such a dramatic increase in protein synthesis, being at most 2 fold. The reasons for this could lie in the different activation pathways involved in cell stimulation by these 2 types of complexes (Robinson *et al*, 1994). This may represent activation of different receptors in response to these IC. FcγRIIIb, for example, may be involved in the binding of small, soluble IC while FcγRII may bind primarily insoluble IC (Edwards, 1994). FcγRIIIb is a glycosyl phosphatidyl inositol (GPI) linked protein which lacks an intracellular domain, and hence it is unknown how it generates intracellular signals following binding of IC (reviewed by Edwards, 1995). It is often thought to work in concert with FcγRII. The difference in the actions of soluble and insoluble IC can be seen in their different abilities to activate the NADPH oxidase (Robinson *et al*, 1992a, 1992b, 1993). Insoluble IC can activate the respiratory burst in resting neutrophils but, soluble IC cannot activate the respiratory burst unless the cell has been previously primed. Additionally, phagocytosis is only triggered by binding of opsonised particles (including immune complexes) to FcγRII.

Having performed these experiments with synthetic IC, it was then necessary to test whether the immune complexes present in SF from rheumatoid joints were capable of activating neutrophil protein synthesis in the same way. As it is impossible to isolate soluble IC from their SF environment without altering their molecular properties, an alternative approach to study their effects was adopted. SF was depleted of insoluble

IC (by centrifugation) and then soluble IC were removed with Pansorbin. Then, by subtraction, the contribution of these IC to protein synthesis by whole SF could be deduced.

SF containing soluble IC was able to induce a great increase in protein synthesis in normal neutrophils at 4 hours and this increase was greater than that observed with synthetic soluble IC. The ability of whole SF to stimulate protein synthesis was as great as that of SF depleted of insoluble IC, (ie that containing only soluble IC). SF which had been depleted of all IC had a similar ability to stimulate protein synthesis when compared to whole SF. Insoluble IC, both synthetic and from SF, induced similar amounts of protein synthesis which was not much greater than observed in control cells. However, this does not preclude the possibility that the synthesis of some important proteins is induced by insoluble IC, even though the overall rate of protein synthesis was not high.

The results demonstrated that immune complexes found *in vivo* appeared have a more minor role than the cytokines found in SF with regard to the total amount of protein synthesis stimulated. However, immune complexes may have a role in stimulating the synthesis of individual proteins. Resting or primed neutrophils may enter the joint from the bloodstream, attracted by chemotactic factors such as IL-8 and C5a. These cells would then encounter immune complexes and cytokines in the SF and become activated. Not only will the respiratory burst be activated, but protein synthesis will also be activated. The neutrophil may then produce cytokines and other proteins such as receptors and oxidase components. These receptors will allow the cell to respond to further activating agents including immune complexes. One such receptor known to be actively synthesised by activated neutrophils is FcγRIIIb. Increased synthesis of oxidase components will allow the generation of a sustained respiratory burst and if these oxidants are secreted, will lead to greater damage to the cartilage and bone in the joint.

In addition to synthesis of receptors and oxidase components, the synthesis of cytokines can lead to the neutrophil directing the immune response within the joint. The secretion of IL-8 can attract fresh neutrophils to the joint causing a positive feedback cycle which perpetuates inflammation. IL-1β and TNF-α secretion can indirectly attract neutrophils to the joint by up-regulating adhesion molecules on endothelial cells. IL-1β can also act on other cell types in the joint including macrophages and synoviocytes leading to damaging consequences, including the resorption of bone and cartilage.

In addition to known proteins, there may also be as yet unknown proteins synthesised and secreted by neutrophils. This is a likely probability as neutrophil protein synthesis is a little studied event. Hence, an unknown protein being synthesised by neutrophils in the rheumatoid joint may be of pathological importance.

CHAPTER 4

NEUTROPHIL SYNTHESIS OF IL-1β AND TNF-α

4.1 INTRODUCTION

IL-1β and TNF-α are pleiotropic cytokines i.e. they have varied effects on many cell types, and many of these effects are common to both cytokines. IL-1β was originally described as endogenous pyrogen as it was found to cause fever (Atkins, 1960). The mature polypeptide is a 17kDa molecule which exists as a monomer that is synthesised by many cell types including monocytes, synoviocytes and nerve cells (reviewed by Espat *et al*, 1994; Dinarello, 1991). IL-1β is synthesised as an inactive 35kDa precursor called pre-IL-1β which is cleaved by a cysteine protease known as Interleukin-1β Converting Enzyme (ICE) to form the mature 17kDa polypeptide (Kostura *et al*, 1989; Cerretti *et al*, 1992; Thornberry *et al*, 1992). Pro-IL-1β has no signal sequence and unlike most secretory proteins is not glycosylated. Hence, it is not thought to be externalised via the usual endoplasmic reticulum and Golgi route. Few other proteins share this property but examples include Endothelial Cell Growth Factor (Jaye *et al*, 1986) and a lactose binding lectin (Cooper & Barondes, 1990). Studies have shown the location of ICE to be in the cytosol (Ayala *et al*, 1994) and mature IL-1β has also been detected in the cytosol (Perregaux *et al*, 1992). Agents which alter the levels of K^+, such as nigericin induce the externalisation of mature IL-1β suggesting that perturbation of the K^+ level is a necessary step in this process (Perregaux *et al*, 1992; Perregaux & Gabel, 1994). Also, the extra

amino acids in the precursor protein appear to regulate secretion because as more of the precursor sequence is removed, more of the IL-1β is secreted (Siders & Mizel, 1995). ICE has been detected in neutrophils and many other cell types, including monocytes and placenta (Cerretti *et al*, 1992).

ICE has homology with a cell death gene *ced-3* found in the nematode (Yuan *et al*, 1993) and IL-1β secretion has been demonstrated to be associated with apoptosis in monocytes (Hogquist *et al*, 1991). Alnemri *et al* (1995) have cloned several alternatively spliced forms of ICE which have different effects on apoptosis (reviewed by Thornberry & Molineaux, 1995).

There are 2 receptors for IL-1β, type I and II which both bind IL-1 with equal affinity. However, the type II receptor is not thought to be active in transducing signals into the cell and is thought to act as a decoy receptor by binding IL-1β and preventing it binding to the active type I receptor (Colotta *et al*, 1993). It has been shown that the type II receptor is shed from the cell surface in response to IL-4 and IL-13 (Colotta *et al*, 1994) while still retaining its binding capacity and in this way also it can prevent IL-1 binding to cell surface receptors and hence neutralize its effects (Colotta *et al*, 1993). There have been clinical trials attempting to use recombinant IL-1R as a therapy in rheumatoid arthritis but these have had limited success with only one patient showing clinical improvement (Drevlow *et al*, 1996).

TNF-α was originally described as a factor found in serum following treatment with endotoxin which could cause necrosis of tumours (Carswell *et al*, 1975). It was also named cachexin as it induces the wasting condition, cachexia. Like IL-1β, it is a 17kDa

protein but exists *in vivo* as a 51kDa homotrimer. It is also synthesised and secreted by a wide range of cell types and can exist as a type II membrane protein. There are 2 receptors for TNF-α , p55 and p75 both of which can be shed from the cell surface to become soluble receptors which retain their binding capacity for TNF-α (Lien *et al*, 1995; Lantz *et al*, 1994). This shedding can be induced by the binding of L-selectin (CD62L) (Richter & Zetterburg, 1994). In this way, the soluble receptors are thought to inhibit TNF-α activity by binding to it and preventing its interaction with cell surface receptors (Ferrante *et al*, 1991). Alternatively, it has been suggested that this interaction with soluble receptors actually stabilises TNF-α in solution for a longer period (Lantz *et al*, 1994). Both the p55 and p75 receptors in soluble form have been detected in synovial fluid from rheumatoid arthritis patients and these may be clinically relevant (Lopez *et* al, 1995; Steiner *et al*, 1995; Roux-Lombard *et al*, 1993).

IL-1β and TNF-α are thought to be important cytokines present in the rheumatoid joint (reviewed by Espat *et al*, 1994; Duff, 1993). They stimulate the production of other inflammatory cytokines such as IL-6 (Ogilvie *et al*, 1996; Cicco *et al*, 1990), GM-CSF (Alsalameh *et al*, 1994), IL-8 (Fujishima *et al*, 1993; Rathanaswami *et al*, 1993) and ENA-78 (Koch *et al*, 1994) from a range of cells including monocytes, chondrocytes and synoviocytes. They also stimulate their own production (Seid *et al*, 1993; Fujisawa *et al*, 1996). IL-1β induces the influx of neutrophils into the joint by enhancing the expression of adhesion molecules on endothelial cells. Both IL-1β and TNF-α suppress cartilage matrix synthesis (Rédini *et al*, 1993), activate chondrocytes to secrete collagenase (Pianon *et al*, 1994), suppress chondrocyte and synovial cell protease inhibitor production, stimulate prostaglandin production by synovial fibroblasts and activate osteoclasts to resorb

bone (Panagakos *et al*, 1994). In a murine models of RA, neutralization of IL-1β and TNF-α reduces neutrophil infiltration and cartilage destruction (van Lent *et al*, 1994; Zhang *et al*, 1995a). Hence, if neutrophils are producing these cytokines within the rheumatoid joint they could contribute to the pathogenesis of RA by directing the immune response and stimulating destructive pathways from other cell types within the joint.

Among the cell types known to produce IL-1β and TNF-α are neutrophils. Several authors have shown neutrophil synthesis and secretion of IL-1β in response to LPS, IL-1β itself, TNF-α and GM-CSF (Tiku *et al*, 1986; Lindemann *et al*, 1988; Lord *et al*, 1991; Marucha *et al*, 1991; Malyak *et al*, 1994). TNF-α synthesis and secretion by neutrophils has also been demonstrated in response to LPS and opsonised yeast particles and non opsonised *Candida albicans* (Djeu *et al*, 1990; Bazzoni *et al*, 1991; Haziot *et al*, 1993; Wei *et al*, 1993). In contrast to cytokine synthesis by monocytes, which can continue for periods greater than 24 h, cytokine synthesis by neutrophils is transient, generally peaking within hours after stimulation and returning to baseline levels by 24 h.

I have, in this Chapter, used an *in vitro* model of the rheumatoid joint environment to determine if neutrophils, which invade the joint space in huge numbers, could be synthesising and secreting physiologically relevant amounts of IL-1β and TNF-α. In addition to this I have attempted to identify the potential agents present within joint synovial fluid which could be stimulating this synthesis. In order to do this I have incubated neutrophils with cell free SF from RA patients and also with synthetic immune complexes. IC are found in abundance in rheumatoid SF and are considered to be the major neutrophil stimulating agents.

4.2 RESULTS

4.2.1 *Synthesis of cell associated IL-1β by neutrophils incubated with GM-CSF, TNF-α or LPS*

Inital experiments in this Chapter were designed to confirm reports of IL-1β synthesis by neutrophils in response to cytokines and LPS. Hence, normal blood neutrophils were incubated in the presence and absence (control) of GM-CSF (14 ng/ml), TNF-α (25 ng/ml) or LPS (5 μg/ml) for periods of up to 24 h in medium containing ^{35}S-methionine. At the specified time points, cell associated IL-1β was immunoprecipitated using an anti-IL-1β antibody followed by addition of Pansorbin.

Figure 4.1A shows fluorographs of radiolabelled IL-1β immunoprecipitated from neutrophils incubated with GM-CSF, TNF-α or LPS. The 3 agents induced the synthesis of IL-1β by neutrophils but to different degrees. Synthesis was not up-regulated by TNF-α. The peak of production is at 5 h for GM-CSF and 6 h for LPS. The amount of IL-1β detected by fluorography was quantified by densitometry and plotted as a graph (Figure 4.1B). In LPS and TNF-α stimulated cells, synthesis returned to baseline by 24 h, however some synthesis can still be seen in the GM-CSF stimulated cells at 24 h. LPS was the most effective, stimulating levels IL-1β, 3.5-fold greater than control cells, followed by GM-CSF (approximately 2-fold) and TNF-α was least effective (levels of

synthesis equivalent to control cells). The amount of IL-1β detected in control cells may be due to partial activation of the cells during purification.

4.2.2 *Secretion of IL-1β by neutrophils incubated with GM- CSF, TNF-α or LPS*

It was then necessary to determine if the newly-synthesised IL-1β was being secreted, as secretion is necessary for the cytokine to have an effect upon other cells. Hence, radiolabelled IL-1β present in the culture supernatant of control cells and those incubated with LPS, GM-CSF or TNF-α was immunoprecipitated. Figure 4.2A shows a fluorograph of the IL-1β immunoprecipitated from culture supernatants. LPS stimulated the greatest quantity of IL-1β secretion, approximately 11-fold greater than that seen when neutrophils were stimulated with either GM-CSF or TNF-α where approximately equal amounts of IL-1β were secreted. LPS-induced secretion began at 3 h, peaking at 5 h and returning to baseline by 24 h. The densitometry values are plotted on the graph in Figure 4.2B. In GM-CSF and TNF-α stimulated neutrophils, secretion can be detected at 5 h for GM-CSF and 4 h for TNF-α and secretion returned to baseline by 7 h. No secretion of IL-1β could be detected in control suspensions. The secretion of IL-1β is therefore a very transient event in response to these stimuli.

4.2.3 *Synthesis of cell associated IL-1β by neutrophils incubated with synovial fluid or immune complexes*

In order to find if neutrophils could be synthesising IL-1β in the rheumatoid joint, an *in vitro* model of the rheumatoid joint environment was used. Cells were incubated with cell free SF (5% v/v) collected from RA patients or with synthetic IC (10% v/v) (HSA and anti-HSA antibodies, prepared as described in 2.4). IC are thought to be the major neutrophil stimulating agent in SF and I have shown that they stimuluate protein synthesis in neutrophils (see 3.2.3). Figure 4.3A shows fluorographs of IL-1β immunoprecipitated from neutrophils stimulated for up to 24 h with SF from 5 different patients (patients 1,2 and 5 had RA, patient 3 had both RA and Ankylosing spondylitis and patient 4 had Psoriatic arthritis) and both soluble and insoluble IC.

All 5 SF samples induced synthesis of cell associated IL-1β by neutrophils, although to differing degrees indicating a wide range of biological variation. This variation may arise from different levels of cytokines and IC within the different samples, or may be the result of drugs used by the patients affecting the measurements. SF 1 induced the greatest increase in cell associated IL-1β synthesis compared to control suspensions (19-fold increase). The remaining SFs stimulated lower increases in cell associated IL-1β synthesis: SF 2 was 16-fold greater; SF 3, 7-fold greater; SF 4, 3-fold greater and SF 5, 4-fold greater. IL-1β could be detected at 2 h, reached a peak at 5-7 h and in SF 2 and 3 returned to baseline by 24 h while, in SF 1, 4 and 5 some IL-1β could still be detected at 24 h.

Both types of IC also stimulated synthesis of IL-1β by neutrophils. Synthesis could be detected at 2 h, peaked at 6 h and returned to baseline by 24 h. Insoluble IC were more effective than soluble IC at inducing IL-1β synthesis. Insoluble IC increased synthesis by 1.3-fold compared to control cells, while soluble IC stimulated a level similar to control levels. The IL-1β detected on fluorographs was quantified by densitometry and the values are shown on the graph on figure 4.3b Interestingly, while soluble IC induced the greatest increase in total protein synthesis as described in chapter 3 (3.2.3), insoluble IC were more effective at inducing IL-1β production.

4.2.4 *Secretion of IL-1β by neutrophils incubated with synovial fluid or immune complexes.*

It was then necessary to determine whether newly synthesised IL-1β was being secreted as this would indicate a role for neutrophil-derived cytokines in extracellular effects within the diseased joint. Because SF already contains IL-1β, the radio-labelling and immunoprecipitation technique was used to distinguish between newly-synthesised, neutrophil-derived IL-1β and that already present in the fluid. Figure 4.4A shows fluorographs of IL-1β immunoprecipitated from the culture supernatants of neutrophils incubated with cell free SF from 3 patients (patient 3 RA, patient 4 RA and Ankylosing spondylitis and patient 5 Psoriatic arthritis). These 3 SF induced secretion of IL-1β from neutrophils although it was of lower magnitude (12-24%) than that secreted by LPS stimulated neutrophils. However, secretion from SF stimulated neutrophils was not detected until 24 h unlike that from neutrophils stimulated with cytokines or LPS which

was detected at much earlier times. Both soluble and insoluble IC failed to stimulate any secretion of IL-1β from neutrophils within 24 h. The amount of IL-1β secreted was measured by densitometry and is shown in graph form in figure 4.4B.

4.2.5 *Neutrophil synthesis of TNF-α*

TNF-α is another important cytokine in RA with similar effects to IL-1β. Hence, I have studied the synthesis of this cytokine in response to LPS, SF or IC. Neutrophils were incubated in the absence (control) and presence of LPS (5 μg/ml), SF (5% v/v), soluble or insoluble IC (10% v/v) in medium containing ^{35}S-methionine for periods of up to 24 h.

Figure 4.5A shows fluorographs of TNF-α immunoprecipitated from these neutrophils. TNF-α synthesis could be detected at 1 h, peaking at 8 h and still detectable at 24 h in LPS, SF and soluble IC stimulated neutrophils but not in insoluble IC stimulated cells. Some synthesis was also seen in control cells and this was assumed to be due to partial activation of the cells during purification. LPS and SF were equally effective stimulators of TNF-α synthesis, enhancing levels to 4.5-fold greater than control suspensions. However, unlike IL-1β synthesis where insoluble IC was a more effective stimulus, soluble IC stimulated the greatest level of synthesis of TNF-α (7.6-fold greater than control) and no synthesis of TNF-α was detected in insoluble IC stimulated neutrophils. The amount of cell associated TNF-α detected is shown in Figure 4.5B, as a graph of densitometry measurements. No secretion of TNF-α could be detected at any time.

4.2.6 *Synthesis of IL-1β by SF and blood neutrophils from RA patients*

In order to determine if IL-1β is synthesised and secreted by neutrophils within the rheumatoid joint, it was necessary to analyse the synthesis of IL-1β in neutrophils isolated from RA patients. Both blood and SF neutrophils were obtained from 3 patients with RA. The cells were then incubated for periods of up to 16 h without further stimulation in the presence of ^{35}S-methionine to radiolabel any newly-synthesised IL-1β. Due to lack of available cells, the synthesis of TNF-α by the neutrophils of patient 3 could only be analysed at 2 time points (4 h and 16 h) while those of patients 1 and 2 were analysed at 4 time points (0 h, 1 h, 4 h and 16 h).

Figure 4.6 shows fluorographs of the IL-1β immunoprecipitated from neutrophils isolated from the SF and blood of RA patients. Both blood and SF neutrophils synthesised IL-1β during incubation which was detected at 1 h and increased through 4 h and 16 h of incubation. Interestingly, blood neutrophils from 2 of the RA patients produced much more IL-1β that SF neutrophils. The level of IL-1β detected by fluorography was quantified by densitometry and the level of IL-1β synthesised by SF neutrophils was found to be only 50% of the peripheral blood levels in patient 1 and 88% of blood levels in patient 2. However, the SF neutrophils of patient 3 synthesised 290% of the IL-1β level found in peripheral blood neutrophils of the same patient.

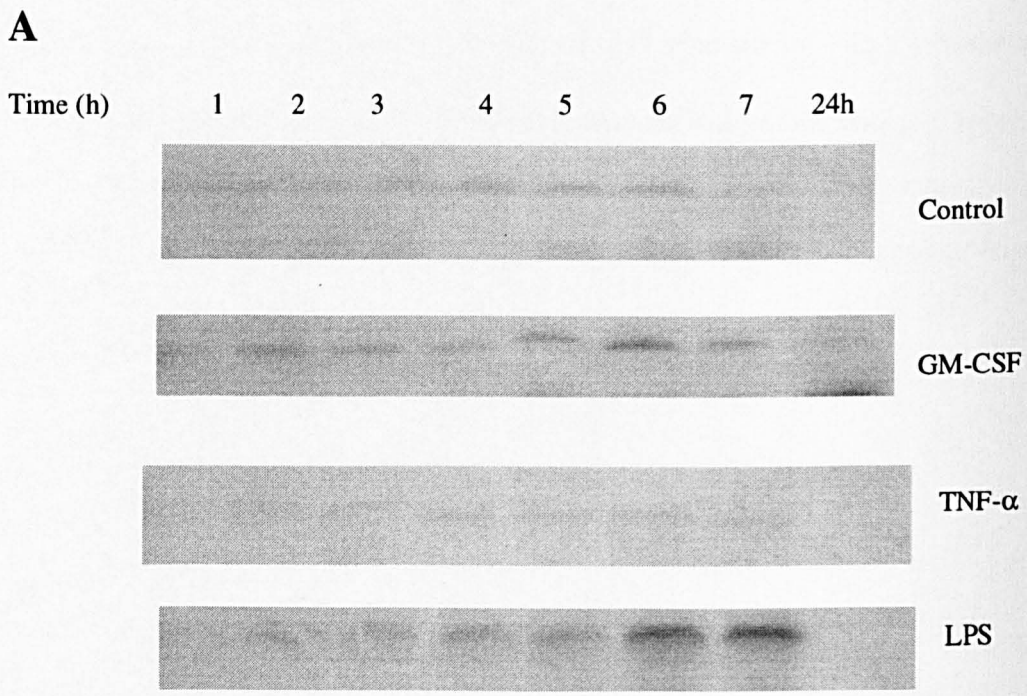
No secretion of IL-1β could be detected from the neutrophils of patients 1 and 3. However, newly synthesised IL-1β was detected in the culture supernatant of the blood neutrophils of patient 2. The secretion accounted for 50% of the peak level of cell associated IL-1β. These results would indicate that neutrophils in the blood of RA patients are primed as are those in the SF. The differing levels of IL-1β detected may be attributable to several variables including severity of inflammation, drugs being taken by the patient and levels of activating agents in the blood and SF.

