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A PUTATIVE ROLE FOR LEUCOCYTE PRODUCTS IN THE REGULATION OF ACUTE INFLAMMATION

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SUBMITTED IN PARTIAL FULFILLMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

TO THE COUNCIL FOR NATIONAL ACADEMIC AWARDS

NOTTINGHAM POLYTECHNIC AND MEDICAL RESEARCH CENTRE,
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AUGUST 1991

ABSTRACT

A Putative Role for Leucocyte Products in the Regulation of Acute Inflammation

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The acute inflammatory response involves the recruitment of leucocytes from the peripheral circulation to sites of injury/infection. Mononuclear cells (MNC) activated at the inflammatory foci are capable of affecting PMN function through the secretion of certain cytokines. We have shown that, *in vitro*, TNF is produced maximally after by MNC 5-6 hours in culture with 1ug ml^{-1} bacterial lipopolysaccharide (LPS). This TNF primed polymorphonuclear leucocytes (PMN) for a dramatic, and irreversible, increase in respiratory burst activity when stimulated with zymosan-activated serum (ZAS) (323.29% $\pm$ 57.94%, n=15 $\pm$ SEM).

Direct stimulatory activity of MNC-conditioned medium (MNCM) was not due to TNF, IL-1beta or GM-CSF. Sephadex G-75 and G-50 filtration demonstrated at least one low molecular weight (<12.3kD) factor in MNCM which directly stimulated PMN superoxide anion production. NAP-1/IL-8 was thought involved in this bioactivity as antiserum to this peptide reduced PMN superoxide anion production by 46.64% $\pm$ 11.46% (n=4 $\pm$ SEM, $p<0.025$). However, only one of three recombinant human NAP-1/IL-8 preparations induced a dose dependent increase in PMN respiratory burst activity. It was possible that NAP-1/IL-8 secreted *in vitro* from LPS stimulated MNC had greater activity than the recombinant forms, or that other low molecular weight factors were involved.

Recombinant human TNF induced increased CD11b and CD18 expression concomitant with lactoferrin (Lf) release, suggesting that this cytokine could potentially increase PMN adhesiveness. The role of Lf released at the same time may act as a negative regulator of inflammation. Adding 10^{-10}M 50%-Fe-saturated Lf to MNC cultures stimulated with LPS, resulted in significant reduction of TNF ($p<0.025$) and IL-1beta ($p<0.05$) production. Lf also inhibited the proliferation of 3-way mixed lymphocyte cultures (MLC) at 24, 48 and 72 hours, together with inhibition of TNF and IL-1beta production.

Finally, the effects of pentoxifylline (PTOX) were investigated with respect to PMN function. *Ex vivo* data obtained after administration of 1 x 400mg slow release PTOX revealed that depressed superoxide anion production in response to ZAS and formyl-methionylleucylphenylalanine (FMLP) correlated with levels of metabolite V (MET V) ($p<0.04$). *In vitro* data confirmed MET V to be the most effective at reducing PMN respiratory burst, Lf release and CD11b/CD18 expression. These findings suggested that PTOX may reduce PMN associated tissue damage, in conditions such as varicose ulceration, via its metabolites rather than the parent drug.

ACKOWLEDGEMENTS

This work was supported by grants from the Leukaemia Research Fund and Hoechst UK.

I would also like to thank:

Prof. John Fletcher for his support, patience and guidance.

Dr. Peter Blaxhall, particularly for his administrative skills.

Dr. Ian Daniels for setting up the column work, but also for his sense of humour.

Dr. Mark Lindsay for all the work on intracellular calcium concentrations and his proof reading and positive criticism.

Christine Porter for all those hours on the FACS, analysing my samples.

Dr. Kevin Slater for his enthusiasm and confidence in my abilities.

All my colleagues at the Medical Research Centre for their willingness to donate blood, and in particular to Janet Amoah for her friendship and encouragement.

Duncan Llewelyn for his patience and understanding.

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ABBREVIATIONS

ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CGD	Chronic granulomatous disease
cGMP	Cyclic guanine monophosphate
DAG	Diacylglycerol
DMSO	Dimethylsulphoxide
EDTA	Ethelene diaminetetra acetic acid
EGTA	Ethelene dioxy-diethyl-enedinitrolo-tetra acetic acid
ELAM-1	Endothelial leucocyte adhesion molecule-1
ELISA	Enzyme-linked immunosorbant assay
FAD	Flavin adenine dinucleotide
FCS	Foetal calf serum
FMLP	Formyl-methionylleucylphenylalanine
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
G-protein	Guanine nucleotide binding protein
GTP	Guanine triphosphate
ICAM-1	Intracellular adhesion molecule-1
IFN	Interferon
IP_3	Inositol triphospate
IL	Interleukin

kD	Kilo dalton
LBP	Lipopolysaccharide-binding protein
Lf	Lactoferrin
LPS	Lipopolysaccharide
LTB_4	Leukotriene B_4
MAG	Monoacylglyerol
MHC	Major histocompatibility
MLC	Mixed lymphocyte culture
MNC	Mononuclear cells
MNCM	Mononuclear cell conditioned medium
NAP-1/IL-8	Neutrophil-activating peptide-1/interleukin-8
NADPH	Nicotinamide adenine dinucleotide phosphate
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PGE_2	Prostaglandin E_2
PIP_2	1-phosphatidyl-D-myo-inositol 4,5-biphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PLD	Phospholipase D
PMN	Polymorphonuclear leucocytes
PTOX	Pentoxifylline
RT	Room temperature
SEM	Standard error about the mean
SOD	Superoxide dismutase
TNF	Tumour necrosis factor alpha

ug	micro gram
ul	micro litre
uM	micro molar
VCAM-1	Vascular adhesion molecule-1
ZAS	Zymosan-activated serum

CHAPTER ONE: INTRODUCTION

Inflammation is the result of the host responding to an insult such as bacterial invasion or tissue damage. This response comprises a number of events involving the blood and tissue of the microcirculation, the desired result being to eliminate the injurious agent and to repair the damaged tissue. The initiation of the inflammatory response by, for example, bacterial invasion results in a number of rapid changes in the microcirculation. The release of soluble chemical mediators by interaction between the invading micro-organisms and plasma causes dilatation of the local arteriolar vessels. This allows for increased blood flow through the microvasculature, which in turn brings increased numbers of leucocytes, together with plasma proteins, oxygen and nutrients to the affected area. Oedema results from an increase in vascular permeability, the resulting loss of fluid from the circulation to the tissues causes haemoconcentration with the slowing of blood flow and reduction of shear forces which allows leucocytes to adhere to the endothelium and then migrate from the circulating pool to the site of invasion. This complex series of events is controlled by a number of factors including chemical mediators from the bacteria themselves, anaphylatoxins produced as a result of activating the complement system, and also monokines and lymphokines secreted from activated leucocytes. In addition to causing this efflux of cells from the peripheral circulation to the tissues there must be a number of control mechanisms to

prevent the severe vascular damage which may result if this process of leucocyte margination, migration and activation is allowed to continue unchecked. These inhibitory mechanisms are less clearly understood but the events involved are no doubt as complex as those which promote inflammation.

In order to combat an infection it is important that increased numbers of leucocytes are delivered to the affected area. The proliferative organ that supplies these cells is the bone marrow which is capable of producing 2×10^9 polymorphonuclear leucocytes (PMN) per Kg body weight per day, the response to infection is to increase this output by approximately ten fold. During periods when the host is not subjected to insult the numbers of leucocytes are maintained within a narrow range and are therefore subject to strict control mechanisms. The output of cells from the bone marrow can depend upon the type of infection; in bacterial invasion there is a predominant increase in PMN whilst a fungal disease will induce production of increased numbers of monocytic cells. These two cell types are myeloid cells and they are induced to differentiate to maturity in the bone marrow in response to a number of colony-stimulating factors (CSFs). In acute inflammation the mature cells are released into the peripheral circulation where they are then available to respond to inflammatory stimuli through recruitment into the tissues.

Activation of the inflammatory response involves a number of different cell types which upon activation have differing effector functions. However, these cells and their secreted products do not work in isolation of each other but participate in complex series of events which lead to the rapid eradication of invading micro-organisms and repair to damaged tissue.

1.2. Polymorphonuclear Leucocytes

The predominant population of cells in the circulating pool are PMN, of which the major cell type is the neutrophil. Basophils and eosinophils belong to this group and are also involved in inflammation although they are found in much smaller numbers in the peripheral circulation. During inflammation neutrophils are thought to be the first cells to migrate into the tissues, followed by monocytes and then lymphocytes. Neutrophils are phagocytic cells and are thought to be the host's first line of defence against invading micro-organisms.

Over a century ago Metchnikoff formulated the phagocytic theory of host defences. Experimenting with marine invertebrates he noticed that certain strains of an invading fungus were ingested and destroyed by phagocytic cells. Other strains remained unaffected with the result being a disseminated and fatal disease in the host. A century later this concept was commonly demonstrated by the development of overwhelming bacterial and fungal infection in conditions resulting from

severe quantitative or functional deficiencies of circulating neutrophils. These observations confirm the importance of these phagocytic cells in the ability of the host to combat microbial infection.

In the peripheral circulation PMN are physiologically inactive. These "resting" cells express only a limited number of receptors on the cell surface. During inflammation PMN may be activated by a whole range of stimuli through binding to these specific cell surface receptors. Apart from those receptors involved in the binding of micro-organisms, PMN possess receptors for soluble chemical mediators. These include receptors for a number of cytokines, including tumour necrosis factor-alpha (TNF) and neutrophil activating peptide-1/interleukin-8 (NAP-1/IL-8). In addition neutrophils also have specific receptors for the complement component C5a, formyl peptides, leukotriene B_4 (LTB_4) and platelet activating factor (PAF). The action of many of these factors is to cause the upregulation of other receptors, such as adhesion molecules, and also to initiate a number of cell functions including degranulation and respiratory burst activity. Therefore, the exposure of PMN to cytokines and chemotactic factors in the circulation will result in adherence of cells to the endothelial wall and migration into the tissues where they may respond to the inflammatory insult.

1.2.1. Margination and Migration

Neutrophils and other leucocytes are induced to marginate in response to chemotactic agents. The concept of margination and exudation of neutrophils dates to the early nineteenth century when Dutrochet first described the egress of "vesicular globules" (leucocytes) from the vascular lumen to the extravascular space. Cohnheim emphasised the apparent adherence of white blood cells to the vessel wall and suggested that endothelial cells participate actively in this process. Both PMN and endothelial cells have a negative surface charge, yielding net electrostatic forces that oppose cell to cell adherence. Chemotactic factors cause a small but significant reduction in the neutrophil surface charge and augment adherence to endothelial cells in vitro (Gallin 1980).

Human C5a and C5a des arg anaphylatoxins are potent chemotaxins causing the accumulation of neutrophils under stimulated in vivo conditions (Fernandez et al 1978). It is suggested from quantitative measurements that the predominant chemotactic activity generated in human serum during complement activation is associated with the C5a des arg molecule. C5a des arg would therefore appear to play a predominant and important role as a major humoral chemotactic factor. Other agents capable of inducing an influx of neutrophils from the circulation to the tissues include formyl-methionylleucylphenylalanine (FMLP) and also leucocyte derived products which exert their effects on

either PMN, the endothelium or both. These include LTB_4, secreted from activated neutrophils (Movat et al 1984), PAF (Ingraham et al 1987), TNF, IL-1 (Mantovani et al 1990) and the recently discovered NAP-1/IL-8 (Furata et al 1989). In order for these agents to induce margination and migration they must first induce the expression of adhesion molecules on both leucocytes and endothelial cells.

1.2.2. Adhesion Molecules

Adhesion molecules are found on both leucocytes and endothelial cells, these molecules belong to a superfamily of integrins and are heterodimers comprised of alpha and beta subunits. Each subfamily is defined on the basis of a common beta subunit and beta1, beta2 and beta3 represent the very late antigens (VLA), leucocyte integrins and cytoadhesins respectively. Netrophils express integrins of the beta2 subfamily, the beta sub-unit is common to all three members and will non-covalently with one of the three distinct alpha sub-units to give three unique adhesion molecules. These adhesion molecules are the lymphocyte functional antigen (LFA-1), the C3bi receptor or MAC-1 and the p150,95 protein. Their cluster determinant classifications are CD11a, CD11b and CD11c respectively, with CD18 representing the common beta sub-unit. There is a strong body of evidence to show that these adhesion molecules are located in the membrane of the secondary (Bainton et al 1987) and tertiary (Lacal et al 1988) granules of mature human neutrophils. Concentrations of

agents such as FMLP, which have been shown to be chemotactic are capable of inducing secondary degranulation and increasing the expression of these adhesion molecules (Lacal et al 1988). Cytokines including TNF (Griffin et al 1990) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Neuman et al 1990), both products of stimulated mononuclear cells, have also been shown to induce increased expression of these adhesion proteins. Neutrophil adherence to endothelium can be inhibited by antibodies to CD11a,b,c and CD18 after the neutrophils have been stimulated with FMLP, PAF, LTB_4, the calcium ionophore A23187 and TNF (Zimmerman and McIntyre 1988). These findings, in addition to those from other investigators, confirm the importance of these molecules in neutrophil adherence.