4.2.7 *Synthesis of TNF-α by SF and blood neutrophils from RA patients*

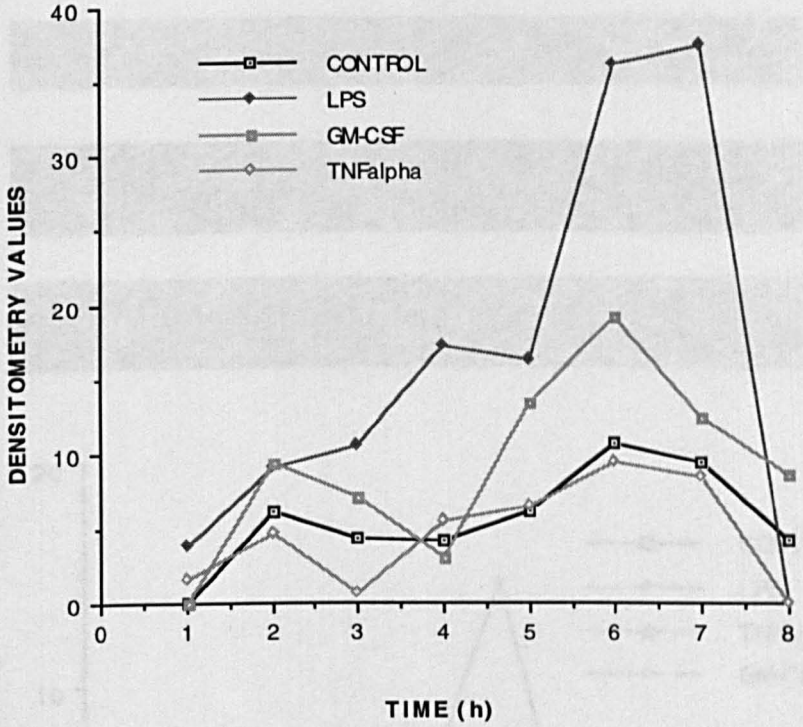
The neutrophils isolated from patients 1-3 above were also analysed for synthesis and secretion of TNF-α. Again, due to lack of available cells, the synthesis of TNF-α by the neutrophils of patient 3 could only be analysed at 2 time points (4 h and 16 h) while those of patients 1 and 2 were analysed at 4 time points (0 h, 1 h, 4 h and 16 h). Figure 4.7 shows fluorographs of TNF-α immunoprecipitated from neutrophils and their culture supernatants after isolation from SF and blood of RA patients and incubation without further stimulus. Similarly to IL-1β production, TNF-α was detected in these cells at 1 h and increased through 4 and 16 h of culture. Again, blood neutrophils synthesised more TNF-α than SF neutrophils in patients 1 and 2 with the level of synthesis by SF neutrophils being only 37% and 62% of that synthesised by blood neutrophils from the same patient. Patient 3 showed greater synthesis of TNF-α by SF neutrophils (204% of that synthesised by blood neutrophils from the same patient).

No secretion could be detected by either blood or SF neutrophils from patient 1 but secretion of TNF-α was detected in blood and SF neutrophil suspensions of patient 2 and blood neutrophils of patient 3. For patient 2, the TNF-α secreted by blood neutrophils accounted for 59% of the peak level of cell associated TNF-α while that of SF neutrophils accounted for 65% of the peak cell associated level. For patient 3, the level of TNF-α secreted by blood neutrophils accounted for 77% of the peak cell associated level. Again, this variation in levels of synthesis and secretion may be accounted for by differences in disease course, drugs taken and levels of inflammatory mediators in blood and SF.

FIGURE 4.1: SYNTHESIS OF CELL ASSOCIATED IL-1β BY NEUTROPHILS INCUBATED WITH GM-CSF, TNF-α OR LPS



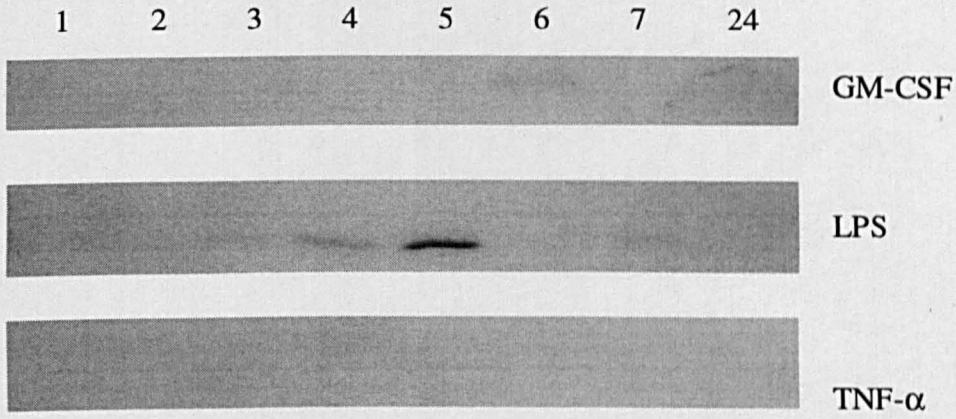
B



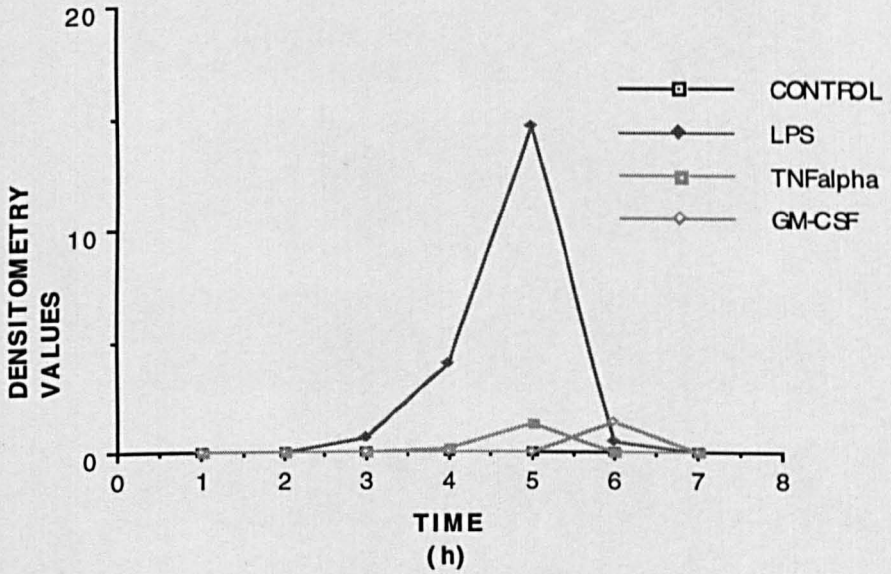
Neutrophils were incubated for periods of up to 24 h in the presence of 60 μCi/ml ^{35}S-methionine and in the presence or absence of GM-CSF (14 ng/ml), TNF-α (25 ng/ml) and LPS (5 μg/ml). At the time points shown, 1 x 10^7 cells were removed and cell-associated IL-1β was immunoprecipitated as described in 2.6 and analysed by SDS-PAGE and fluorography as described in 2.7.1. Figure 4.1A shows the fluorographs obtained and Figure 4.1B shows the densitometry values calculated from the fluorographs.

FIGURE 4.2: SECRETION OF IL-1β BY NEUTROPHILS INCUBATED WITH GM-CSF, TNF-α OR LPS

A



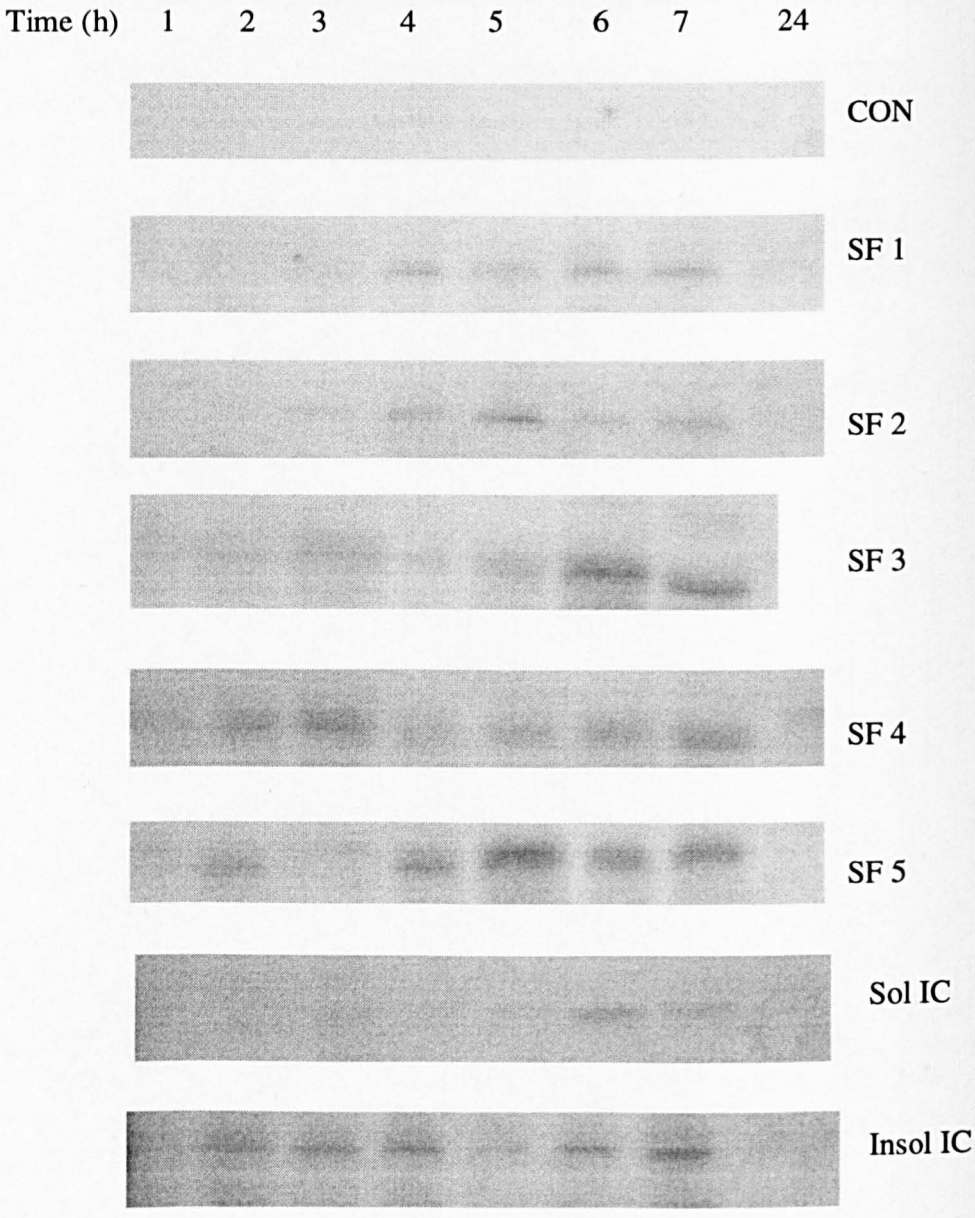
B

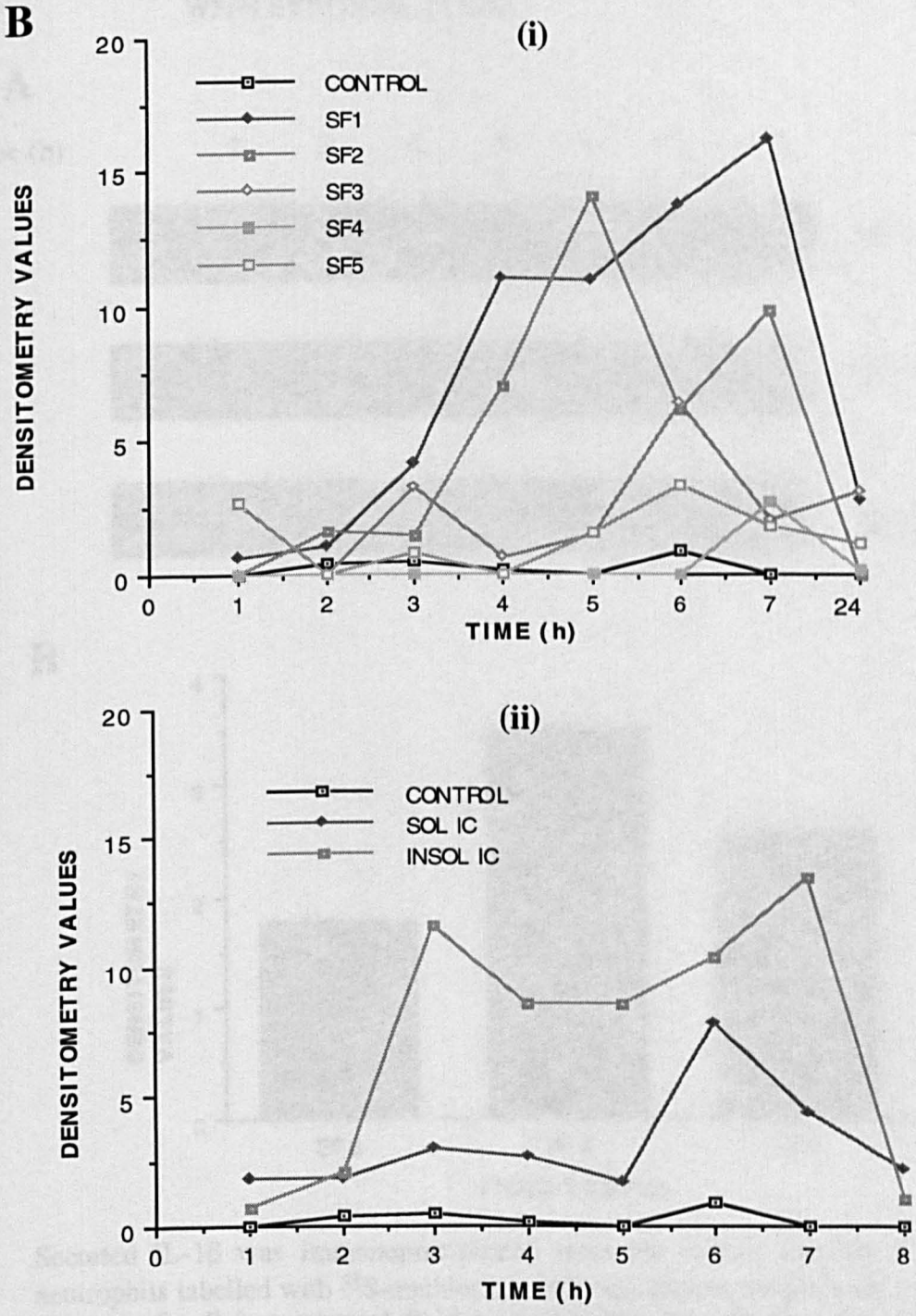


Neutrophils labelled with ^{35}S-methionine were incubated in the presence and absence of GM-CSF (14 ng/ml), LPS (5 μg/ml) or TNF-α (25 ng/ml) for periods of up to 24 h as described in 2.2. Following incubation, IL-1β secreted into the medium was immunoprecipitated as described in 2.6 and analysed by SDS-PAGE and fluorography. Figure 4.2A shows the time of incubation along the top and the stimulating agent at the right of the fluorographs. Figure 4.2B shows the densitometry values obtained from the fluorographs.

FIGURE 4.3: SYNTHESIS OF CELL ASSOCIATED IL-1β BY NEUTROPHILS INCUBATED WITH SYNOVIAL FLUID OR IMMUNE COMPLEXES

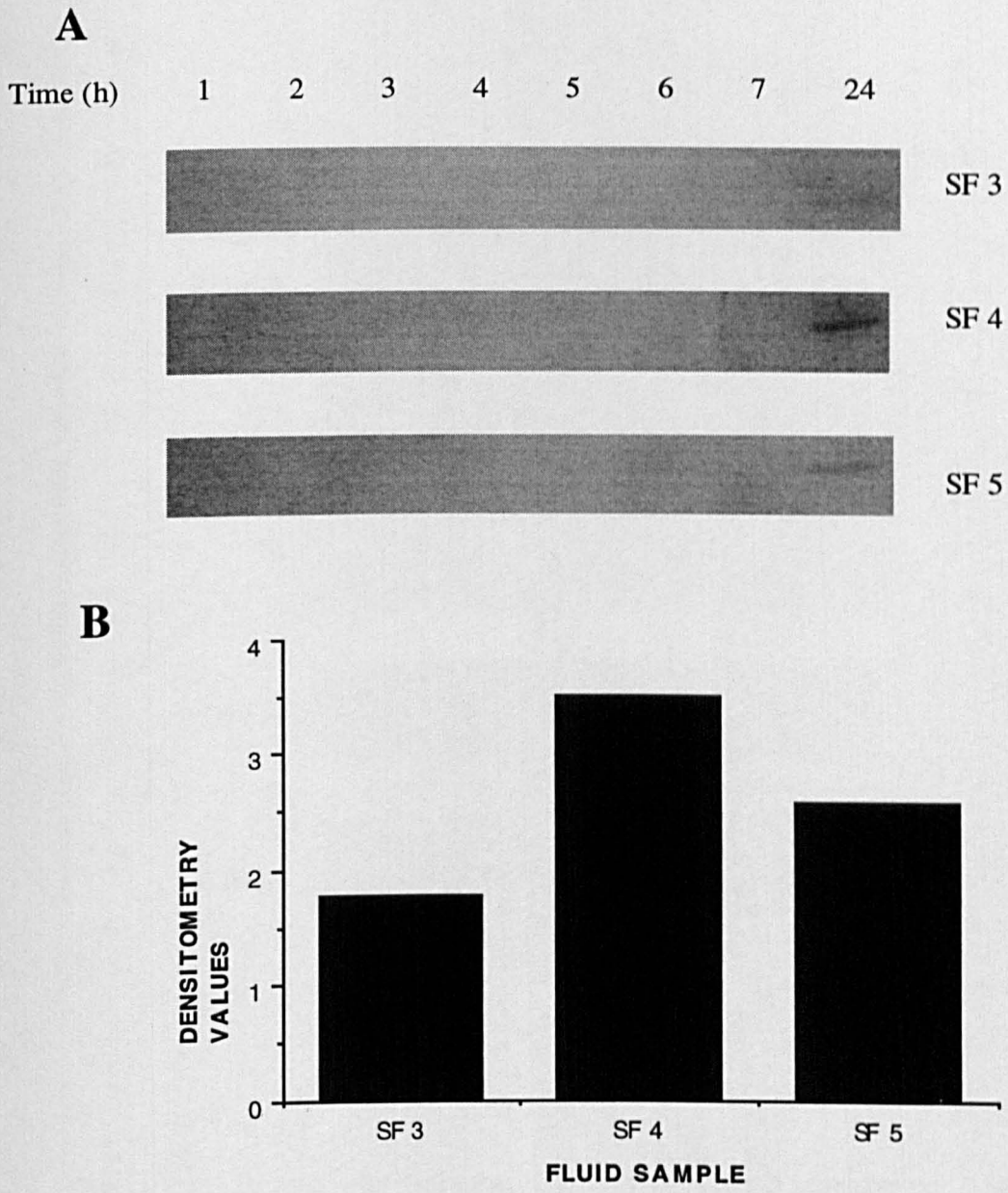
A





Neutrophils were labelled with ^{35}S-methionine as described in 2.2 and incubated with or without cell free synovial fluid (5% v/v) from 5 separate patients, (1,2 and 5 suffered from RA, 3 had both RA and ankylosing spondylitis and 4 had psoriatic arthritis), soluble or insoluble IC (10% v/v). After incubation, cell-associated IL-1β was immunoprecipitated from the samples as described in 2.6 and analysed by SDS-PAGE and fluorography. The fluorographs are shown in Figure 4.3A and the densitometry values measured on those fluorographs in Figure 4.3B (SF stimulated values in (i) and IC stimulated values in (ii)).