The suggestion that adhesion molecules are associated with the membrane of secondary granules (Bainton et al 1987) is partially confirmed by the association of increased CD11b/CD18 expression with the exocytosis of secondary granule contents (Arnaout et al 1984). However, more recent evidence suggests that PMN adhesion can occur independently of degranulation and that increased cellular adhesion is independent of cell surface expression of MAC-1 (CD11b/CD18) (Schleiffenbaum et al 1989). Adhesion itself may be partially responsible for the induction of degranulation. The role of degranulation in neutrophil adhesiveness has become a contentious issue, and one that has yet to be fully resolved, partly due to the differing experimental conditions employed. The importance of all these

molecules has been demonstrated in animal models, where the transient neutropaenia caused by chemoattractants is blocked by the presence of anti-CD18 antibodies in the circulation (Lundberg and Wright 1990).

The increased expression of adhesion molecules on the cell surface is not the only requirement for the induction of adhesion. Hogg (1989) reports a requirement for the binding of divalent cations to stabilize the interaction between alpha and beta subunits. Binding of Mg^{2+}, for example, will result in an "active" form of the adhesion molecule.

Despite the contention as to how neutrophils increase their expression of these molecules there is no doubt that on activation these cells rapidly increase their cell surface expression of CD11b,c/CD18 by several fold. This probably occurs by translocation of intracellular granules to the cell surface and fusion of the granule membrane with the plasma membrane. Little or no CD11a/CD18 is stored intracellularly (Arnaout et al 1984) hence no increase in this adhesion molecule is observed. Another adhesion molecule present on neutrophils is associated with binding to high endothelial venules, the leucocyte adhesion molecule-1 (LAM-1). This molecule belongs to a different family of adhesion proteins and is regulated in a different manner. The LAM-1 contains a lectin-like molecule which binds to a specific glycoconjugate on target cells.

Treatment of neutrophils with GM-CSF induces rapid and complete loss of LAM-1 which is associated with upregulation of CD11b (Griffin et al 1990), therefore the same stimulus appears capable of differentially regulating the expression of these molecules. TNF is also capable of inducing increased expression of CD11b/CD18 with a concomitant loss of LAM-1 molecules (Spertini et al 1991). The loss of LAM-1 has been associated with adhesion to activated human umbilical vein endothelial cells (HUVEC) (Smith et al 1991) and it has been suggested that LAM-1 is critical for the initial binding event between unstimulated neutrophils in the circulation and inflamed endothelium, whereas CD11a/CD18 and CD11b/CD18 are thought to be important for adhesion strengthening and transendothelial migration (Smith et al 1991). However, there is also evidence to suggest that CD11a/CD18 is also associated with the binding of unstimulated neutrophils to endothelium activated to express intracellular adhesion molecule-1 (ICAM-1) (Smith et al 1991).

As yet less is known about the LAM-1 molecule although it appears that it may also contribute in the shift of progenitor cells from the marrow to the blood (Griffin et al 1990). Adhesion molecules therefore play a very important role in the movement of neutrophil traffic from the bone marrow to the circulating pool and from there to the tissues.

In order for the neutrophils and other leucocytes to interact with and adhere to the endothelial cells the latter must also

be activated to express adhesion molecules that act as ligands for those receptors present on the surface of the leucocytes. Bevilacqua et al (1985) demonstrated that pretreatment of HUVEC in vitro with IL-1 induced synthesis of cell surface activity that increased leucocyte adherence. Gamble et al (1985) demonstrated a similar effect when HUVEC were treated with recombinant human TNF, which promoted neutrophil adherence by an endothelial-dependent mechanism requiring de novo RNA and protein synthesis. Bacterial lipopolysaccharide (LPS) has also been shown to induce increased expression of endothelial cell surface factors that promote neutrophil adherence (Schleimer and Ruttledge 1986). The surface factors involved are members of a different integrin from those found on leucocytes. The agents mentioned will induce increased expression of endothelial leucocyte adhesion molecule-1 (ELAM-1) (Bevilacqua et al 1987), ICAM-1 (Hogg 1989) and vascular cell adhesion molecule-1 (VCAM-1) (Schwartz et al 1990).

An inherited deficiency of CD11/CD18 leads to patients with marked neutrophilic leucocytosis, presumably due to deficient margination. Elucidation of the underlying defect has greatly advanced the knowledge of the molecular basis of normal leucocyte adhesion. The functional defect involves synthesis of the beta chain (CD18), with the severity of clinical infection correlating with the degree of receptor abnormality (Kishimoto et al 1987).

1.2.3. Neutrophil Granules

PMN are often referred to as granulocytes due to the number of granules present in their cytoplasm. Neutrophils possess three different types of granules, the primary (or azurophil) granules, the secondary (or specific) granules and the tertiary granules. Each granule type contains different functional proteins (see table 1). The first granules to appear during neutrophil maturation are the primary granules which comprise approximately one third of all the granules in the mature cell. Primary granules function predominantly in the intracellular milieu where they are involved with the killing and digestion of micro-organisms. The secondary granules appear at approximately the metamyelocyte stage of neutrophil maturation. Neutrophil secondary granules are preferentially released during diapedesis (as discussed in section 1.2.2) and migration into the tissues. As table 1 shows, the secondary granules contain a number of different proteins with diverse actions. Like the primary granules the specific granules also contain lysozyme, the functions of the other secondary granule constituents are less well understood. These other constituents, unique to these granules, include vitamin B_{12}-binding protein, cytochrome b and lactoferrin. Little is known about vitamin B_{12}-binding protein, but the cytochrome b is an essential part of the NADPH-oxidase, therefore patients deficient in secondary granules show reduced respiratory burst activity.

Table 1: Neutrophil Granule Contents

Function	Primary	Secondary	Tertiary
Microbicidal agent	Myelo-peroxidase Lysozyme Defensins Cationic proteins	Lysozyme Lactoferrin	
Serine proteases	Elastase Cathepsin G		
Metallo-proteinases	Proteinases	Collagenases	Gelatinase
Acid hydrolases	N-acetyl glucosamine Cathepsins B/D Glucuronidase Glycerophosphatase Mannosidase		
Others		Mac-1 complex B12 binding protein Cytochrome b_{-245} Histaminase FMLP receptor	Mac-1 complex

Lactoferrin (Lf) is an iron-binding protein with a molecular weight of 77kD. Lf is found in many secretions in association with IgA and lysozyme resulting in a potent bacteriostatic combination (Bortner et al 1986). Neutrophils are the sole source of Lf found circulating in the plasma, and there is a correlation between plasma levels of Lf and the number of circulating neutrophils (Baynes et al 1986). In addition Lf has been shown to inhibit granulopoiesis (Bagby et al 1983), suppress antibody production (Duncan and McArthur 1981) and regulate natural killer cell activity (Nishiya and Horwitz 1982). Therefore, it would appear that Lf is involved in the modulation of a number of inflammatory and immune responses. Lf has been associated with increased neutrophil adhesiveness through decreases in negative charge (Gallin 1980).

Patients with neutrophil secondary granule deficiency constitute an important model illustrating the critical role that these granules play in the evolution of the inflammatory response. Secondary granule products activate the complement cascade to generate C5a and the opsonin C3b, in these patients there is a failure to generate C5a from the serum.

Not only are the contents of these granules vital for various aspects of neutrophil function, but they also act as an intracellular source of receptors. The secondary granules, in addition to acting as an intracellular store for adhesion molecules, have also been shown to contain receptors for the

tripeptide FMLP (Fletcher and Gallin 1983). This peptide has been shown to be secreted by certain strains of Escherichia coli (Schiffmann et al 1975); at low concentrations it acts as a potent chemotaxin while at high concentrations it is a stimulator of neutrophil respiratory burst activity (Gallin 1984). Therefore, agents that cause the liberation of these granule contents during exocytosis will also induce increased expression of these receptors at the cell surface. PMN isolated and maintained at 4°C express a single high affinity population of FMLP receptors, warming to 37°C causes a marked increase in the expression of these receptors (Tennenberg et al 1988). Therefore a very mild stimulus is sufficient to induce increased expression of FMLP receptors. Neutrophils from patients with congenital deficiency of secondary granules have impaired upregulation of FMLP receptors, an impaired respiratory burst to certain stimuli and deficient bacterial killing (Gallin 1985).

Secondary granules may be the intracellular store for CD14, which has been associated with the binding of the LPS-LPS binding protein (LBP) complex. Cytokines such as TNF and GM-CSF upregulate the expression of CD14 and increase cell responsiveness to LPS (Wright et al 1991). This has been suggested as a positive mechanism for the clearing of gram-negative bacteria. All of these observations add weight to the proposal for neutrophil secondary granules being an important intracellular store of receptors.

The tertiary granules were first identified as being discrete from secondary granules through the observation of a distinct sub-cellular distribution and release of gelatinase, the metallo-proteinase contained within this granule type (Dewald et al 1982). As with secondary degranulation, extremely mild stimuli are sufficient to induce exocytosis of gelatinase; both granule types will discharge their contents in the absence of respiratory burst activity (Dewald et al 1982). These mild stimulatory conditions are likely to occur in a chemotactic factor gradient, the secondary and tertiary granules may play a role in the early events of neutrophil mobilization into the tissues.

1.2.4. Chemotaxis

Following adherence the leucocytes move across the endothelium by as yet poorly understood mechanisms. However, this process occurs without any obvious damage to either endothelial cells or basement membrane. Once the cells have passed across the endothelium into the extravascular space they move along a chemotactic gradient towards the site of infection or injury.

Chemotaxis is essentially an in vitro concept and the distance and degree to which leucocytes "chemotax" in vivo is uncertain. However, studies using animal models and skin blisters in man have shown that neutrophils, in particular, will respond to a

chemotactic stimulus and move from the circulation and into the affected area (Issekutz and Movat 1980, Movat 1984).

In vitro studies of chemotaxis with respect to neutrophils, have shown that on exposure to a chemotactic stimulus the "resting" cell is transformed from a spherical cell with a smooth plasma membrane to a polarized, elongated cell with extensive membrane ruffling, a broad anterior lamellipodium, and a rounded uropod in the rear. These events are associated with actin polymerization in the advancing granule free lamellipodium, posterior displacement of the nucleus and anterior disposition of the microtubules organizing centre and cytoplasmic granules. In moving cells circumferential contraction waves, which move from the front to the back of the cell, appear to be important for anterior propulsion (Haston and Shields 1984). These changes are also seen in monocytes in response to chemotactic stimuli (Cianciolo and Snyderman 1981).

In order for an agent to qualify as a chemotaxin it has to induce directed movement of leucocytes in vitro. In addition to the morphological changes that occur in response to these agents, leucocytes also undergo modulation of surface receptor expression. Modulation of chemotactic receptors contributes to directed migration; in the polarized cell, differential receptor density appears to result from translocation of the intracellular pool of receptors to the leading edge. These include receptors for FMLP (Sullivan et al 1984), Fc and C3bi

(Walter et al 1980, Wilkinson et al 1980). Receptor expression is not ligand specific, for example FMLP, in addition to the redistribution of FMLP receptors, also causes increased expression of both Fc and C3bi receptors on neutrophils (Sullivan et al 1984). The redistribution of receptors will occur on cells held in suspension, when cells are allowed to adhere to a suitable surface, locomotion in the direction of polarization is seen. Polarity is therefore independent of adhesion and locomotion, in leucocytes this polarity is temporary and is lost when the agonist is removed. If cells are placed in a uniform concentration of chemoattractant they will migrate at random, if there is a gradient cells will polarize and move along this gradient. The cell will continue to move along the gradient through the redistribution of receptors to the head of the cell.

In the same way that the concept of chemotaxis is an in vitro one, the events which halt the migration of these cells is also based on in vitro data. It has been suggested that neutrophil emigration into inflammatory lesions is terminated by inactivation or inhibition of the chemotaxins. Chemotactic factor inactivators in serum have been reported (Kreutzer et al 1979, Hupp et al 1982) however, observations on tachyphylaxis, whereby cells will not respond to the same concentration of stimulus once they have been previously exposed to it, does not support an in vivo role for these inactivators. This would

suggest that chemotaxis is halted through its effects upon the cells and not the chemotaxis inducing agonist.