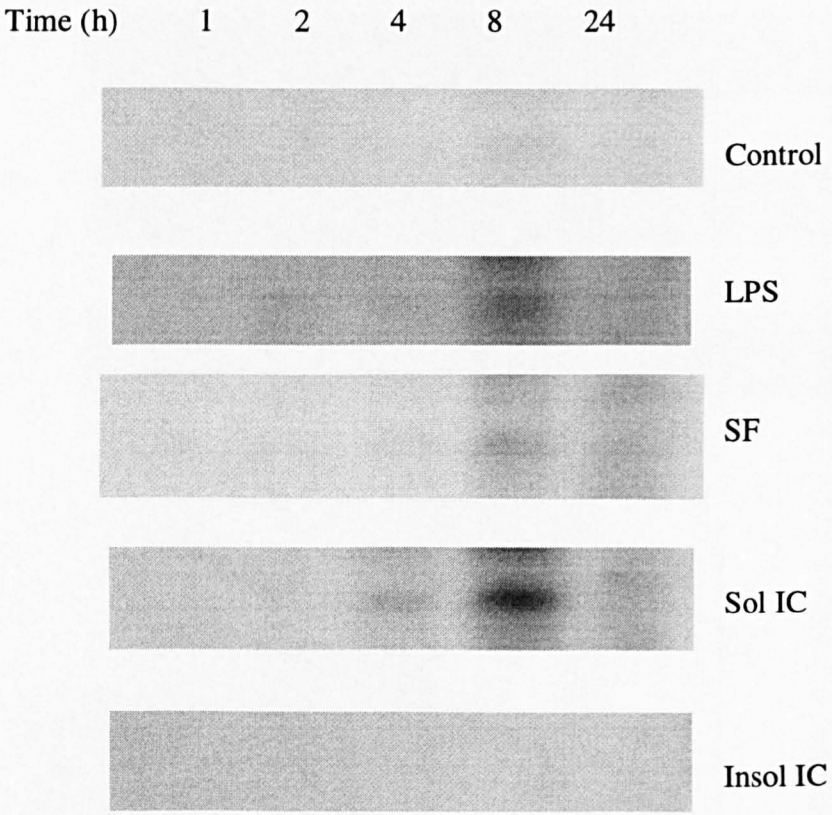
FIGURE 4.4: SECRETION OF IL-1β BY NEUTROPHILS INCUBATED WITH SYNOVIAL FLUID



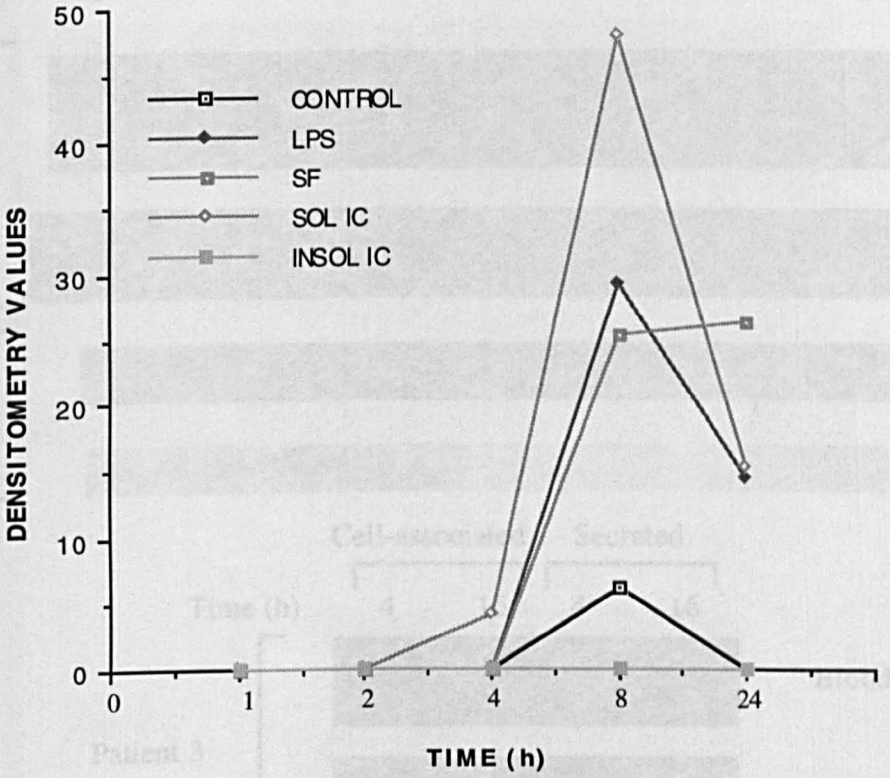
Secreted IL-1β was immunoprecipitated from the culture medium of neutrophils labelled with ^{35}S-methionine and incubated in the presence or absence of cell free synovial fluid collected from RA patients. In this experiment only 3 SF samples were used to treat the cells. These correspond to SF 3, 4 and 5 in figure 4.3. Figure 4.4A shows the fluorographs and figure 4.4B shows the densitometry values measured at 24 h.

FIGURE 4.5: SYNTHESIS OF CELL ASSOCIATED TNF-α BY NEUTROPHILS INCUBATED WITH LPS, SF OR IMMUNE COMPLEXES

A

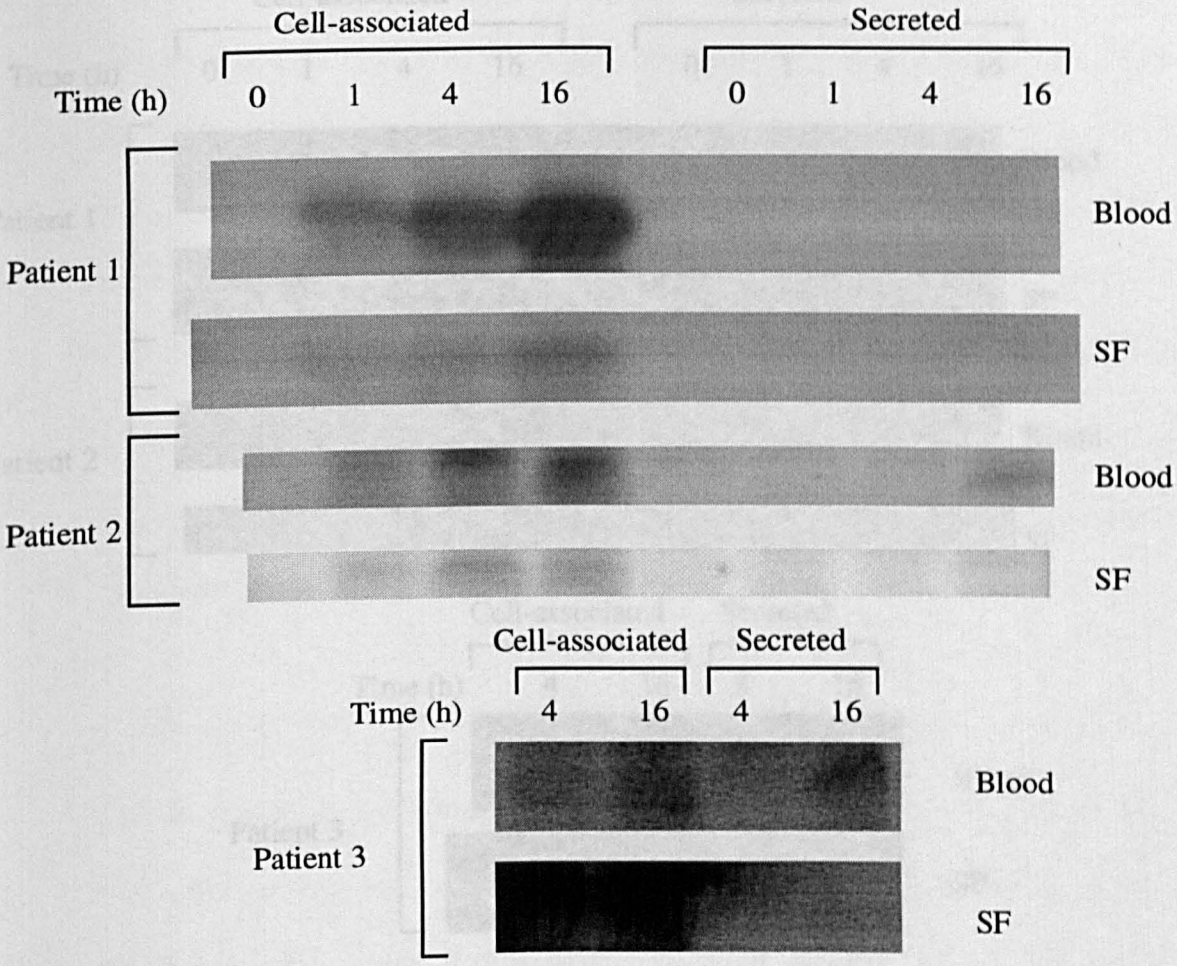


B



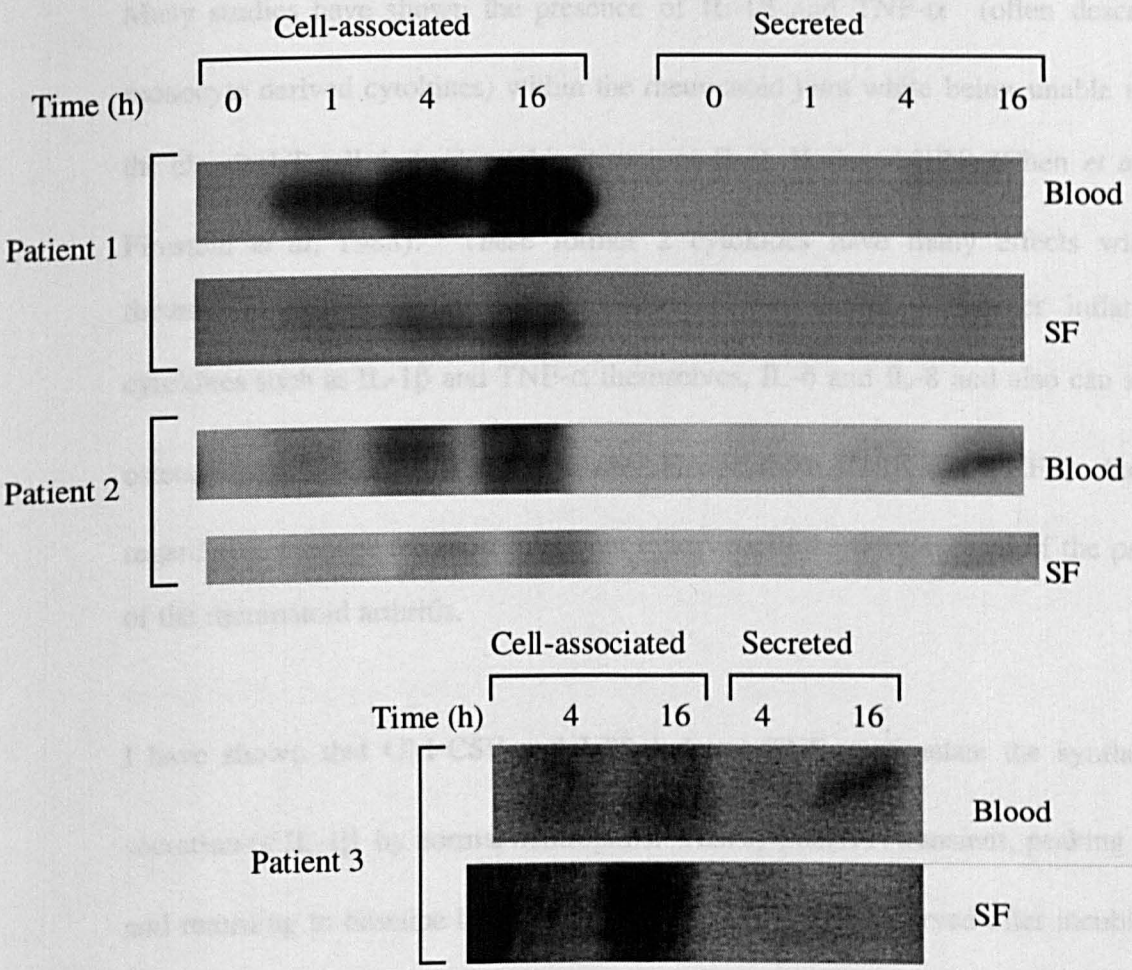
The figure shows fluorographs on which TNF-α synthesised by radiolabelled neutrophils has been analysed. The TNF-α was immunoprecipitated from cells incubated in the presence of ^{35}S-methionine and in the presence and absence of LPS (5 μg/ml), SF, obtained from an RA patient (5% v/v), soluble or insoluble IC (10% v/v). Culture conditions are described in 2.2 and the immunoprecipitation technique is described in 2.6. 4.5A shows the fluorographs on which TNF-α was detected and 4.5B shows the densitometry values calculated from the fluorographs.

FIGURE 4.6: SYNTHESIS OF IL-1β BY BLOOD AND SYNOVIAL FLUID NEUTROPHILS FROM RHEUMATOID ARTHRITIS PATIENTS



Neutrophils were obtained from the blood and synovial fluid of 3 RA patients and incubated in the presence of ^{35}S-methionine without further stimulus for periods of up to 16 h. Both cell associated and secreted IL-1β was then immunoprecipitated from the samples and analysed by SDS-PAGE and fluorography. In the case of Patients 1 and 2, the time points analysed were 0, 1, 4 and 16 h while those analysed for Patient 3 were 4 h and 16 h due to a lower number of cells obtained from the joint.

FIGURE 4.7: SYNTHESIS OF TNF-α BY BLOOD AND SYNOVIAL FLUID NEUTROPHILS FROM RHEUMATOID ARTHRITIS PATIENTS



Neutrophils were obtained from the blood and synovial fluid of 3 RA patients and incubated in the presence of ^{35}S-methionine without further stimulus for periods of up to 16 h. Both cell associated and secreted TNF-α were then immunoprecipitated from the samples and analysed by SDS-PAGE and fluorography. The time points studied were identical to those in figure 4.9 for all 3 patients.

4.3 DISCUSSION

Many studies have shown the presence of IL-1β and TNF-α (often described as monocyte derived cytokines) within the rheumatoid joint while being unable to detect the classical T cell derived cytokines such as IL-2, IL-3 and IFNγ (Chen *et al*, 1993; Firestein *et al*, 1988). These former 2 cytokines have many effects within the rheumatoid joint including the stimulation of production of further inflammatory cytokines such as IL-1β and TNF-α themselves, IL-6 and IL-8 and also can stimulate osteoclasts to resorb bone and cartilage. Hence, both IL-1β and TNF-α have been regarded as perhaps the most important cytokines in the development of the pathology of the rheumatoid arthritis.

I have shown that GM-CSF and LPS but not TNF-α stimulate the synthesis and secretion of IL-1β by normal neutrophils. This synthesis is transient, peaking at 3-5 h and returning to baseline levels by 24 h. Secretion was observed after incubations of between 3 h and 7 h. This is in contrast to monocytes which continue to synthesise cytokines for periods greater than 24 h. These results are in agreement with Lord *et al* (1991) and Marucha *et al* (1991) but not with Malyak *et al* (1994) who showed only IL-1β mRNA up-regulation following stimulation by GM-CSF and TNF-α, but no protein biosynthesis could be detected in these latter studies. This may be a result of synthesis being below the detection level for their assay, or else the degradation of IL-1β during the assay.

I have also shown the synthesis and secretion of IL-1β following stimulation with cell free synovial fluid obtained from the joints of patients with RA and other arthropathies.

Once again cellular synthesis is transient, peaking at 3-5 h and returning to baseline levels by 24 h. However, secretion of this cytokine cannot be detected until 24 h after stimulation. When cells were stimulated with synthetic soluble and insoluble IC, cellular synthesis of IL-1β was seen once again peaking at 3-5 h and returning to baseline levels by 24 h. However, whilst soluble IC stimulated a greater degree of total cellular protein synthesis and secretion than insoluble IC (Figs 3.4, 3.5), insoluble IC are more efficient at inducing IL-1β synthesis than soluble IC. It is thought that the 2 types of IC activate neutrophils by binding to different receptor/intracellular signalling systems. Secretion of IL-1β could not be detected following stimulation with IC. This may occur because IC are not sufficient stimuli to induce both the synthesis and the secretion of IL-1β: it may be that a second signal, present in synovial fluid, is necessary to induce the secretion of IC-induced IL-1β in neutrophils.

This is the first demonstration of IL-1β synthesis and secretion by neutrophils stimulated with SF from RA patients. The result suggests that neutrophils could be synthesising and secreting IL-1β in the rheumatoid joint. It could be argued that neutrophils only produce about 10% of the amount of IL-1β produced by activated monocytes and hence their contribution is unimportant. However, the numbers of neutrophils present in the rheumatoid joint could make this small production per cell significant. Neutrophils make up around 90% of the cells in the synovial fluid with only a few T cells and monocytes present. These overwhelming numbers can thus make the small per cell synthesis of IL-1β by neutrophils reach significant levels when calculated as an amount made by the entire population.

I have also demonstrated the synthesis of TNF-α by normal blood neutrophils in response to LPS with peak synthesis at 4 h and returning to baseline levels by 24 h. Again this is in agreement with previously published data (Palma *et al*, 1992; Cassatella *et al*, 1993). However I was unable to show secretion of TNF-α protein unlike the 2 groups mentioned.

I have shown synthesis of TNF-α in response to stimulation with SF from RA patients. This synthesis is greater than that seen with LPS. Both soluble and insoluble IC were able to induce TNF-α synthesis in neutrophils, with insoluble IC again being more efficient. However, I was unable to detect any secretion of TNF-α. This could be due to a number of reasons. Firstly, secreted TNF-α could be binding to the cells via the TNF-R or be bound in solution by soluble TNF-R which has been shown to be shed from activated neutrophils hence preventing it being recognised by the antibody used in this assay (Lantz *et al*, 1994). Secondly, the neutrophils may need an additional stimulus such as adherence or contact with other cell types for the TNF-α to be released. Thirdly, secreted TNF-α may be degraded in the culture medium by elastase or Cathepsin G which is also released by activated neutrophils (Scuderi *et al*, 1991; VanKessel *et al*, 1991).

Previous work in our laboratory has shown that neutrophils isolated from the rheumatoid joint contain only a small amount of mRNA for IL-1β compared to levels expressed in blood neutrophils stimulated *in vitro* with GM-CSF (Quayle *et al*, 1995). Further, blood neutrophils of RA patients sometimes contain a great deal more mRNA than paired SF neutrophils (Quayle *et al*, 1995). I have shown that this finding is also true in 2 of the 3 patients studied here when measuring protein levels in neutrophils

from the blood and synovial fluid of RA patients. These preliminary results show that blood and SF neutrophils from RA patients synthesise both IL-1β and TNF-α when cultured *in vitro* without further stimulus. This suggests that the neutrophils have been activated in the blood and joint. The amount produced by blood neutrophils is much greater than that produced by SF neutrophils in 2 of the 3 patients.

An explanation for this observation is that neutrophils are activated prior to entering the joint and the signal to synthesise IL-1β and TNF-α is encountered in the bloodstream. Once in the SF, the cells may then quickly release cytokines. The lower expression of IL-1β and TNF-α by SF neutrophils compared to other cells may be explained by the high turnover of neutrophils. Thus, an inbuilt transient expression of IL-1β and TNF-α would mean that no specific signal is required to shut off biosynthesis, ie, neutrophil generation of cytokines is a short-lived, self-limiting phenomenon. The population of SF neutrophils will therefore be very heterogeneous, with only the newly-recruited cells actively synthesising and secreting these compounds and those which have been present in SF longer will have their synthesis down-regulated.

Both IL-1β and TNF-α can have effects on many cells within the rheumatoid joint. They can act upon osteoclasts and osteoblasts to resorb bone and cartilage, inhibit cartilage production by chondrocytes, stimulate cytokine secretion by a number of cell types including macrophages, synoviocytes and neutrophils, and increase the influx of cells by enhancing adhesion molecule expression on endothelial cells. A significant production of these cytokines by neutrophils in the rheumatoid joint could allow these cells to direct the immune response against the joint tissue and bone. In addition to this, both TNF-α and IL-1β induce their own, and each others, synthesis by neutrophils. This has been observed in IFNγ stimulated neutrophils, which synthesise TNF-α

which then acts upon the neutrophils to synthesise IL-8 (Kasama *et al*, 1995). Hence, a positive feedback loop could be set up making the production of these cytokines self-perpetuating and allowing neutrophils continued control over the inflammatory response in the joint.