While it remains a matter of conjecture as to how far neutrophils and other leucocytes migrate in response to a stimulus there is no doubt that in order to perform their required functions they must migrate into the affected area. This may simply involve movement across the capillary endothelium and basement membrane with little further directed movement.

As the cells move from the circulation and through the tissues they become increasingly "primed" as a result of increased receptor expression and affinity. Studies on exudate neutrophils show increased expression of both FMLP and C3bi receptors (Zimmerli et al 1986). These cells react more rapidly and show enhanced response when finally reaching the site of infection or injury.

1.2.5. Phagocytosis and Respiratory Burst.

The function of neutrophils in vivo, is to phagocytose and kill invading micro-organisms. This is brought about by interactions with receptors at the cell surface; the neutrophil expresses on its surface, receptors for the Fc portion of antibodies. There are three different types of receptor for binding gamma type immunoglobulins FcR1, FcR2 and FcR3; antibodies secreted in

response to invasion by foreign particles act as opsonins and bind to the surface of an invading micro-organism. The C3bi receptor or MAC-1 (CD11b/CD18) has already been discussed with respect to PMN adherence, however, this receptor also plays an important role in phagocytosis. Activation of the complement cascade during an inflammatory response leads to the generation of serum proteins capable of opsonising bacteria, the complement components C3b and C3bi bind to the CR1 receptor (CD35) and the CR3 receptor (CD11b/CD18) respectively. As previously mentioned with respect to PMN adherence, the CR3 receptor shows increased expression in response to chemotactic agents and cytokines.

The neutrophil is capable of binding these opsonised particles which are then internalised due to the formation of a phagocytic vacuole surrounding the bound particle. The resulting phagosome then fuses with the primary granules in the cytoplasm of the cell. This fusion activates the release of lysosomal enzymes into the phagosome which are capable of degrading the phagocytosed bacteria. The activation of the respiratory burst produces toxic oxygen radicals which will ultimately kill the invading microbe.

The respiratory burst can also be activated by soluble chemical mediators such as FMLP, the anaphylatoxin C5a and certain cytokines. The increased metabolic activity in response to these stimuli involves a rapid increase in oxygen consumption

followed by the oxidation of glucose via the pentose phosphate pathway. The activated oxidase system is located in the plasma membrane, it consists of a number of membrane bound and cytosolic components, the latter being translocated to the membrane during activation. The cartoon in figure 1 shows the various components of the NADPH-oxidase complex. It acts as an electron transport chain utilising NADPH as the electron donor, in response to stimulation the soluble oxidase components SOCI, SOCII and SOCIII are translocated to the membrane (Ambruso et al 1990). The other members of the complex include cytochrome b_{-245} (Segal and Jones 1978), and FAD containing flavoprotein (Cross et al 1982) and a ubiquinone (Crawford and Schneider 1982).

NADPH-oxidase activity is defective in chronic granulomatous disease (CGD), which provides a model for the role of NADPH-oxidase activity in bacterial killing. The normal respiratory burst is dependent on appropriate receptor function, intact activating pathways, and an integral NADPH-oxidase. The correlation between ligand binding and biological activity suggests that receptor-ligand binding is critical for the activation of metabolic activity within the cell.

Stimulation of neutrophils results in an initial rise in pH in the phagosome due to consumption of protons in the dismutation of superoxide anions to give hydrogen peroxide and molecular oxygen. This provides the optimal pH for the action of cationic