It is interesting to note that unlike other stimuli, SF does not induce the secretion of IL-1β until 24 h after the initial stimulus. It is possible that some agent present in SF retards IL-1β secretion in neutrophils although the reason for this is unknown. It has been shown that macrophages release IL-1β during apoptosis and this may have some relevance here. After 24 h in the rheumatoid joint, the neutrophil may be reaching the end of it's lifespan and at this point will release the newly synthesised IL-1β. This cytokine is known to enhance macrophage phagocytic activity, hence its local release may up-regulate the phagocytosis of apoptotic neutrophils by macrophages in the joint.

CHAPTER 5

PRIMING OF NEUTROPHILS BY IL-1β AND TNF-α

5.1 INTRODUCTION

The neutrophil can exist in at least 3 separate states:

(i) quiescent

(ii) primed

(iii) activated

A quiescent neutrophil is a naive cell which has not been exposed to any inflammatory stimuli. Of this cell population, approximately 50% will circulate in the blood while the other 50% will be marginated (ie loosely adherent) on blood vessel walls.

A primed neutrophil has been exposed to an inflammatory agent, and exists in a state of enhanced responsiveness. A primed cell has the ability to express a greater and more rapid response when exposed to a stimulus. Neutrophils may also be said to exist in a fourth state, ie apoptotic. Priming is a recently defined phenomenon in neutrophils and to date, little work has been done to elucidate the mechanisms involved in this process, while a great deal of investigations have been done into activation mechanisms.

The initial demonstration of neutrophil priming was published by McCall *et* al, (1979). Later, McPhail *et al* (1984) showed that neutrophils incubated with FMLP exhibited a small response of the NADPH oxidase which quickly returned to unstimulated levels.

When these cells were restimulated with a different agent, (eg the calcium ionophore A23187), a second response from the oxidase was obtained. This response was of a much greater magnitude and duration than the initial response. It was also much greater and more rapidly activated than that of cells stimulated with A23187 alone. Similar results were seen if the diacylglycerol analogue PMA was used as either the priming (first stimulus) or activating (second stimulus) agent.

Since this initial work, many inflammatory agents have been identified as neutrophil priming agents. These include, GM-CSF (Weisbart *et al*, 1985; Edwards *et al*, 1989; Yuo *et al*, 1990; Bober *et al*, 1995), IL-8 (Yuo *et al*, 1991; Elbim *et al*, 1994; Reali *et al*, 1995), TNF-α (Elbim *et al*, 1993; Berkow *et al*, 1987, Klebanoff *et al*, 1986); PAF (Biffl *et al*, 1994), IL-1β (Sullivan *et al*, 1989), IL-6 (Borish *et al*, 1989), the neurotransmitter peptide, Substance P (Lloyds *et al*, 1995), LPS (Condliffe *et al*, 1996; Worthen *et al*, 1988) and G-CSF (Yuo *et al*, 1990; Humphreys *et al*, 1991). Adhesion of neutrophils to endothelium via the integrin molecule CD11b/CD18 has also been shown to prime the cells although the priming action has been linked only to the CD18 part of the molecule (Lilies *et al*, 1995; Dapino *et al*, 1993). This may be particularly relevant *in vivo* where cytokines may upregulate adhesion molecules on endothelial cells, which then in turn prime neutrophils in preparation for leaving the bloodstream to go to an inflammatory site where they will be ready to be activated. A recent study has shown that moderate exercise can also prime neutrophils (Smith *et al*, 1996). The majority of these investigations used FMLP as the activating agent.

The effects of priming on the neutrophil are several fold. Firstly, there are short term effects occurring within 15-60 minutes of priming. These include upregulation of surface receptors such as CD11b/CD18 and FMLP-R from intracellular pools and shedding of

others, such as CD62 from the cell surface (Condliffe *et al*, 1996; Berkow *et al*, 1987; Edwards *et al*, 1990). The respiratory burst is enhanced, a response which is most commonly used to test for priming (McPhail *et al*, 1984). Changes in cell shape have also been observed during priming, indicating some alteration in the cytoskeleton (Edwards, 1994). However, one study was unable to demonstrate actin polymerisation during priming by substance P (Lloyds & Hallett, 1993). These short term effects of priming are independent of *de novo* protein synthesis and prepare the neutrophil for a maximal response when the inflammatory site is reached (Edwards *et al*, 1990).

In addition to the short term changes, there are long term effects of priming. These occur over several hours following the priming stimulus. In contrast to the short term effects, these long term effects do require *de novo* protein synthesis by the neutrophil (Hughes *et* al, 1987; Edwards *et al*, 1989). A primed neutrophil has a longer life span, often surviving several days in culture (Edwards, 1994) and the ability of these cells to produce reactive oxidants remains elevated (Edwards *et al*, 1990). The neutrophil also begins to synthesise and secrete a variety of pro-inflammatory cytokines (reviewed by Cassatella, 1995). These effects are suppressed by the protein synthesis inhibitor cycloheximide, demonstrating the need for protein synthesis to produce these cytokines and at least some components of the NADPH oxidase (Edwards *et al*, 1990).

Studies are now being done to elucidate the signal transduction pathways involved in the priming mechanism. It has become apparent that these are distinct from those involved in activation of neutrophils. Activation of the NADPH oxidase is accompanied by a 10 fold increase in intracellular Ca^{2+} (Hallett *et al*, 1990; Al-Mohanna & Hallett, 1988). However, priming does not require an increase in intracellular Ca^{2+} (Lloyds & Hallett, 1993). Evidence is mounting to suggest that protein tyrosine phosphorylation is a major signalling event in priming and many proteins are tyrosine phosphorylated during priming (Gomez-

Cambronero *et al*, 1989; McColl *et al*, 1991; Ohta *et al*, 1992). Protein tyrosine phosphorylation occurs at similar concentrations of agonists and with a similar time course as priming (Lloyds *et al*, 1995). Additionally, priming is suppressed by inhibitors of tyrosine phosphorylation such as Genistein (Akimaru *et al*, 1992). The major proteins phosphorylated are yet to be identified, although the mitogen activated protein kinase (MAPK) is thought to be one of those involved (Gomez-Cambronero *et* al, 1992; Ohta *et al*, 1992; Thompson *et al*, 1993; Nick *et al*, 1996; reviewed by Hallett & Lloyds, 1995). There is also thought to be a role for Phospholipase A_2 in priming (Roberts *et al*, 1996).

Priming offers a highly efficient way in which to regulate the activation of neutrophils. The need for 2 separate signals before full activation can be achieved can prevent against accidental activation of the neutrophil population which could prove disastrous for the host. For example, neutrophils are thought to be the major cell type causing damage in adult respiratory distress syndrome and multiple organ failure following injury, conditions which are often fatal (Botha *et al*, 1995).

Neutrophils in the rheumatoid joint are thought to have been exposed to priming and activating agents *in situ,* as assessed by comparison of the functional states of paired blood and synovial fluid samples (Nurcombe *et al*, 1991; Watson *et al*, 1993). SF can activate the oxidase in GM-CSF primed cells, a property attributed to immune complexes (Robinson *et al*, 1992). However, recent evidence suggests that neutrophils entering the rheumatoid joint may have already been primed as *de novo* synthesis of IL-1β has already begun in the blood neutrophils of RA patients (Quayle *et al*, 1995). This priming may be a result of exposure to IL-1β or TNF-α present in the blood, possibly produced by neutrophils. It has also been suggested that substance P which is present in the diseased joint may be an important priming agent (Lloyds *et al*, 1995).

Priming of neutrophils in RA must be a very important step in the development of the pathology of the disease. In this Chapter, I have shown that 2 cytokines, known to be synthesised by neutrophils, are capable of priming these cells to become activated in response to immune complexes. Most studies on priming in neutrophils use the bacterial peptide FMLP as the second, activating stimulus but this agent is unlikely to be present in the rheumatoid joint. Hence, I have used immune complexes which are thought to be the major neutrophil activating factor present in the rheumatoid joint to stimulate the cells. I have already shown in Chapter 4 that synovial fluid can stimulate synthesis of IL-1β and TNF-α in normal neutrophils, and that neutrophils isolated from synovial fluid are actively synthesising IL-1β and TNF-α. Hence, it is possible that these cytokines act in a paracrine fashion on naive neutrophils to prime them either prior to entering the joint, or immediately after entering the joint.

5.2 RESULTS

5.2.1 *Effect of GM-CSF, TNF-α or IL-1β alone on the neutrophil oxidase.*

In order to discount the possibility that the cytokines were directly activating the oxidase, the effects of these agents alone on chemiluminescence alone was tested. A graph showing the chemiluminescence traces obtained is shown in Figure 5.1 The characteristic patterns of oxidase activity seen with soluble and insoluble IC were not seen under these conditions. Only very low increases in chemiluminescence output above basal levels were observed. Hence, the cytokines alone do not activate the NADPH oxidase under these conditions.

5.2.2 *Priming of neutrophil oxidase with GM-CSF*

GM-CSF is known to prime neutrophils to respond to both soluble and insoluble immune complexes and so this cytokine was used as a positive control for these experiments. Experiments were performed as described in 2.8. In essence, neutrophils were incubated in the presence and absence of cytokines and their ability to generate reactive oxidants or mobilize intracellular Ca^{2+} were measured. Figure 5.2 is a graph showing the effect of GM-CSF on priming the NADPH oxidase for a further response to soluble (A) and insoluble (B) immune complexes. Neutrophils were primed for 1 h with 14ng/ml GM-CSF then the oxidase activated using immune complexes. Previous work has shown these to be optimal conditions for priming with GM-CSF. The graphs show that GM-CSF does indeed prime the neutrophils to give a greater response to subsequent stimulation with soluble but not insoluble IC. It has previously been demonstrated that soluble and insoluble IC induce responses with differing kinetics and this is apparent from these experiments (Robinson *et al*, 1992). Soluble IC stimulate a rapid burst of oxidase activity

in primed cells, peaking and returning to a baseline level within 5 min. Insoluble IC stimulate a much slower, but longer lasting response in either primed or unprimed cells which reaches a peak level at around 8 min and continues at this level for some time. It is noteworthy that the soluble IC failed to activate the respiratory burst in unprimed neutrophils.

5.2.3 *Priming of neutrophil oxidase with TNF-α*

It was then necessary to determine (a) if TNF-α could also prime neutrophils for their response to IC and (b) if so, to establish the optimal incubation conditions required for priming. Neutrophils were incubated either with or without TNF-α (10, 25, 50 ng/ml) for 15 min and 1 h, and then samples were stimulated with either soluble or insoluble IC. Figure 5.3 shows the priming action of TNF-α on the neutrophil NADPH oxidase. TNF-α was shown to prime neutrophils very rapidly. Thus, within 15 min incubation with all concentrations of TNF-α used oxidase activity was primed, but 10 ng/ml was found to be most effective concentration. This priming was effective for stimulation with soluble IC but only cells incubated with 25 ng/ml TNF-α for 15 min were activated by insoluble IC. The priming was still evident in soluble IC stimulated cells after 1 h incubation, although not in insoluble IC stimulated cells. The amount of chemiluminescence detected was greater than that seen with GM-CSF after 1 h incubation ie TNF-α was a more potent neutrophil priming agent under these conditions.

5.2.4 *Priming of neutrophil oxidase with IL-1β*

The potential priming effect of IL-1β on NADPH oxidase activity was then investigated. Neutrophils were thus incubated with or without IL-1β (10, 25, 50 ng/ml) for 15 min and 1 h, and then stimulated with either soluble or insoluble IC. The results of these experiments can be seen in Figure 5.4. Again, IL-1β primed the neutrophil oxidase very rapidly, (within 15 min incubation), the kinetics of priming being similar to TNF-α. However, in this case, the amount of chemiluminescence detected was lower than that seen with TNF-α. 50ng/ml of IL-1β gave the greatest priming response for soluble IC at 15 min but this effect had all but disappeared by 1 hour of incubation. The 50 ng/ml concentration also gave the greatest priming response for insoluble IC. IL-1β had little effect on oxidase activity stimulated by insoluble IC, but greater effects on that stimulated by soluble IC. However, oxidase activity stimulated by soluble IC had decreased after 1 h incubation.

5.2.5 *Measurement of intracellular Ca^{2+} changes in TNF-α and IL-1β primed neutrophils during activation with soluble IC.*

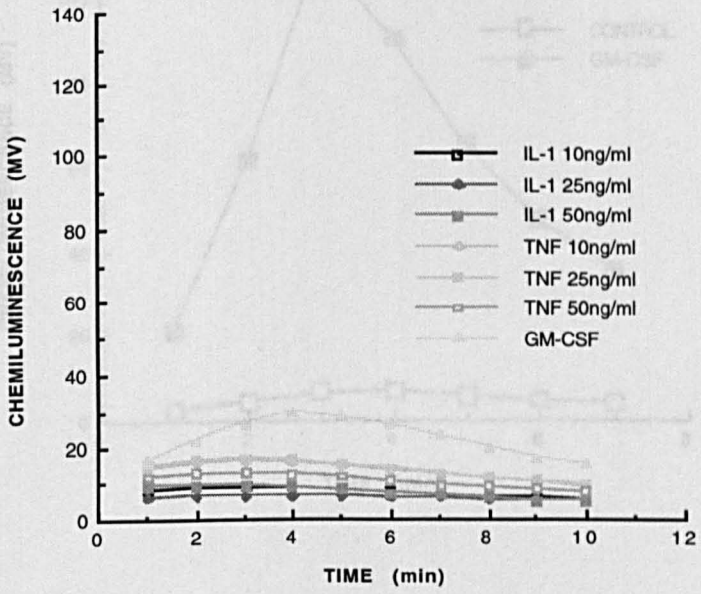
When Fluo-3 loaded neutrophils were placed in a fluorimeter, the basal fluorescence value corresponding to a resting intracellular Ca^{2+} concentration of 109.5 nM (±70, n=6) was obtained (Fig 5.5). When the cells were stimulated with soluble IC, the intracellular Ca^{2+} level increased rapidly and peaked within 60 s of stimulation and then rapidly returned to

pre-stimulation levels. Maximal intracellular Ca^{2+} levels obtained were 341.6 nM (±239.4, n=4). When the suspensions were activated with FMLP (100 nM), the initial rise in intracellular Ca^{2+} was slightly increased, but an "extra" intracellular Ca^{2+} signal was observed 2.5 min after stimulation. Previous work has shown that this extra Ca^{2+} signal arises from Ca^{2+} influx (Merrit *et al*, 1990). When neutrophils were primed with TNF-α (50 ng/ml for 10 min) the kinetics of the intracellular Ca^{2+} transient following stimulation with soluble IC was altered (Fig 5.6A). The magnitude of the initial Ca^{2+} transient was increased and an "extra" Ca^{2+} signal was observed in the TNF-α primed cells.

It was then necessary to determine whether these intracellular Ca^{2+} signals arose from mobilization of intracellular Ca^{2+} stores, or from Ca^{2+} influx. This was investigated by stimulating neutrophils in Ca^{2+} free medium containing EGTA (1mM) (Fig 5.6B). The kinetics of the initial intracellular Ca^{2+} transient seen in the primed and unprimed responses were largely unaltered in Ca^{2+} free medium. This indicates that the initial Ca^{2+} rise is due to mobilization of intracellular stores. However, the "extra" intracellular Ca^{2+} signal seen only in primed cells was not observed in Ca^{2+} free medium. This indicates that the "extra" Ca^{2+} signal induced by priming arises largely from Ca^{2+} influx.

Similar intracellular Ca^{2+} transients were seen in IL-1β-primed neutrophils. In these experiments, the magnitude of the intracellular Ca^{2+} transients were unexpectedly low (Fig 5.7). However, it can clearly be seen that (i) IL-1β priming similarly induces an "extra" Ca^{2+} signal (Fig 5.7a) and (ii) the "extra" Ca^{2+} arises from Ca^{2+} influx (Fig 5.7b).

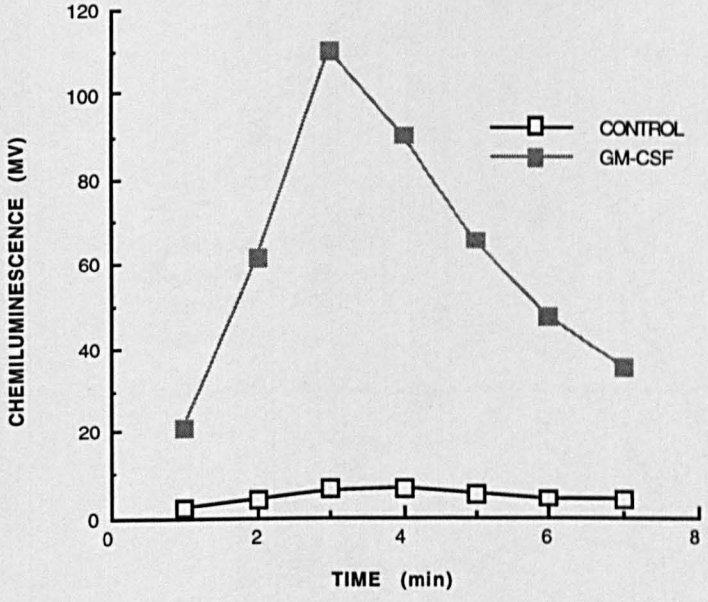
FIGURE 5.1: EFFECT OF PRIMING AGENTS ON NADPH OXIDASE ACTIVITY



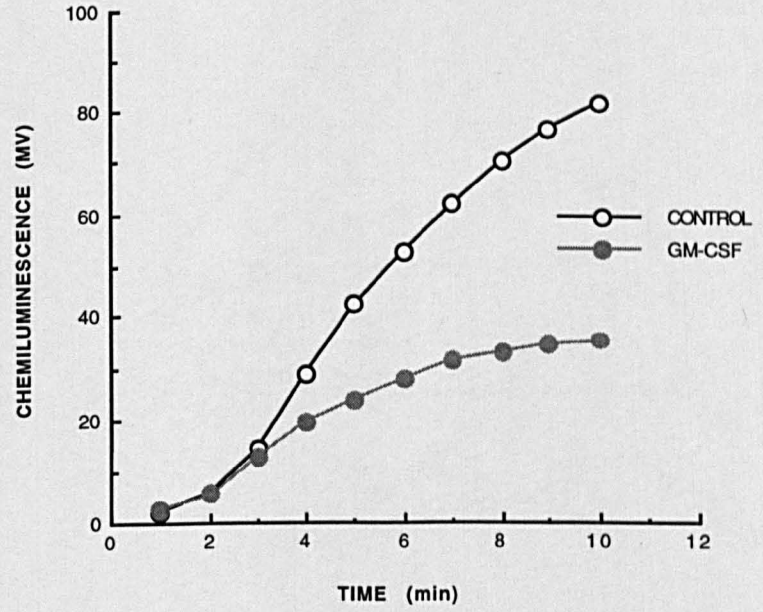
Neutrophils were incubated in the absence (control) and presence of GM-CSF (14 ng/ml), TNF-α (10, 25, 50 ng/ml) and IL-1β (10, 25, 50 ng/ml) for 1 h. Following incubation, oxidase activity was measured using the chemiluminescence assay described in 2.8 but without activating the neutrophils with immune complexes. The data are plotted using axes scales used in Figure 5.2-5.4, for comparison.

FIGURE 5.2: PRIMING OF THE NEUTROPHIL NADPH OXIDASE WITH GM-CSF

A



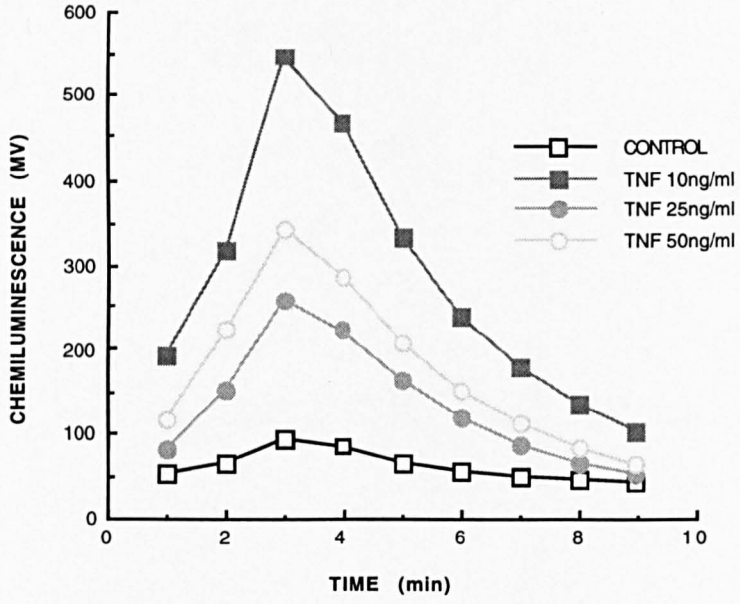
B



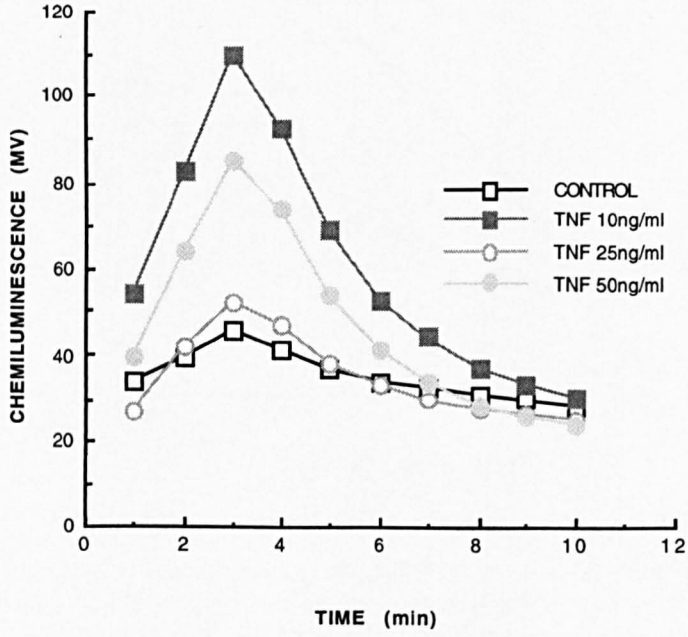
Neutrophils were incubated for 1 h in the absence (control) and presence of GM-CSF (14 ng/ml). After incubation, the oxidase activity following activation with soluble and insoluble IC (10% v/v) was assessed using the luminol chemiluminescence assay as described in 2.8. The results are shown in (A) of stimulation with soluble IC and in (B) of stimulation with insoluble IC.

FIGURE 5.3: PRIMING OF THE NEUTROPHIL NADPH OXIDASE WITH TNF-α

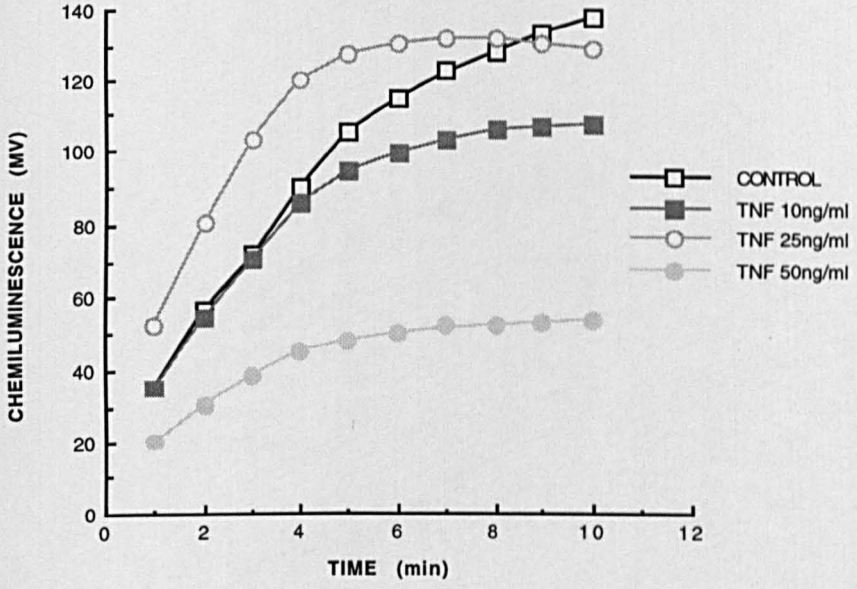
A



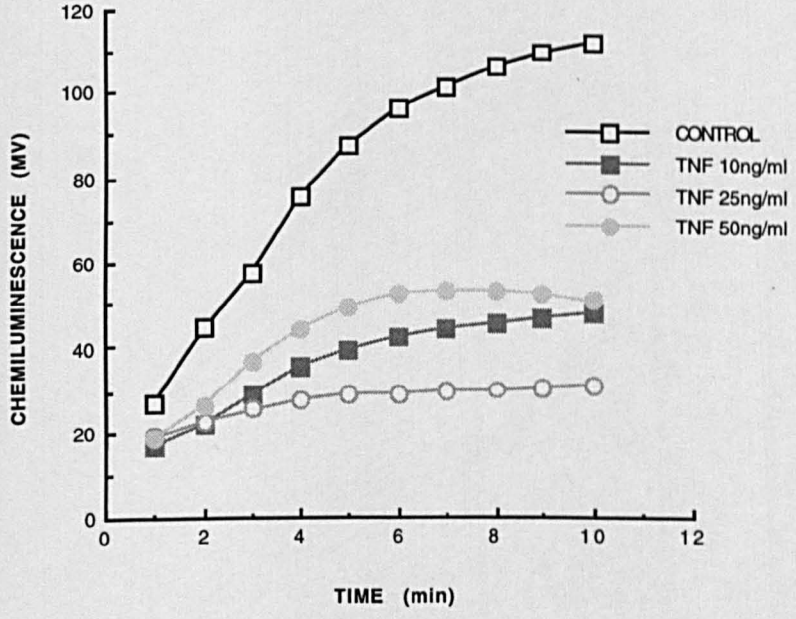
B



C



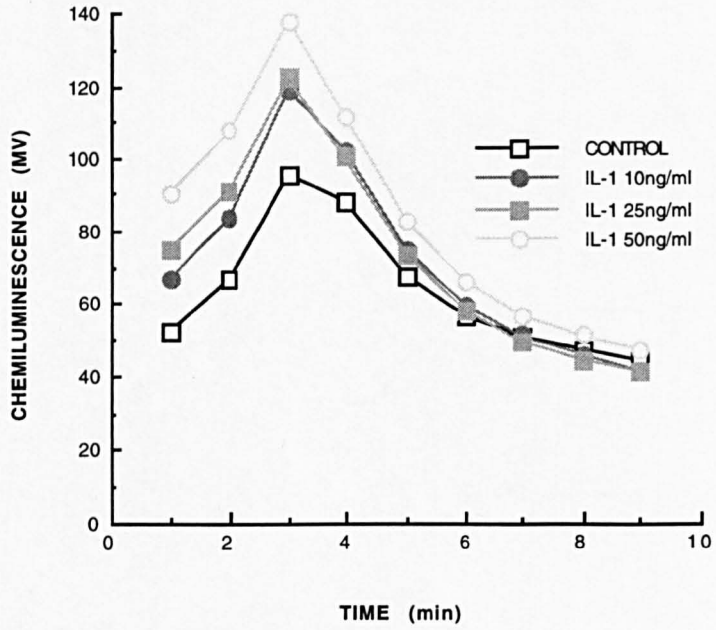
D



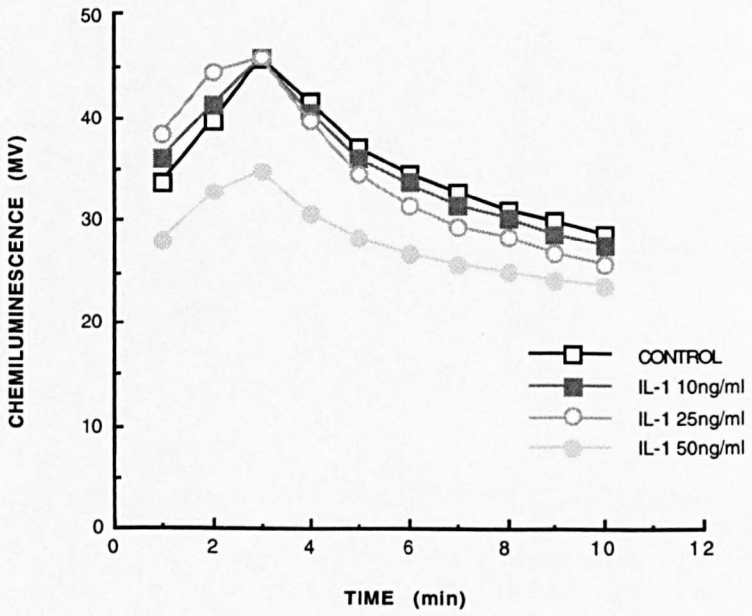
Neutrophils were incubated for 15 min and 1 h in the absence (control) and presence of TNF-α (10, 25 and 50 ng/ml) to prime them. After incubation, the oxidase activity following activation with soluble and insoluble IC (10% v/v) was assessed using the chemiluminescence assay as described in 2.8. The results shown are of stimulation with soluble IC for 15 min (A), and 1 h (B), and of stimulation with insoluble IC for 15 min (C) and 1 h (D).

FIGURE 5.4: PRIMING OF THE NEUTROPHIL NADPH OXIDASE WITH IL-1β

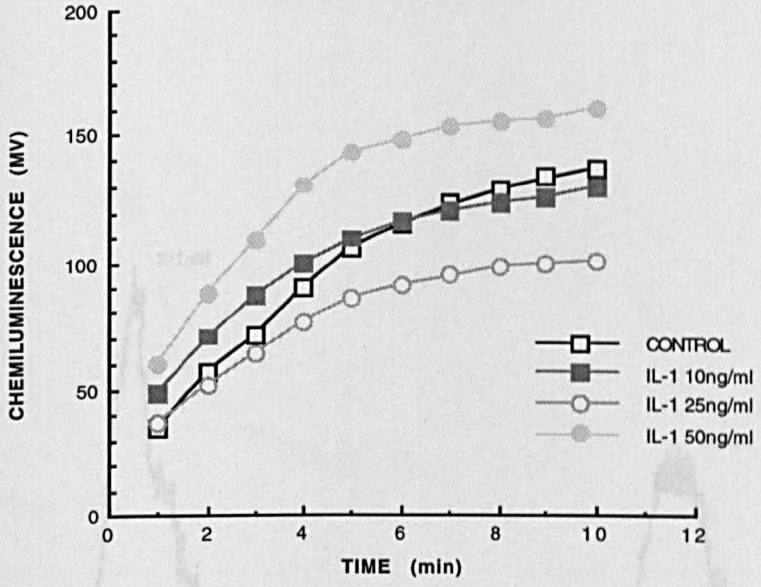
A



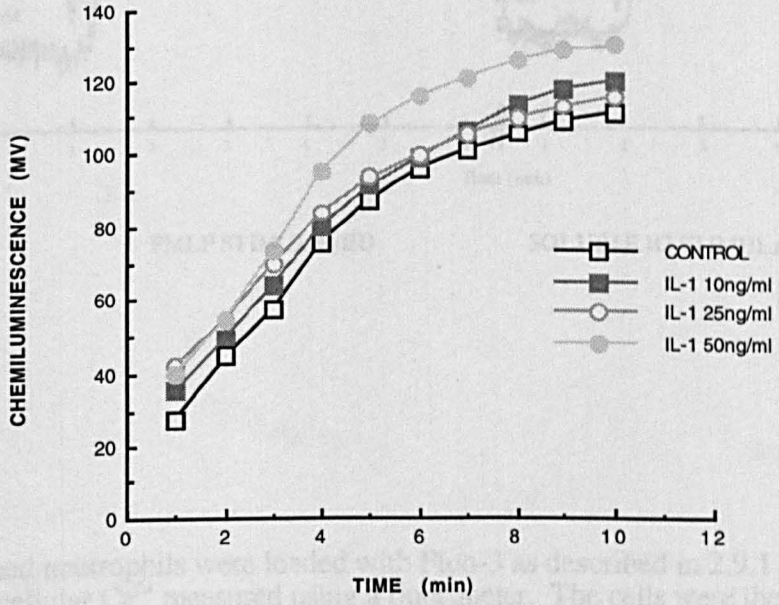
B



C

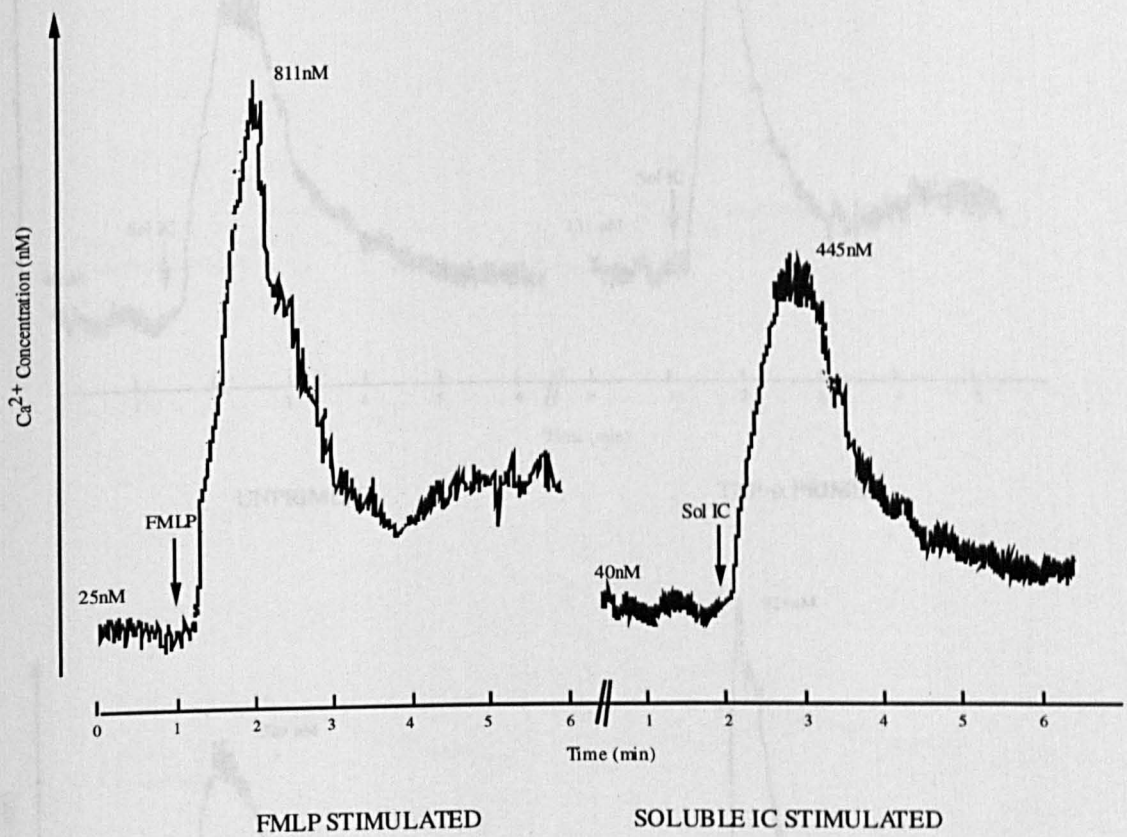


D



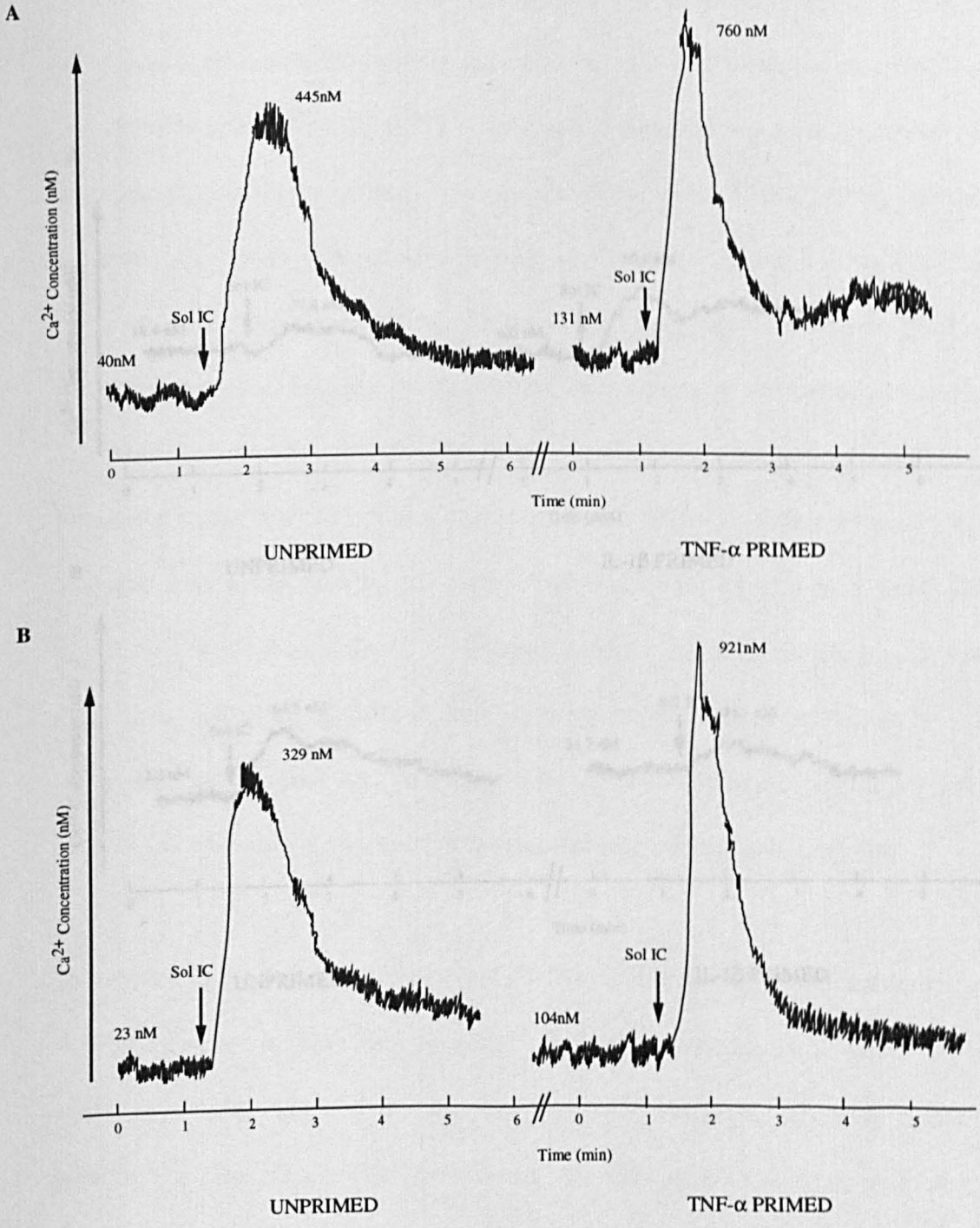
Neutrophils were incubated for 15 min and 1 h in the absence (control) and presence of IL-1β (10, 25 and 50 ng/ml). After incubation, oxidase activity following activation with soluble and insoluble IC (10% v/v) was assessed using the chemiluminescence assay as described in 2.8. The results shown are of stimulation with soluble IC for 15 min (A), and 1 h (B), and of stimulation with insoluble IC for 15 min (C) and 1 h (D).