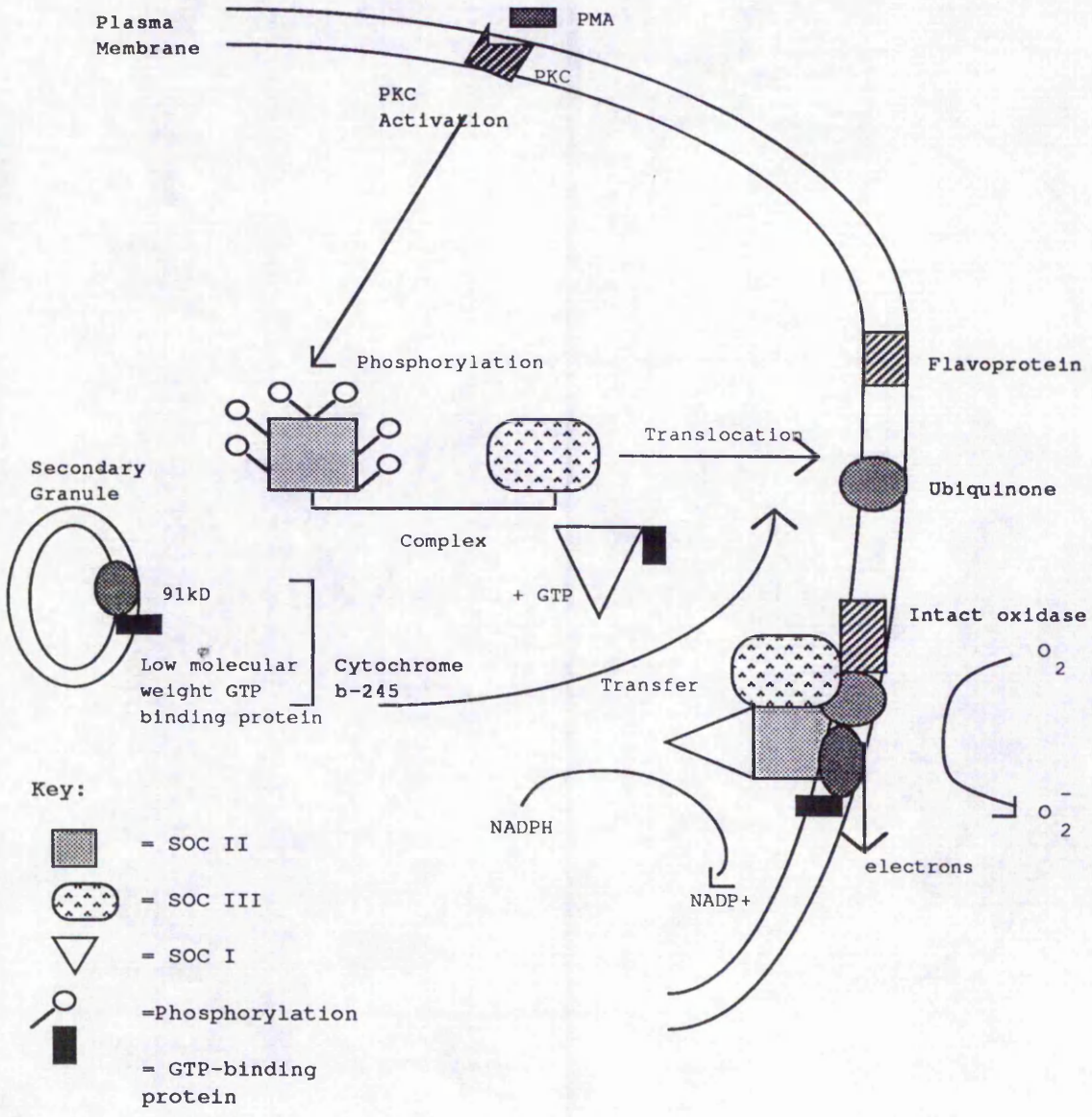

Figure 1: The Neutrophil NADPH-Oxidase. Assembly of components in response to PMA stimulation. Activation leads to phosphorylation of SOCII (47kD) through its six serine phosphorylation sites, SOCII then forms a complex with SOCIII (67kD) and this complex is translocated from the cytosol to the plasma membrane where it binds with a flavoprotein and a ubiquinone. Activation of PKC causes the transfer of cytochrome b_{-245} to the plasma membrane. SOCI, in the presence of GTP is translocated from the cytosol to the plasma membrane to complete the oxidase.

proteins. Then pumping of H^+ from the cytoplasm into the phagosome reduces the pH and provides conditions optimal for the hydrolases released as a result of fusion with the primary granules. In addition, the activation of the oxidase provides oxygen radicals which are also released into the phagosome. The step wise four-electron reduction of O_2 to H_2O_2 produces O_2^-, H_2O_2 and .OH. Superoxide anions are the initial products of respiratory burst and once formed leads to the formation of other radicals or active metabolites. Hydrogen peroxide is formed through spontaneous dismutation then myeloperoxidase released from the primary granules catalyses the formation of hypochlorite from hydrogen peroxide and chloride ions. Hydroxyl radicals are also thought to be produced via the Haber-Weiss reaction and the interaction of hydrogen peroxide and hypochlorite leads to the formation of singlet oxygen. These products of the respiratory burst are toxic to the micro-organism and these together with chloramines (formed through the interaction of hypochlorite and amines), cationic proteins and defensins bring about the destruction of the invading microbe.

The kinetics of both the onset of respiratory burst varies according to the stimulus used. Phagocytic activated respiration may continue for 10-30 minutes fully stimulated, whereas with chemotactic stimuli, the respiratory burst tends to be biphasic, consisting of a highly elevated first phase

lasting only 1-3 minutes, followed by a slower less active phase which may last for more than 30 minutes.

Cationic proteins, effective at neutral pH appear to impede bacterial replication. Lysozyme is a cationic protein which acts by breaking down the proteoglycans found within bacterial cell walls. The release of these proteases into the tissues must be prevented to avoid damage to the host, this is achieved through the action of hypochlorite which in the absence of the other substrates reacts with primary granule enzymes and the NADPH-oxidase inactivating and denaturing them (Weiss 1989).

Neutrophils are therefore ideally equipped with the appropriate machinery and contents to allow for the efficient eradication of a number of micro-organisms with minimal damage being caused to surrounding tissues.

1.2.6. Neutrophil Activation: Signal Transduction Mechanisms

As the previous sections have demonstrated PMN are capable of responding to a number of different stimuli which leads to a range of responses dependent upon the type and concentration of the agonist. The most extensively studied of the PMN cell surface receptors is that for FMLP, and many of the properties exhibited by this receptor with respect to the signal transduction mechanisms, are common.

The FMLP receptor is tightly associated with the membrane, the requirement for bilayer disrupting surfactants to solubilize the protein (Niedel 1981), suggest that it is an integral membrane protein. There is evidence to indicate that guanine nucleotide binding proteins (G-proteins) regulate the affinity of the FMLP receptor, where the interaction of the receptor with a G-protein converts the receptor from a high affinity form to a low affinity form (Lad et al 1985). There may be a relationship between multiple affinity states of the FMLP receptor and the activation of various neutrophil functions. The high affinity state has been associated with chemotaxis and intracellular calcium transients, while the low affinity state of the receptor may be associated with degranulation and respiratory burst activity (Lohr and Snyderman 1982). Sklar et al (1989) reports three states for the FMLP receptor; these are the ternary complex of ligand, receptor and G-protein, the rapidly dissociating occupied receptor (ligand-receptor complex) and a desensitized slowly dissociating guanine-nucleotide-insensitive receptor (desensitized-ligand-receptor complex). These three different states have been characterized by distinct biochemical properties. Upon stimulation with FMLP there is a rapid interconversion among receptor states from a rapidly dissociating form to a slowly dissociating form. Therefore, in intact neutrophils the FMLP receptor can exist in different states, which can be influenced by the binding of ligand.

Having bound the ligand, the FMLP receptor-ligand complex rapidly initiates an increase in intracellular calcium and activation of protein kinase C. Both events occur as a result of the generation of two second messengers, inositol triphosphate (IP_3) and diacylglycerol (DAG). The formation of these second messengers occurs during the breakdown of 1-phosphatidyl-D-myo-inositol 4,5-biphosphate (PIP_2) by an FMLP receptor sensitive phospholipase C (PLC). It is at this level that the pertussis toxin sensitive G-protein is though to act by coupling the PLC and FMLP receptor. Cockcroft and Gomperts (1985) showed that PLC in neutrophil plasma membranes can be activated by the addition of GTP analogues in the presence of calcium. However, the exact mechanisms by which the G-proteins are involved with PLC have not been fully elucidated.

Stimulation of neutrophils also induces release of arachidonic acid which is inhibited by pretreatment of the cells with pertussis toxin (Bokoch and Gilman 1984). The inhibitory effect of pertussis toxin on neutrophil activation by other factors, such as C5a (Becker *et al* 1986), LTB_4 (Goldman *et al* 1985) and PAF (Naccache *et al* 1985) suggest that on binding ligand, these receptors are also coupled to a G-protein. All of these factors act through specific receptors and induce the generation of IP_3 and DAG. Since pertussis toxin reduces neutrophil responsiveness to all these agonists, this suggests that these receptors couple to PLC through a common pathway. It is unknown whether these receptors couple to the same G-protein or PLC.