FIGURE 5.5: MEASUREMENT OF INTRACELLULAR Ca^{2+} CHANGES IN NEUTROPHILS DURING ACTIVATION WITH FMLP AND SOLUBLE IMMUNE COMPLEXES



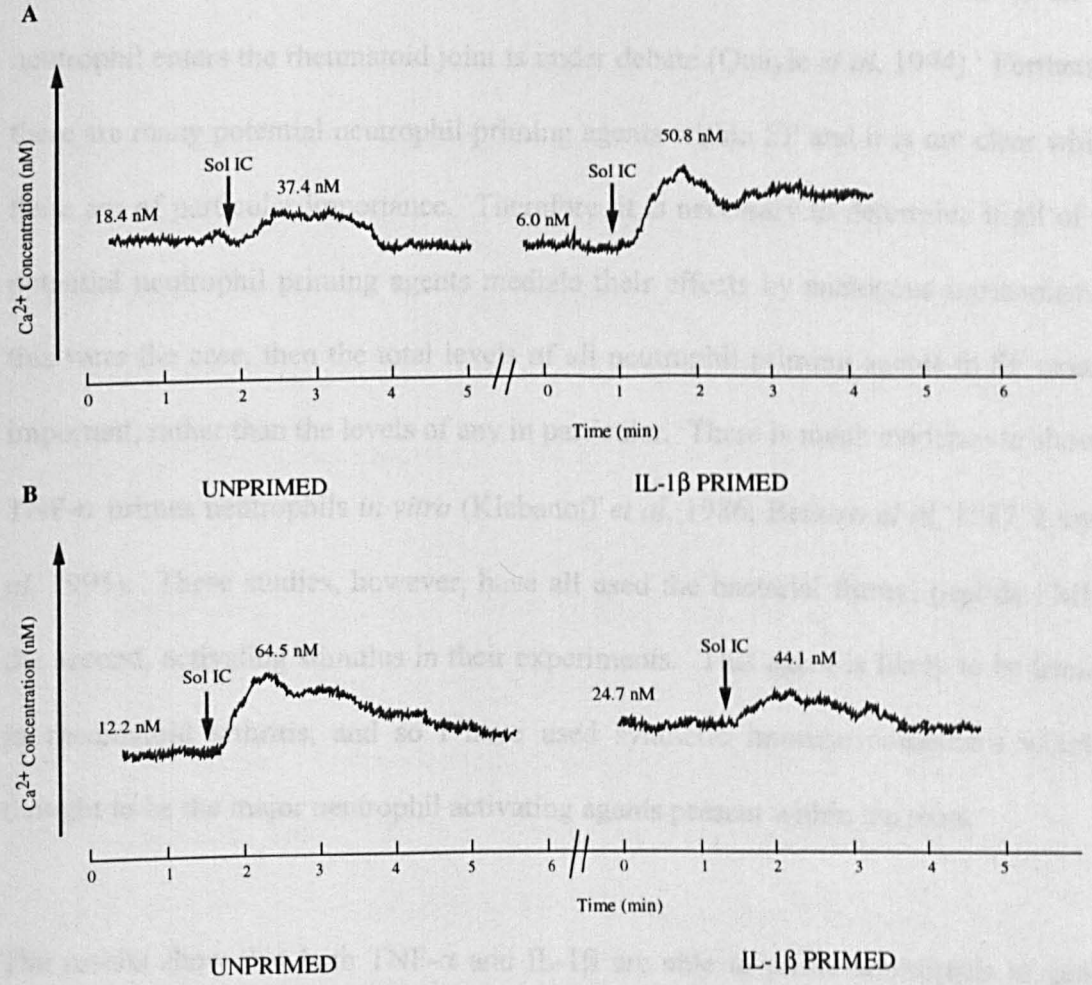
Unprimed neutrophils were loaded with Fluo-3 as described in 2.9.1 and the basal level of intracellular Ca^{2+} measured using a fluorimeter. The cells were then stimulated with either FMLP (100nM) or soluble IC (7% v/v) and the change in intracellular Ca^{2+} levels measured.

FIGURE 5.6: EFFECT OF PRIMING OF NEUTROPHILS WITH TNF-α ON INTRACELLULAR Ca^{2+} CHANGES OBSERVED DURING ACTIVATION BY SOLUBLE IMMUNE COMPLEXES



Neutrophils were loaded with Fluo-3 and then incubated in the absence (unprimed) and presence (primed) of TNF-α (25 ng/ml) for 10 min and in the presence (A) and absence (B) of extracellular Ca^{2+}. The basal level of intracellular Ca^{2+} was measured using a fluorimeter and the cells were then activated with soluble IC (7% v/v). The change in intracellular Ca^{2+} levels was then measured and the results displayed above. (A) shows the trace obtained when neutrophils were suspended in the presence of extracellular Ca^{2+} and (B) shows the trace obtained when neutrophils were suspended in Ca^{2+} free medium containing EGTA (1mM).

FIGURE 5.7: EFFECT OF PRIMING OF NEUTROPHILS WITH IL-1β ON INTRACELLULAR Ca^{2+} CHANGES DURING ACTIVATION WITH SOLUBLE IMMUNE COMPLEXES



Neutrophils were loaded with Fluo-3 and then incubated in the absence (unprimed) and presence (primed) of IL-1β (25 ng/ml) for 10 min and in the presence and absence of extracellular Ca^{2+}. The basal level of intracellular Ca^{2+} was measured using a fluorimeter and the cells were then activated with soluble IC (7% v/v). The change in intracellular Ca^{2+} levels was then measured and the results displayed above. (A) shows the trace obtained when neutrophils were suspended in the presence of extracellular Ca^{2+} and (B) shows the trace obtained when neutrophils were suspended in Ca^{2+} free medium containing EGTA (1mM).

5.3 DISCUSSION

Evidence from the literature strongly indicates that neutrophils within the rheumatoid joint have been exposed to priming and activating stimuli (Dularay *et al*, 1988; Watson *et al*, 1993; Nurcombe *et al*, 1991). However, whether this occurs before or after the neutrophil enters the rheumatoid joint is under debate (Quayle *et al*, 1994). Furthermore, there are many potential neutrophil priming agents within SF and it is not clear which of these are of particular importance. Therefore, it is necessary to determine if all of these potential neutrophil priming agents mediate their effects by analogous mechanisms. If this were the case, then the total levels of all neutrophil priming agents in SF would be important, rather than the levels of any in particular. There is much evidence to show that TNF-α primes neutrophils *in vitro* (Klebanoff *et al*, 1986; Berkow *et al*, 1987; Lloyds *et al*, 1995). These studies, however, have all used the bacterial formyl peptide FMLP as the second, activating stimulus in their experiments. This agent is likely to be irrelevant in rheumatoid arthritis, and so I have used synthetic immune complexes which are thought to be the major neutrophil activating agents present within the joint.

The results show that both TNF-α and IL-1β are able to prime neutrophils to generate ROI in response to immune complexes. I have shown this in 2 ways. Firstly, an enhanced oxidase response to immune complexes is seen following priming, and secondly transient rises in $[Ca^{2+}]_i$ following activation with immune complexes showing priming dependent changes. Priming takes place very rapidly, within 15 minutes after

exposure to TNF-α and IL-1β. This is in contrast to GM-CSF which requires incubation times in excess of 30 min to achieve maximum priming (data not shown). The priming effect on oxidase activity by TNF-α and IL-1β was still apparent after incubation for 1 hour.

Although priming by TNF-α and IL-1β occurred within simular time courses, the degree of functional enhancement was different for these cytokines. TNF-α was a much more potent priming agent than both IL-1β and GM-CSF, under these conditions. Several concentrations of both IL-1β and TNF-α were tested to determine the optimal concentrations required for priming the oxidase. In the case of TNF-α, this was found to be 10 ng/ml. The optimal concentration for IL-1β was found to be at 25-50 ng/ml.

The concentration of 25 ng/ml of TNF-α or IL-1β was used to prime the neutrophils in the second part of the work measuring intracellular Ca^{2+} transients. Although priming does not induce a Ca^{2+} transient itself, subsequent activation of the cell can, depending upon the stimulus, alter the kinetics of these transients. In the case of unprimed cells, activation with immune complexes leads to a rapid and transient increase in $[Ca^{2+}]_i$. The peak is achieved relatively quickly and is unaffected by removal of extracellular Ca^{2+}. This would indicate that this increase in Ca^{2+} levels in unprimed cells comes from the mobilisation of internal stores. After the initial peak, the Ca^{2+} level declines to a level only slightly above the resting level in the presence of extracellular Ca^{2+} and declines to the resting level in the absence of extracellular Ca^{2+}.

When the cells have been primed prior to activation by immune complexes, the pattern of the Ca^{2+} transient is altered. The initial peak is slightly enhanced and instead of the level returning to resting, a second peak is observed. As with unprimed cells, the initial peak is unaffected by the removal of extracellular Ca^{2+}, but, the second peak is totally abolished in the absence of extracellular Ca^{2+}. This would indicate that the second peak seen in primed cells is the result of an influx of extracellular Ca^{2+}.

As with the results observed for priming the oxidase response, measuring $[Ca^{2+}]_i$ levels showed TNF-α to be a more effective priming agent than IL-1β. The peaks of $[Ca^{2+}]_i$ obtained were greater when priming was achieved with TNF-α. The concentration of TNF-α 25ng/ml was effective for priming and pre-incubation times of only 3-5 min could achieve priming.

These results taken together show that TNF-α and IL-1β are capable of priming neutrophils for activation by soluble immune complexes. This is relevant in RA as both IL-1β and TNF-α are present in the SF, and may also be present in the blood of patients. SF also contains immune complexes, both soluble and insoluble which can activate the neutrophils after they become primed by TNF-α or IL-1β.

The presence of IL-1β and TNF-α in the rheumatoid joint makes either agent a good candidate for the neutrophil priming agent. Recent results published by Quayle *et al*,

(1995) showing increased amount of IL-1β mRNA in the blood neutrophils compared to SF neutrophils of RA patients, and also those presented in Chapter 4 suggest that IL-1β and TNF-α may be synthesised by neutrophils in the bloodstream of rheumatoid arthritis patients. This suggests that the neutrophils have already been primed in the bloodstream, and if they secrete the cytokine prior to leaving the bloodstream this provides a route by which further resting neutrophils can be primed before entering the joint.

The priming of neutrophils in RA is likely to contribute to the pathogenesis of the disease. Primed neutrophils are able to respond more quickly and aggressively to stimuli such as the immune complexes present in the synovial fluid. Several receptors are up-regulated either via mobilisation of internal stores or *de novo* protein synthesis which increases the neutrophil's ability to respond to stimuli. Some of the components of the NADPH oxidase are synthesised and the magnitude of the respiratory burst is increased. These effects of priming can be detected several hours after the original stimulus and the lifespan of the neutrophil is dramatically increased from 8-20 h to several days. Hence, priming of the neutrophils in the joint, or before entering the joint, can perpetuate the chronic inflammation that characterises rheumatoid arthritis. It will then be of interest to determine if IL-1β and TNF-α also induce neutrophil protein synthesis, which would result in a further up-regulation of neutrophil function in disease.

CHAPTER 6

NEUTROPHIL APOPTOSIS IN THE RHEUMATOID JOINT

6.1 INTRODUCTION

The neutrophil is a short lived cell with an estimated half life in the circulation of approximately 8-20 hours, although this can be much longer if the cell is recruited into tissues during inflammation. Neutrophils constitutively undergo a process termed apoptosis or programmed cell death. This is distinct from necrotic cell death which involves swelling of the cytoplasm and eventual cell rupture. In contrast, apoptosis, first described in 1972, does not cause cell rupture. Instead the apoptotic cells are cleared by phagocytic cells and so degradative cellular constituents (eg proteases) are not released into tissues where they might otherwise cause damage (Kerr *et al*, 1972).

Apoptosis is characterised by condensation of the chromatin in the nucleus, cell shrinkage and fragmentation of the nucleus into apoptotic bodies. The DNA is cleaved by an endogenous endonuclease into small fragments of approximately 200 base pairs (Kerr *et al*, 1972). In addition to this, the functional capability of the cell is decreased. Many functions are impaired in apoptotic neutrophils including spreading on surfaces, phagocytosis, chemotaxis, NADPH oxidase activity and the ability to degranulate (Whyte *et al*, 1993).

Some of this loss of functional capacity can be attributed to a decrease in cell surface receptors. The classical receptor studied during neutrophil apoptosis is the low affinity IgG receptor CD16 (FcγRIIIb). Surface expression of this molecule is decreased in

apoptotic neutrophils (Dransfield *et al*, 1994). Other receptors which are downregulated on apoptotic neutrophils are CD35 (CR1), the selectins CD43 and sialyl CD15 and one of the α chains of the β2 integrins CD11a. The remaining β2 integrins appear to be in an inactive form (Dransfield *et al*, 1995).

Several of these morphological and functional changes can be used as markers for neutrophil apoptosis. The most common measure of apoptosis is morphology, as the cells become smaller and the nucleus loses its characteristic multi-lobed shape and becomes rounder and more densely stained. Other markers for apoptosis that are often used include the loss of CD16 expression from the cell surface, impaired NADPH oxidase activity and the cleavage of DNA into 200 bp fragments which can be detected as a ladder pattern on an agarose gel.

After apoptotic neutrophils have become functionally inactive, they are recognised and phagocytosed by macrophages and other professional phagocytic cells (Kerr *et al*, 1972; Savill *et al*, 1989). The method by which macrophages recognize apoptotic neutrophils is distinct from other phagocytic mechanisms, as the ingestion of an apoptotic neutrophil does not elicit the release of inflammatory mediators seen when macrophages ingest pathogens (reviewed by Haslett *et al*, 1994; Savill, 1994). The uptake of apoptotic neutrophils by macrophages has been demonstrated in several inflammatory diseases and is thought to control the resolution of inflammation. Macrophages containing apoptotic neutrophils have been observed in synovial fluid from several inflammatory arthritis conditions including RA (Jones *et al*, 1993; Savill *et al*, 1989). Recognition of apoptotic neutrophils by macrophages is thought to be mediated by integrins, in particular the $\alpha_v\beta_3$ vitronectin receptor (reviewed by Savill, 1994). Monoclonal antibodies to this receptor have been shown to inhibit uptake of apoptotic neutrophils (Savill *et al*, 1990). Recognition can also be inhibited by acidic conditions and cationic particles. At an inflammatory site, the pH is acidic which may

inhibit the uptake of apoptotic neutrophils. This inhibition may allow the neutrophils to progress beyond apoptosis and into necrosis, leading to the release of the cellular contents which includes many cationic particles. This may then lead to the further inhibition of macrophage uptake, forming a vicious circle and perpetuating inflammation (reviewed by Haslett, 1992).

In many respects, neutrophils have been shown to be different to other cells undergoing apoptosis. This may be because these cells constitutively undergo apoptosis whereas other cell types such as thymocytes must be induced to undergo apoptosis. Studies have shown that an elevation in $[Ca^{2+}]_i$ delays the onset of apoptosis in neutrophils (Cousin *et al*, 1995; Whyte *et al*, 1993). This is in contrast to thymocytes in which an elevation in $[Ca^{2+}]_i$ has been shown to be a necessary, if not a directly stimulating event for apoptosis (McConkey *et al*, 1990; Kluck *et al*, 1994). Other studies have shown that an increase in cAMP mediated by cAMP elevating agents such as Prostaglandin E_2 or cAMP analogues retard apoptosis in neutrophils (Rossi *et al*, 1995) while promoting it in thymocytes (McConkey *et al*, 1990; reviewed by Schwartzman & Cidlowski, 1993).

Protein synthesis has been demonstrated to be necessary for the onset of apoptosis in almost all cell types (reviewed by Schwartzman & Cidlowski, 1993). This suggests that the cell must transcribe a "death gene" in order to initiate the apoptotic process. Such genes include members of the *bcl-2* gene family and the *fas* gene. Again, in contrast, the neutrophil requires active protein synthesis to rescue it from apoptosis (Stringer *et al*, 1996; Brach *et al*, 1992).

Much work has been done to find factors which can rescue neutrophils from apoptosis. Most of these factors are inflammatory agents found *in vivo* but there is also an interest in identifying drugs which can delay apoptosis in neutrophils. These drugs may be

important in treating diseases in which neutropenia is a symptom or side effect of treatment.

Apoptosis can only be delayed, but not completely prevented in neutrophils. Agents which have been shown to achieve this delay include GM-CSF (Brach *et al*, 1992; Yamamoto *et al*, 1993), IL-1β (Colotta *et al*, 1992), IL-2 (Pericle *et al*, 1994), LPS (Lee *et al*, 1993; Yamamoto *et al*, 1993), IFNγ (Colotta *et al*, 1992; Klebanoff *et al*, 1992), G-CSF (Colotta *et al*, 1992) and the platelet derived products ATP, and diadenosine phosphates, Ap_3A and Ap_4A (Gasmi *et al*, 1996). Some drugs have been shown to delay apoptosis, such as glucocorticoids, although only those with anti-inflammatory activity (Lilies *et al*, 1995) and also sodium butyrate is effective (Stringer *et al*, 1996).

TNF-α has been shown by different authors to have opposing effects on neutrophil apoptosis. Takeda *et al* (1993) have shown that TNF-α actually enhances apoptosis and suggest that a similarity of the TNF receptor to the Fas antigen may be involved in this effect. Conversely, Colotta *et al* (1992) have shown that TNF-α rescued neutrophils from apoptosis. There are also conflicting results on the effects of other agents on apoptosis. For example, G-CSF, C5a, FMLP and IL-6 have all been shown to delay apoptosis in some studies (Lilies *et al*, 1995; Lee *et al*, 1993; Biffl *et al*, 1996) but not in others (Brach *et al*, 1992; Colotta *et al*, 1992). These conflicting results may be due to differences in experimental technique.

Little is known about the genetic control of neutrophil apoptosis. To date no group have been able to detect expression of the gene family *bcl-2* in mature neutrophils. The *fas* gene is thought to be of more importance in neutrophils. Fas is a protein of the TNF-R/nerve growth factor (NGF) receptor family expressed on many cell types which

mediates cell death via apoptosis after interaction with the Fas ligand or anti-Fas antibodies. Normal neutrophils are very susceptible to Fas mediated death (Lilies *et al*, 1994). Neutrophils express the Fas ligand, a member of the TNF/NGF family (Iwai *et al*, 1994) and so it is possible that co-expression of Fas and FasL could mediate autocrine cell death (reviewed by Lilies & Klebanoff, 1995). It is also known that neutrophils express the cysteine protease, IL-1 converting enzyme (ICE). This gene product may also have a role in apoptosis as it has been shown to have sequence homology with the death gene *ced-3* of the nematode *Caenorhabditis elegans* (Yuan *et al*, 1993). Other groups have demonstrated the processing and release of IL-1β during apoptosis of macrophages (Hogquist *et al*, 1991).

Neutrophil apoptosis and clearance by macrophages is thought to be an important process in the resolution of inflammation. Hence, the regulation of apoptosis in the chronic inflammatory disease RA may be in some way impaired as the inflammation is not resolved in this disease, but persists for many years. In the experiments described in this Chapter, I have mimicked the conditions in the rheumatoid joint in order to study their effects on neutrophil apoptosis. A recently published study has shown that synovial fluid, rather than enhancing the neutrophil's lifespan, actually promotes apoptosis (Bell *et al*, 1995). I have repeated this study to confirm the results and then I have gone on to examine the effects of hypoxia and the ability of the neutrophil to produce reactive oxygen intermediates on apoptosis. ROI have been implicated as a cause of apoptosis in neutrophils (Watson *et al*, 1995). Other studies have concluded that hypoxia delays apoptosis in neutrophils (Hannah *et al*, 1995; Mecklenburgh *et al*, 1996). The rheumatoid joint is a hypoxic environment, containing only 10-20% of the O_2 tension of air (Edwards *et al*, 1984). I have examined the possibility that in this hypoxic environment, synovial fluid does not have the devastating effect on neutrophil survival that is seen when they are cultured in an O_2 rich laboratory environment.

6.2 RESULTS

6.2.1 *Effect of synovial fluid on neutrophil apoptosis*

SF can activate neutrophil protein synthesis, a process known to be necessary to rescue neutrophils from apoptosis. It is also a rich source of cytokines that when used experimentally, can delay neutrophil apoptosis *in vitro*. Hence I have incubated neutrophils with different concentrations of heterologous SF and then examined the effects on apoptosis. Neutrophils were incubated for 18-20 h in the presence of 0% or 5-75% (v/v) SF (as described in 2.10), and then the proportion of apoptotic cells assessed by morphology and a functional assay, namely NADPH oxidase activity.

6.2.1.1 *Morphology*

Figure 6.1 shows the results obtained from 4 separate experiments The percentage of apoptotic cells in control suspensions incubated for 18 h in culture ranges from 67.6-76.5% (mean value 71.8% ± 3.7). This value was similar when cells were incubated with 5% and 10% v/v SF (mean values of 70.1%, ± 6.6 and 69.1%, ± 6.5 respectively) ie these low concentrations of SF did not enhance or delay apoptosis. However, when the volume of SF was increased to 25% (v/v) the number of apoptotic cells increased to 81.1% (± 6.3) and as the volume of SF is increased again to 50% and 75% (v/v), the percentage of apoptotic cells increases still further (96.5%,± 2.9 and 99.2%, ± 0.9 respectively). These high concentrations of SF significantly accelerated neutrophil death by apoptosis.

6.2.1.2 *NADPH Oxidase activity*

Figure 6.2 shows a graph of the oxidase activity of the neutrophils after incubation for 18-20 h, measured by the chemiluminescence assay (as described in 2.12). These results parallel those of the morphological assays described in Figure 6.1. Control cells and those incubated with 5% and 10% (v/v) SF showed similar levels of oxidase activity which began to decrease when 25% (v/v) SF was added. When the volume of SF in the culture was increased to 50% and 75% (v/v), the oxidase activity was very low. This result mimicked the data obtained in the morphology results, in which a high proportion of cells were apoptotic and hence would be predicted to have impaired functional responses.

6.2.2 *Effect of oxidant scavengers on neutrophil apoptosis*

ROI are thought to be responsible, at least in part, for the onset of apoptosis. However, under hypoxic conditions, neutrophils produce decreased amounts of ROI due to the limited supply of O_2. Rather than attempt to incubate neutrophils under hypoxic conditions, I have mimicked hypoxia by using ROI scavengers to remove any oxidants that the neutrophils may produce. The scavengers used were superoxide dismutase (SOD) which scavenges O_2^-, and catalase which scavenges H_2O_2.

6.2.2.1 *Morphology*

Figure 6.3 shows the percentage of apoptotic neutrophils following 18-20 h incubation with or without SOD (250 and 500 μg/ml) and catalase (200 and 400 μg/ml). The mean value of apoptotic cells in control cultures was 71.4% (± 6.4) (n=5). The addition of 250 and 500 μg/ml SOD reduced the proportion of apoptotic cells to 59.9% (± 8.3) (n=3) and 57.9% (± 7.2) (n=4) respectively. Catalase reduced the numbers of

apoptotic cells still further to 47.8% (± 11.0) (n=3)(at 200 μg/ml) and 47.7% (± 13.2) (n=4)(at 400 μg/ml). These results were found to be statistically significant when catalase alone or catalase in combination with SOD were used ($p<0.02$). Using both SOD and catalase together did not decrease the number of apoptotic cells any further.

6.2.2.2 *NADPH Oxidase activity*

Figure 6.4 shows a graph of the oxidase activity of neutrophils incubated for 18-20 h in the presence and absence of SOD and catalase. The stimulus for the oxidase was PMA. The results once again, parallel those of morphology although in this case they are more marked with the cells incubated with scavengers having much greater levels of oxidase activity than control cells. Again, catalase was more effective in delaying apoptosis than SOD and the addition of both together did not enhance the effect of catalase alone. Only the addition of catalase (both 200 and 400 μg/ml) was found to produce a statistically significant result. This would indicate that H_2O_2, rather than O_2^-, may play a role in neutropil apoptosis.

6.2.3 *Effect of both synovial fluid and oxidant scavengers on neutrophil apoptosis*

In these experiments, I tested whether scavengers could rescue neutrophils from the accelerated apoptosis seen when they are incubated with SF. To do this, I tested low and high concentrations of SF, (5% and 50% v/v) and cells were incubated for 18-20 h in the presence and absence of SF and in the presence and absence of SOD and catalase. Once again, apoptosis was assessed by morphology and oxidase activity.

6.2.3.1 *Morphology*

Figure 6.5 shows a table and graph depicting the percentage of apoptotic cells present after 18-20 h incubations. Control cells contained 71.4% (± 6.4) apoptotic neutrophils. Those cells incubated with 5% (v/v) SF contained 72.7 (± 5.1) apoptotic cells. When SOD was also included in the incubation medium, 67% (± 4.0) of the neutrophils were apoptotic. However, when catalase was included, 55.4% (± 8.3) of the neutrophils were apoptotic. Again, the addition of both scavengers together did not significantly enhance the effect of catalase alone (mean value of 54.6% ± 7.8). The results obtained from these experiments were not found to be statistically significant. The results obtained when cells were incubated with 50% (v/v) SF were quite different. In this case, neither SOD nor catalase were able to rescue the neutrophils from apotosis. Figure 6.6 shows the proportion of apoptotic cells observed in these cultures. The percentage of apoptotic neutrophils in cultures incubated with 50% (v/v) SF was 96.9% (± 3.2), compared to a control level of 71.4% (± 6.4). When SOD was included in the incubation medium, the number of apoptotic cells was found to be 99.4% (± 0.7). When catalase was substituted for SOD this number was found to be very similar at 98.6% (± 2.3). The addition of both catalase and SOD together did not improve the survival of the neutrophils any further (99.5% ± 0.6).

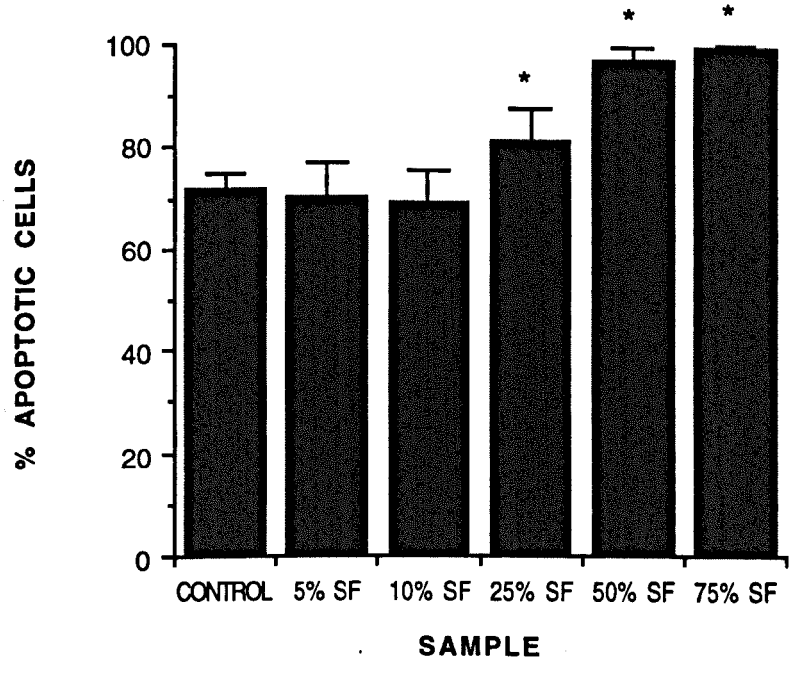
6.2.3.2 *NADPH Oxidase activity*

Figure 6.7 shows a graph of the oxidase activity of neutrophils in response to PMA. This was assessed following incubation for 18-20 h in the presence and absence of 5% (v/v) SF and in the presence and absence of SOD and catalase. These results differ slightly from those observed by assessing morphology. The oxidase activities measured in control and 5% (v/v) SF treated neutrophils were similar. The activity in cells incubated with both 5% (v/v) SF and SOD was also very similar to control cells.

However, when the cells were treated with both 5% (v/v) SF and catalase, the oxidase activity was greater than control or 5% (v/v) SF treated cells alone. The cells treated with 5% (v/v) SF, and SOD and catalase together, also had significantly greater oxidase activity than control cells, although it was not as great as was observed with catalase alone. These results would suggest that although the cells display apoptotic morphology, they are not yet functionally inactive and may have only just begun to undergo apoptosis.

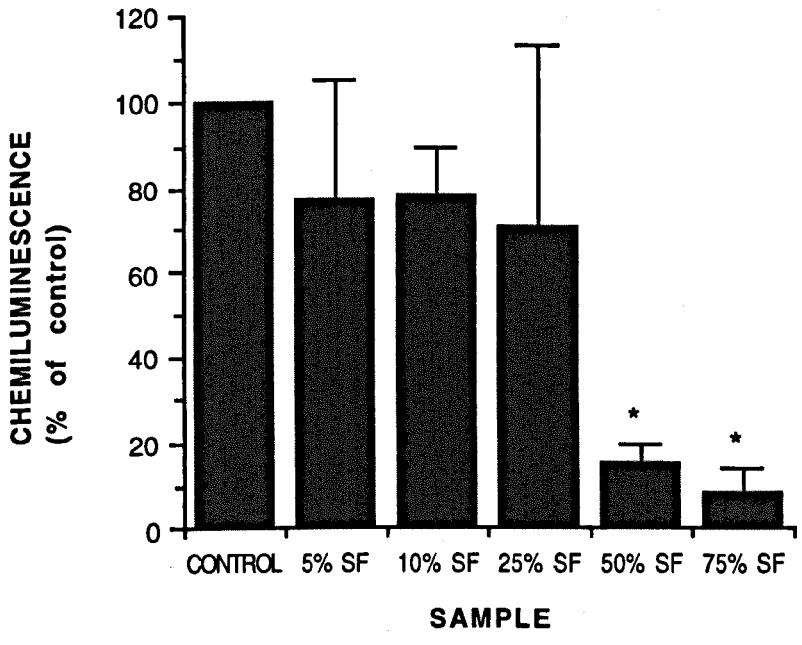
The results seen when the concentration of SF was increased to 50% (v/v) parallel those observed with morphology. These can be seen in figure 6.8. Neutrophils incubated with 50% (v/v) SF alone had only 13% of the oxidase activity of control cells. Those cultures in which SOD and catalase are included had slightly less oxidase activity, being approximately 10% of control cells. Thus, when incubated with higher concentrations of SF, the oxidant scavengers SOD and catalse cannot protect against neutrophil apoptosis.

FIGURE 6.1: EFFECT OF SYNOVIAL FLUID ON NEUTROPHIL APOPTOSIS: MORPHOLOGY



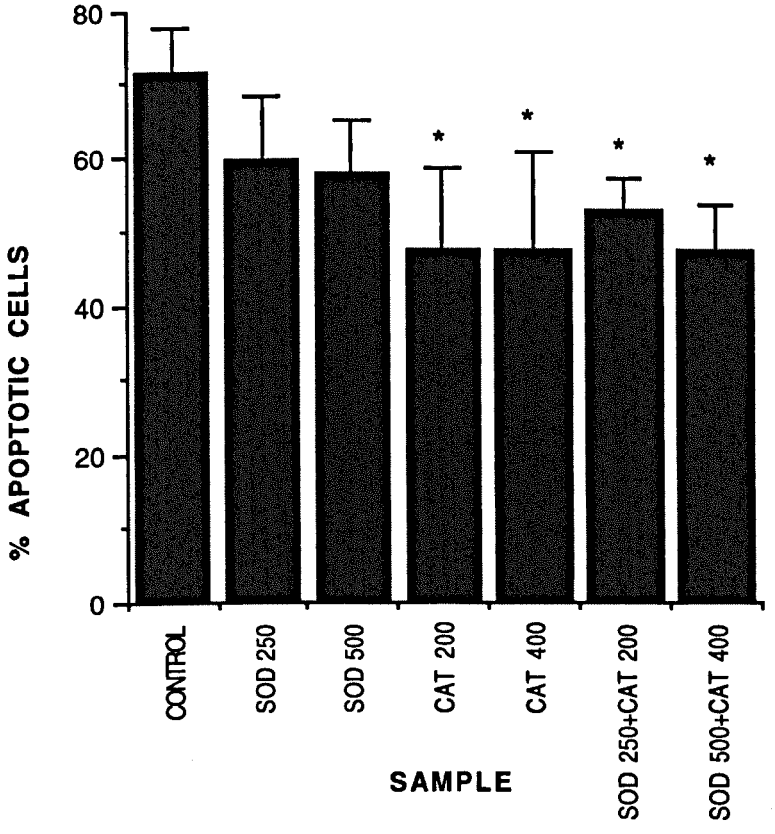
The graph shows the number of apoptotic neutrophils following overnight culture with concentrations of SF ranging from 5% to 75%. Cytospins were prepared from the cultures and at least 500 cells in random fields counted on each slide. Apoptotic neutrophils were determined as described in 2.11. The * indicate values which are significantly higher than control values ($p<0.001$).

FIGURE 6.2: EFFECT OF SYNOVIAL FLUID ON NEUTROPHIL APOPTOSIS: FUNCTIONAL ASSAY



The above graph shows the maximum chemiluminescence of neutrophils incubated overnight with varying concentrations of SF (5%-75% v/v) measured as described in 2.12. Following overnight incubation, 5 x 10^5 cells were stimulated with PMA and the oxidase response measured by chemiluminescence assay. Only the maximum value obtained is displayed which is expressed as a percentage of the control value, taken to be 100%. The * indicates values which are significantly lower than control levels ($p<0.001$).

FIGURE 6.3: EFFECT OF OXIDANT SCAVENGERS ON NEUTROPHIL APOPTOSIS: MORPHOLOGY



The graph shows the number of apoptotic neutrophils deterimined on cytospins prepared from overnight cultures of neutrophils containing the ROI scavengers SOD and catalase. Cytospins were prepared and assessed as described in 2.11. The incubation mixture was 1 ml. The * indicate values which are significantly lower than control levels ($p<0.02$).

Control - no additions

SOD 250 - culture contained 250μg of SOD

SOD 500 - culture contained 500μg of SOD

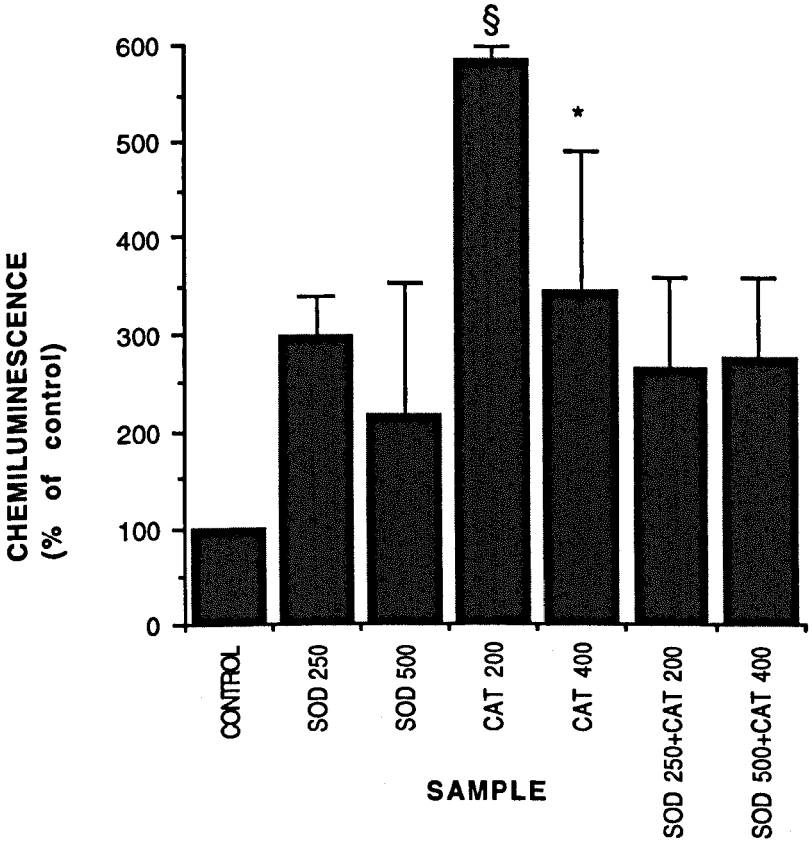
CAT 200 - culture contained 200μg of catalase

CAT 400 - culture contained 400μg of catalase

SOD 250+CAT 200 - culture contained both SOD (250 μg) and catalase (200μg)

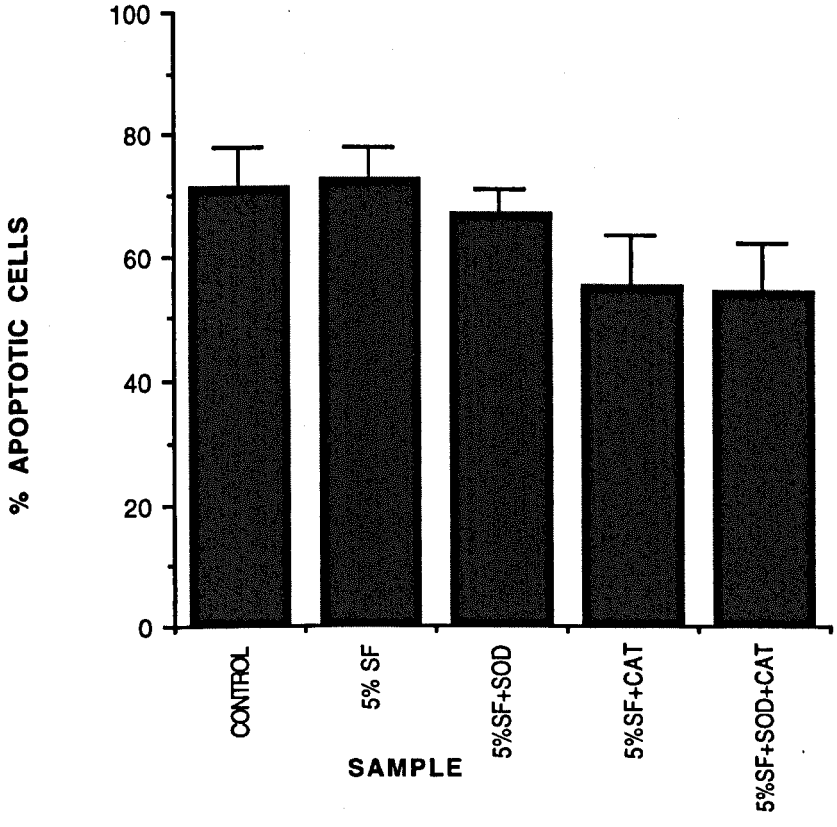
SOD 500+CAT 400 - culture contained both SOD (500μg) and catalase (400μg)

FIGURE 6.4: EFFECT OF OXIDANT SCAVENGERS ON NEUTROPHIL APOPTOSIS: FUNCTIONAL ASSAY



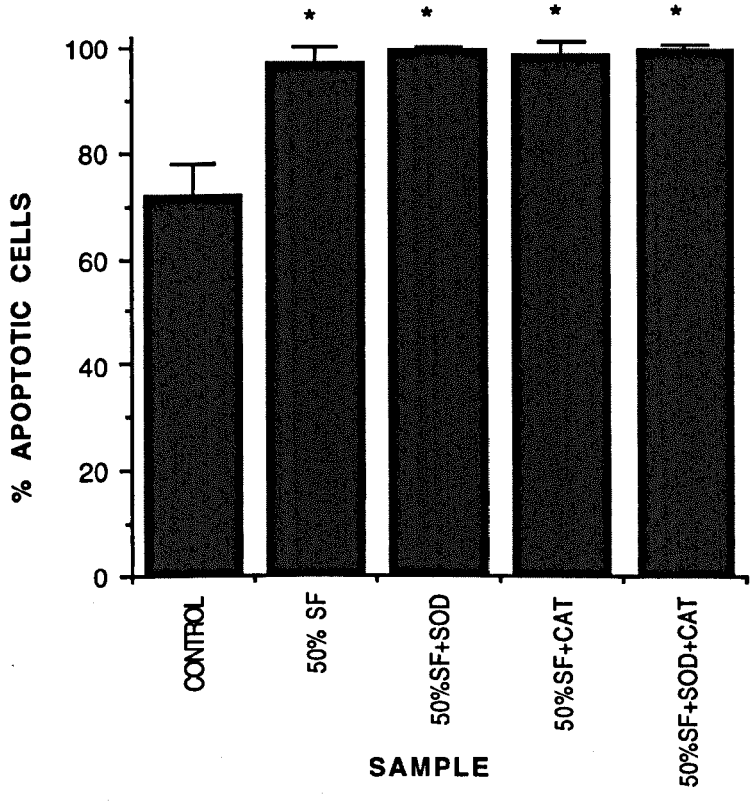
The above graph shows the amount of ROI produced by neutrophils incubated overnight in the presence and absence of the scavengers SOD (250 and 500μg/ml) and catalase (200 and 400μg/ml). The amount of oxidants was measured using a chemiluminescence assay as described in 2.12. The amounts shown on the graph are the maximum values obtained when the cells were stimulated with PMA over a 10 min time period. The samples are as listed in Figure 6.3. The * and § indicate values that are significantly greater than control levels ($p<0.04$ and $p<0.02$ respectively).