The active receptor-ligand complex stimulates PLC via a G-protein. PLC then hydrolyses PIP_2, PIP and PI resulting in IP_3, IP_2 and IP respectively, or inositol cyclic phosphates and DAG. DAG can be rapidly metabolised either by a diacylglyceride lipase to a monoacylglycerol and arachidonic acid, or phosphorylated to phosphatidic acid through the action of a diacylglycerol kinase; phosphatidic acid is a precursor for many phospholipids. Of these metabolic products of phosphoinositide metabolism IP_3 and DAG have been shown to be important as signalling mediators. IP_3 causes the release of calcium from intracellular stores (Prentki et al 1984) and DAG stimulates protein kinase C (PKC) (Helfman et al 1983).

An insight into the importance of PKC has been provided with the use of phorbol esters; these act as analogues for the endogenously produced diacylglycerol. Phorbol esters, such as phorbol myristate acetate (PMA) bind directly to and activate PKC and allows for elucidation of the pathway leading from the activation of PKC to NADPH-oxidase activity. PKC activated in this way shows multifunctional catalytic activity and appears to play roles in transmembrane control of protein phosphorylation. PMA stimulates an increase in membrane PKC activity and a decrease in cytosolic PKC activity. PMA activation has been shown to cause the phosphorylation of the 47kD SOCII component of the NADPH-oxidase (Ambruso et al 1990), which, as previously described, is then translocated from the cytosol to the membrane.

The PKC dependent pathway is not the sole cascade for activation of the oxidase system. N-formyl peptides such as FMLP cause marked changes in the permeability of the neutrophil membrane to calcium ions (Korchak et al 1984a). These peptides induce a very rapid release of intracellular, membrane associated calcium. The intracellular calcium levels rise from approximately 1-2 x 10^{-7}M to a stimulated level of 10^{-6}M or more. The changes in membrane permeability to calcium may be dependent on the mobilization of intracellular calcium. On the binding of FMLP to its cell surface receptor, kinetic studies indicate that the rise in intracellular calcium is one of the earliest measurable events (Korchak et al 1984b). This finding is consistent with the hypothesis that increases in cytosolic calcium serve as a signal for the activation of subsequent physiological responses. Sklar and Oades (1985) presented evidence showing that the levels of superoxide anion production and degranulation, in calcium depleted cells, correlated with the amount of calcium made available to the cells at the time of stimulation. This also suggests that both calcium and a signal dependent upon receptor occupancy must be available at the same time. Hallett et al (1990) working with single cells showed that the heterogeneity of the oxidase response was due to the variability in timing and magnitude of the calcium response. Also that there was a threshold requirement for an intracellular concentration of 250 nmoles above which oxidase activity occurred.

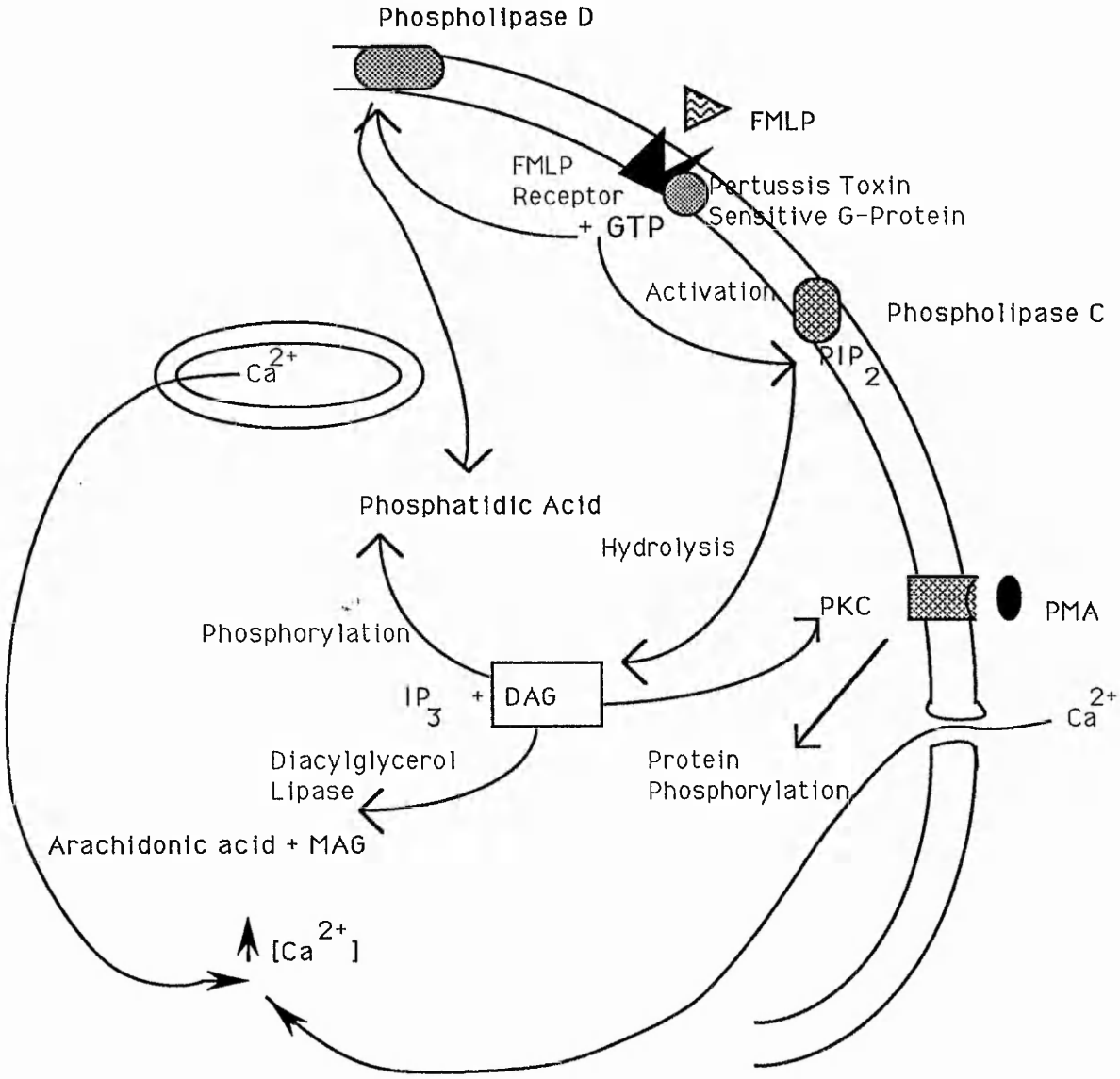

Figure 2: Neutrophil Activation: Involvement of second messengers. Binding of FMLP to its receptor is associated with the activation of a pertussis toxin sensitive G-protein. In the presence of GTP this activation leads to the hydrolysis of PIP_2 by PLC leading to IP_3 and DAG formation. IP_3 releases Ca^{2+} from intracellular stores: DAG can either bind to PKC to open calcium channels and induce phosphorylation of cellular proteins or be phosphorylated to phosphatidic acid or by the action of diacylglycerol lipase be converted to arachidonic acid and MAG