FIGURE 6.5: COMBINED EFFECT OF ROI SCAVENGERS AND LOW CONCENTRATIONS OF SYNOVIAL FLUID ON NEUTROPHIL APOPTOSIS: MORPHOLOGY



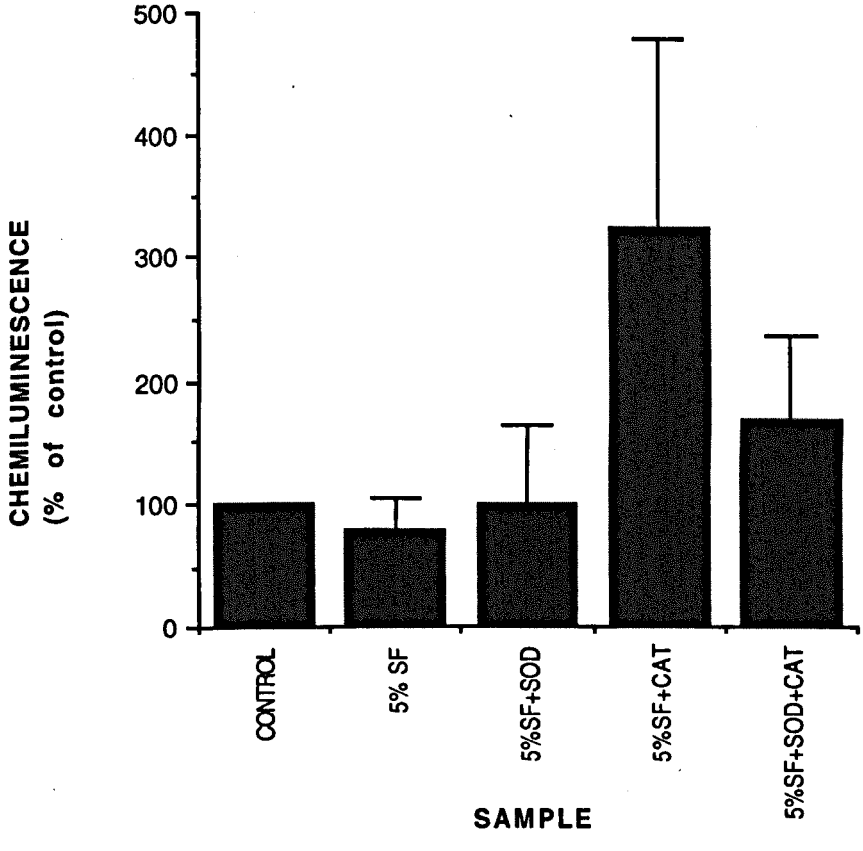
Neutrophils were incubated in the presence and absence (control) of SF (5% v/v), SOD (500 μg/ml) and catalase (400 μg/ml) for 18 h. After incubation, cytospins were made and stained as described in 2.11. The number of apoptotic and non apoptotic neutrophils was then assessed by microscopy. At least 500 cells in random fields were counted on each slide. None of the additions significantly altered the number of apoptotic cells present in the cultures.

FIGURE 6.6: COMBINED EFFECT OF ROI SCAVENGERS AND HIGH CONCENTRATIONS OF SYNOVIAL FLUID ON NEUTROPHIL APOPTOSIS: MORPHOLOGY



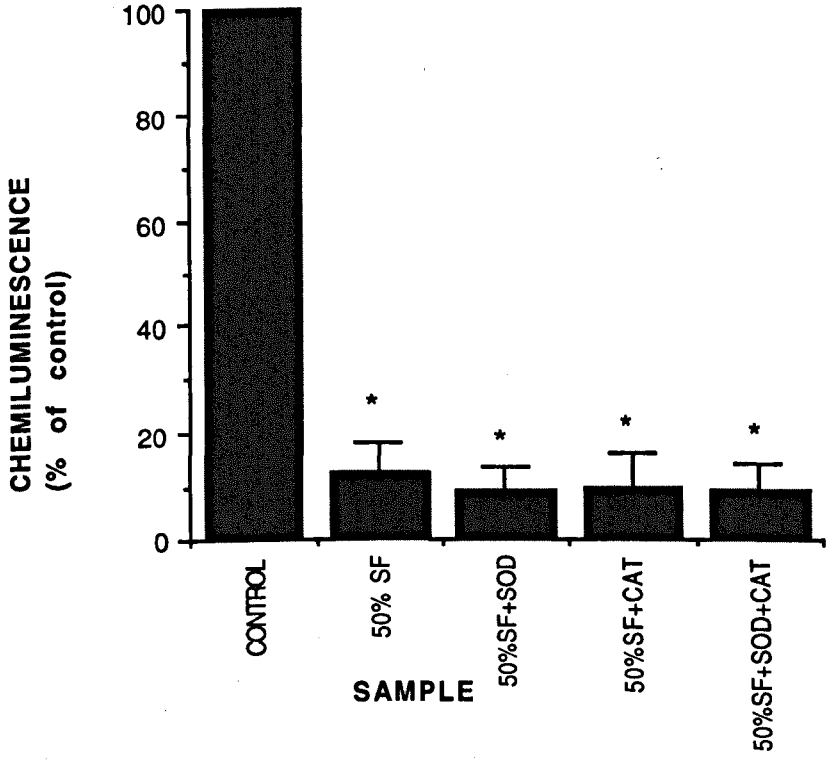
Neutrophils were incubated in the presence and absence (control) of SF (50% v/v), SOD (500 μg/ml) and catalase (400 μg/ml) for 18 h. After incubation, cytospins were made and stained as described in 2.11. The number of apoptotic and non apoptotic neutrophils was then assessed by microscopy. At least 500 cells in random fields were counted on each slide. The * indicate values which are significantly greater than control values ($p<0.02$).

FIGURE 6.7: COMBINED EFFECT OF ROI SCAVENGERS AND LOW CONCENTRATIONS OF SYNOVIAL FLUID ON NEUTROPHIL APOPTOSIS: FUNCTIONAL ASSAY



Neutrophils were incubated in the presence and absence (control) of SF (5% v/v), SOD (500 μg/ml) and catalase (400 μg/ml) for 18 h. After incubation, the ability of the neutrophils to generate reactive oxidants in response to PMA was assessed using the chemiluminescence assay described in 2.12. None of the additions significantly altered the ability of neutrophils to produce an oxidase response following overnight culture.

FIGURE 6.8: COMBINED EFFECT OF ROI SCAVENGERS AND HIGH CONCENTRATIONS OF SYNOVIAL FLUID ON NEUTROPHIL APOPTOSIS: FUNCTIONAL ASSAY



Neutrophils were incubated in the presence and absence (control) of SF (50% v/v), SOD (500 μg/ml) and catalase (400 μg/ml) for 18 h. After incubation, After incubation, the ability of the neutrophils to generate reactive oxidants in response to PMA was assessed using the chemiluminescence assay described in 2.12. The * indicate values which are significantly lower than control levels ($p<0.001$).

6.3 DISCUSSION

There are currently many groups investigating apoptosis in a number of cell types. These studies have highlighted a striking difference between neutrophils and other cell types undergoing apoptosis. The reason for this difference is most likely to lie in the fact that neutrophils are committed to die from the moment they leave the bone marrow. While other cells require a signal to initiate apoptosis, the neutrophil needs a signal to prevent it from undergoing apoptosis.

Many studies have shown that this signal is likely to be an inflammatory mediator such as a cytokine. There are many different cytokines and other inflammatory mediators present in rheumatoid synovial fluid, and hence one would expect synovial fluid to protect neutrophils from apoptosis. However, the results shown here and those of another group suggest that this is not the case (Bell et al, 1995).

I have shown that incubating neutrophils with increasing concentrations of synovial fluid accelerates apoptosis in these cells. Control cells show approximately 70% apoptotic cells after culture overnight. Although low concentrations of SF (5% and 10%) did not significantly affect the number of apoptotic cells, higher concentrations induced apoptosis in almost the whole population (99% apoptosis when 75% SF was added).

The reason for this apparent anomaly may lie in the activating potential of synovial fluid. The synovial fluid of an RA patient contains many inflammatory mediators including several cytokines. It may be that the neutrophils become so vigourously activated (eg by immune complexes) that they quickly become exhausted leading to a more rapid onset of apoptosis in these cells. The activated neutrophils will be producing large quantities of reactive oxidants which have been implicated in apoptosis (Watson et al, 1995) and this may also contribute to the rapid cell death. Additionally,

proteolytic enzymes are thought to contribute to rapid apoptosis in neutrophils (Trevani *et al*, 1996). Hence, enzymes released from the neutrophils own granules, such as elastase may accelerate apoptosis in the rheumatoid joint.

Another recent paper has implicated TNF-α in acceleration of apoptosis (Takeda *et al*, 1993). This paper also suggests that neutrophil activation accelerates apoptosis, a theory which would fit with the evidence presented here. The TNF-R is structurally similar to the Fas antigen, a cell surface protein causally associated with apoptosis. The Fas ligand is structurally similar to TNF-α and is expressed on the surface of neutrophils but not monocytes and eosinophils. Neutrophils are especially susceptible to Fas mediated death and the structural homology of Fas with the TNF-α family may provide a clue to the role of TNF-α in accelerating apoptosis. TNF-α is abundant in SF and may be one factor involved in neutrophil apoptosis in the rheumatoid joint.

Another recent study has shown that hypoxia delays the onset of apoptosis in neutrophils (Hannah et al, 1995). This study used both hypoxic conditions and a system to mimic the effect of hypoxic conditions to study apoptosis. The system used employed ROI scavengers to mimic the inability of neutropils to produce ROI under hypoxic conditions by removing those ROI from the medium. I have utilised this study to examine the effect of hypoxia on synovial fluid neutrophils. I have used superoxide dismutase (SOD) which scavenges $O_2^{\cdot}$, and catalase which scavenges H_2O_2 to remove ROI produced by neutrophils during incubation. The reasoning behind this set of experiments was the environment in the rheumatoid joint which is hypoxic. The concentration of O_2 available in the inflamed joint is approximately 10-20% that of the atmosphere (Edwards et al, 1984) and hence, the low O_2 tension may limit the generation of ROI. Hence, if hypoxia rescues neutrophils from apoptosis, perhaps the

results seen when neutrophils are incubated with synovial fluid in air are artefactual and not a true representation of the conditions within the joint.

The results show that, in agreement with Hannah et al (1995), catalase delays apoptosis to a significant degree. However, I have also shown that SOD can delay apoptosis while Hannah and co-workers found no effect. The effect seen with SOD is of lesser magnitude than that of catalase and no additive effect is seen when both scavenging enzymes are used together.

I have also shown that catalase and to a lesser extent SOD, can delay apoptosis in neutrophils treated with 5% heterologous SF, although the results were not found to be statistically siginificant. However, these enzymes were unable to rescue cells treated with 50% SF. It is possible that greater concentrations of SF lead to more complete activation of the neutrophils, and a greater concentration of scavenger may be needed to counteract this.

Several authors have examined apoptotic neutrophils in the synovial fluid of RA patients. These studies have shown that the percentage of apoptotic neutrophils found in freshly isolated synovial fluid is between 0 and 22% (Savill *et al*, 1989; Bell *et al*, 1995). Hence, a relatively small population of neutrophils in the joint are actually apoptotic in an environment where they are exposed to 100% synovial fluid. In an *in vivo* situation, the neutrophils should be ingested by macrophages and replaced by fresh, naive neutrophils from the circulation. This may account for the low number of apoptotic neutrophils observed in synovial fluid compared to the large proportion which become apoptotic when incubated with SF *in vitro*.

The IL-1β converting enzyme (ICE) is now being investigated with respect to apoptosis. It has been shown to have homology to the *C. elegans* gene *ced-3*, a "death gene" which induces apoptosis during the development of the organism (Yuan *et al*,

1993). IL-1β has been shown to be processed and secreted during apoptosis of monocytes and this may be a result of the activity of the ICE enzyme (Hogquist *et al*, 1991). Neutrophils are known to express ICE and I have shown that incubation with synovial fluid induces IL-1β synthesis and secretion in neutrophils. Interestingly, this secretion occured later than with any other stimulus at 24 h which would coincide with cells becoming apoptotic. Hence, the synthesis of IL-1β could be an important factor in the accelerated apoptosis in neutrophils exposed to synovial fluid.

The role of apoptosis in rheumatoid arthritis is a complex one. It would be expected that a neutrophil in the inflamed joint would have an enhanced lifespan due to the many activating agents present in synovial fluid. However, the results presented here would contradict this hypothesis. The role of priming should be considered here. In my experiments I used unprimed, naive neutrophils but earlier results suggest that neutrophils entering the joint are already primed in the blood. Priming could render the neutrophil more resistant to apoptosis as it enhances protein synthesis, a factor which protects neutrophils from apoptosis under some circumstances.

CHAPTER 7

GENERAL DISCUSSION

The role of the neutrophil in rheumatoid arthritis is often thought to be restricted to that of an effector cell which discharges its proteolytic enzymes and reactive oxidants into the joint thereby causing damage. However, while this remains an important mechanism of destruction in the rheumatoid joint, the results shown in this thesis demonstrate the possibility of another role for the neutrophil in the direction of the immune response within the joint.

I have shown that normal blood neutrophils stimulated with the cell free synovial fluid of RA patients show enhanced *de novo* protein synthesis. This synthesis is differential as certain proteins are upregulated by a large degree while others are only slightly upregulated. When neutrophils were stimulated with synthetic soluble immune complexes, an even greater enhancement of protein synthesis was seen. Insoluble IC did not induce a great increase in protein synthesis. Soluble IC also induced a large degree of protein secretion when compared to control cells and also to SF or insoluble IC stimulated cells. When compared to IC derived from SF, the synthetic IC had similar effects.

Two of these proteins synthesised are the inflammatory cytokines IL-1β and TNF-α. The synthesis of these cytokines is stimulated by both SF and synthetic immune complexes which mimic those found in SF. No secretion of TNF-α could be detected in these studies, although this could be due to a number of reasons including the breakdown of TNF-α by proteases that are also released by activated neutrophils. However, secretion of

IL-1β was detected when the stimulus used was SF but not IC, either soluble or insoluble. It may be that IC can only provide an initial signal to the neutrophil to synthesise the cytokines and a second signal, which may be present in SF, is needed to induce secretion.

In addition to stimulating normal neutrophils, cells were obtained from the blood and SF of RA patients and incubated without further stimulus. These cells were found to synthesise both IL-1β and TNF-α. Blood neutrophils from RA patients synthesised a greater amount of IL-1β and TNF-α than SF neutrophils in 2 out of 3 patients. This would suggest that blood neutrophils of RA patients are already primed prior to entering the joint and have begun synthesising cytokines which may be secreted once they have entered the inflamed joint. The lesser level of synthesis in SF neutrophils may reflect a down-regulation of cytokine synthesis once cells are in the joint. Some secretion was detected from the blood neutrophils of 2 patients and the SF neutrophils of 1 patient.

Further studies were carried out into the ability of IL-1β and TNF-α to prime neutrophils. The reasoning behind this was that if neutrophils were synthesising and secreting these cytokines, they may have an important paracrine role in the stimulation of naive neutrophils entering the joint or in the bloodstream, prior to entering the joint, thereby perpetuating inflammation. It was found that both IL-1β and TNF-α were able to prime neutrophils to respond to immune complexes, a stimulus found in the rheumatoid joint.

An enhanced oxidase response was detected in primed cells, and TNF-α was a more effective priming agent than IL-1β. This priming effect was also detected when intracellular Ca^{2+} changes were measured during activation with soluble IC. Hence, these

2 cytokines may form a positive feedback cycle, being synthesised by neutrophils in the joint then priming fresh neutrophils entering the joint which then go on to be activated by IC or other agents present in SF.

Finally, I have investigated the effect of SF on apoptosis in neutrophils. Recent evidence suggests that SF accelerates apoptosis. One would predict that due to the high concentrations of inflammatory agents present in SF, it would actually retard apoptosis.

In confirmation of the report by Bell *et al* (1995), it was found that SF does indeed accelerate apoptosis in neutrophils. However, another report suggested that hypoxia can rescue neutrophils from apoptosis (Hannah *et al*, 1995). The rheumatoid joint is a hypoxic environment, and hence I studied the effects of SF on apoptosis in the presence of oxidant scavengers in order to mimic a hypoxic environment. When low concentrations of SF are used under these conditions, neutrophil apoptosis is retarded, but, when higher concentrations are used, there is no effect. It may be that there was not a sufficiently high concentration of scavengers to counteract the effect of the higher concentration of SF. The reason for this apparently anomalous effect of SF on neutrophil apoptosis could lie in the amount of inflammatory mediators present. The neutrophil could be fully activated so that it is quickly exhausted and becomes unable to sustain activation, therefore it undergoes apoptosis.

The results discussed above suggest a more direct role for the neutrophil in RA. The cell once thought incapable of, and with no need for, protein synthesis does in fact synthesise a range of proteins, many of which may be important in RA. Certain of these proteins may be as yet undescribed agents which could be having an effect in the rheumatoid joint. Work is now underway to sequence both proteins and mRNA from activated neutrophils in order to identify novel genes. Additionally, it would be useful to study the receptors

through which soluble and insoluble IC bind, as this may give an insight into their differing actions.

IL-1β and TNF-α are acknowledged as very important cytokines in the pathogenesis of RA. They have multiple effects within the joint, both on neutrophils and a wide variety of other cell types. IL-1β and TNF-α stimulate bone resorption, as well as chondrocyte and synoviocyte production of further inflammatory cytokines including both IL-1β and TNF-α themselves, IL-6 and IL-8.

In addition to effects on other cells, these cytokines may set up a positive feedback cycle in which their production by neutrophils induces priming of further neutrophils which are then activated to produce further amounts of IL-1β and TNF-α. A system such as this may perpetuate the inflammation in the joint and will induce the continuing influx of fresh neutrophils into the inflamed joint. IL-1β causes the upregulation of adhesion molecules on endothelial cells, in addition to the synthesis of IL-8 by cells within the joint, perhaps by the neutrophils themselves. These conditions increase the influx of neutrophils from the bloodstream into the joint to continue the cycle of chronic inflammation.

The question of where priming of neutrophils takes place in RA is unanswered. Neutrophils were originally thought to be primed upon entering the joint by factors in SF. However, the enhanced synthesis of IL-1β and TNF-α by neutrophils in the blood of RA patients which is greater than that seen in SF neutrophils, would argue against this theory. This suggests that neutrophil involvement in RA is a more systemic event as opposed to being localized in the joint space.

The cells have already been primed prior to entering the joint and have begun to synthesise IL-1β and TNF-α which may be released upon entering the joint. Hence, perhaps agents present in the blood of RA patients are responsible for priming neutrophils which are then fully activated by IC or other factors in SF. It is possible that some of the IL-1β and TNF-α are secreted in the bloodstream by neutrophils leading to the priming of naive cells in the blood. Further work which should be done includes investigating the synthesis of other cytokines by neutrophils such as IL-6, IL-8 and IL-1ra and the role of ICE in IL-1β secretion and apoptosis. The ability of these cytokines to prime neutrophils and their effects along with those of IL-1β and TNF-α on other cells present in the rheumatoid joint, are also important aspects to be investigated.

Both TNF-α and IL-1β are effective priming agents for further stimulation by immune complexes. These are thought to be the major neutrophil stimulating agents present within the joint. The synthetic immune complexes used in these experiments have been compared to those derived from synovial fluid for their ability to stimulate protein synthesis. It has been found that similarly to synthetic insoluble IC, SF derived insoluble IC have little effect on protein synthesis by neutrophils. Soluble IC have a much more dramatic effect on protein synthesis, inducing up to 12x control levels of synthesis. When comparing these to SF derived IC, it was impossible to isolate the soluble IC alone from SF without their breakdown. Hence, SF containing only soluble IC and other soluble agents was used and compared not only with control and synthetic soluble IC stimulated cells but also with cells stimulated with SF from which all IC had been removed. Soluble IC, both synthetic and SF derived, were able to induce a similar increase in protein synthesis when compared.

Hence, it is valid to make the assumption that effects seen with synthetic IC are likely to also be seen in the rheumatoid joint.

Very little work has been done into neutrophil apoptosis within the rheumatoid joint. It is assumed that the neutrophils, once they become apoptotic, are phagocytosed by macrophages and cleared from the joint space. Indeed, reports have been published showing macrophages which have ingested apoptotic neutrophils in the joint.

It has been assumed that due to the high levels of inflammatory agents in SF, that the neutrophil's lifespan will be extended. However, this may not be the case. Neutrophils incubated with SF show accelerated apoptosis which increases with the concentration of SF. A factor that has not been taken into consideration here is that the rheumatoid joint is a hypoxic environment with an oxygen concentration approximately 10-20% that of air. Recent work has shown that hypoxia delays apoptosis. The mechanism by which hypoxia delays apoptosis may involve reactive oxidants. In an hypoxic environment, the neutrophil would produce decreased levels of oxidants due to lack of substrate, ie oxygen. Other reports have suggested that ROI are involved in causing apoptosis. Hence, an inability to produce ROI could retard apoptosis. Using oxidant scavengers to remove any ROI produced and hence mimic a hypoxic environment did delay apoptosis in neutrophils exposed to a low concentration of SF but had no effect at higher concentrations. This may be the result of too low a concentration of scavengers if a high level of ROI are being produced. Further work is necessary to assess the effect of manipulating the atmospheric conditions on the survival of neutrophils incubated with SF.

Another factor which may be of importance in apoptosis of neutrophils is the IL-1β Converting Enzyme (ICE) which has been shown to have apoptotic activity in macrophages

associated with the secretion of IL-1β. IL-1β is not seen secreted by SF stimulated neutrophils until 24 h. By this time the neutrophil may be apoptotic as a result of the activity of ICE processing IL-1β for secretion.

The results presented here suggest a less passive role for the neutrophil in RA than has often been thought. The neutrophils may not only enter the joint and empty its toxic contents into the SF by degranulation. It may also be controlling the immune response within the joint and possibly systemically by the secretion of at least 2 cytokines, IL-1β and TNF-α which have divergent effects on many cell types. There may also be as yet uncharacterized proteins being secreted with the capacity to direct the immune response.

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