Although the most studied, calcium and DAG dependent pathways are not the only signal transduction mechanisms at work in these cells upon activation. In addition both cAMP and cGMP dependent signal transduction mechanisms play a role, as well as other protein kinases. However, due to the lack of definitive information on these pathways it remains difficult to elucidate the exact mechanisms at work on activation of these cells.

Human neutrophils have been shown to respond to C5a resulting in cell aggregation (Craddock et al 1977), release of granule contents (Henson et al 1978) and induction of the respiratory burst. These responses, in addition to chemotaxis, are mediated by C5a receptors expressed on the cell surface. There are 1-3 x 10^5 binding sites per cell with an affinity of 10^9 M^{-1} (Chenoweth and Hugli 1978). C5a is generated as a result of complement activation, both C5a and C5a des arg will bind to the neutrophil receptor, although the des arg form of the molecule is much less active with respect to neutrophil activation. Henson et al (1978) showed that the interaction of C5a with neutrophils resulted in a concentration dependent rapid desensitization to the agonist. However, cells could still be induced to release granule enzymes in response to complement coated zymosan particles. Chenoweth and Hugli (1980) suggested that on binding of C5a, the ligand-receptor complex is internalised and degraded by the neutrophils. Both C5a and FMLP receptors may be internalised, recycled and re-expressed

(Van Epps et al 1990). FMLP receptors were re-expressed more rapidly than receptors for C5a with the half maximal re-expression time being 5-10 minutes and 18-60 minutes respectively.

Cytokines have been shown to upregulate response to FMLP either by changing the affinity (Atkinson et al 1988) and/or the number (Weisbart et al 1986) of receptors. More recently GM-CSF and TNF have been shown to enhance responses to FMLP by enhancing the signal transduction pathway (McColl et al 1990). The regulation of C5a receptors is less clearly understood. Dahinden et al (1988) showed that GM-CSF could prime neutrophils for increased LTB_4 secretion in response to C5a stimulation. Only a single class of high affinity C5a receptors has been defined in neutrophils, and the suggestion is that GM-CSF primes through its effect on lipid mediators after binding of the agonist.

The activation of neutrophils by the binding of different agonists, brings about a number of complex biochemical changes which eventually lead to the initiation of a variety of cell functions. These differing functions can be modulated through increased receptor numbers, differences in affinity and also varying the concentration of the agonist, such as FMLP.

1.3. Mononuclear phagocytes

The mononuclear phagocyte system is comprised of peripheral blood monocytes, their bone marrow precursors and tissue macrophages. Monocytes and macrophages have many important and diverse functions in addition to being phagocytic cells. Human monocytes circulating in the peripheral blood have a half life of approximately three days. On reaching the tissues the monocyte is referred to as a macrophage, with tissue macrophages exceeding the number of circulating monocytes. The life span of tissue macrophages is unknown, but is in the region of months rather than days. These cells are found predominantly in the spleen, lymph nodes, pulmonary alveoli and peritoneum. Those present in the liver are termed Kupffer cells and in the skin as Langerhans cells. In addition to being found in these areas macrophages are found throughout the body. Once having been induced to migrate into the tissues they do not return to the circulation.

Like neutrophils, monocytes/macrophages act as phagocytic cells and will migrate towards an infected area in response to a number of chemotactic agents. After arriving at the inflammatory focus within the tissue, the cells phagocytose the infectious agent much in the same way as neutrophils. Mononuclear phagocytes express receptors for the Fc portion of immunoglobulins of the IgG class, and also receptors for the third component of complement (C3bi). Opsonised organisms are

recognised by these receptors with the C3bi receptor also possibly recognising LPS (Wright et al 1989). On attachment to the cell the organism is ingested by endocytosis, and again in a manner similar to neutrophils, the organism is then killed by oxygen-dependent and oxygen-independent mechanisms.

In the acute phase of an infection monocytes are stimulated, for example by LPS, to secrete TNF, IL-1 and IL-6. These monokines can mediate fever and TNF and IL-6 have been implicated in the induction of the acute phase response. IL-6 is thought to stimulate the production of C-reactive protein, while TNF is involved in the induction of endotoxic shock. TNF has also been shown to upregulate a number of neutrophil functions, the actions of TNF will be discussed later on in this section.

Mononuclear phagocytes are also capable of modulating the functions of T and B lymphocytes. They act as antigen presenting cells to these lymphocytes which are not capable of recognising free antigen themselves. Monocytes/macrophages also play an important role in inducing and regulating the immune response.

Macrophages will infiltrate tumours and bring about the lysis of tumour cells. Macrophage products including reactive oxygen metabolites, neutral proteases and TNF have been implicated in this cytotoxicity.

An important function of macrophages in the tissues is repair and remodelling. As already mentioned macrophages are relatively long-lived cells within the tissues and they can be found present in wounds immediately after injury. Here they secrete collagenase (Werb and Gordon 1975a) and elastase (Werb and Gordon 1975b) which will act to break down connective tissue. Factors released by these cells induce the proliferation of fibroblasts and promote neovascularisation.

All these described functions are the result of activating these cells with an appropriate stimulus. Macrophages in tissues are quiescent cells that resemble peripheral blood monocytes in morphology. Many stimuli can activate these cells including LPS, complement components and T cell products such as GM-CSF, IL-3 and interferon gamma (IFNgamma). The use of these agents in vitro has brought about a greater under standing of the processes involved in monocyte activation. Warren and Ralph (1986) showed that macrophage colony-stimulating factor (M-CSF) primes monocytes to secrete IL-1, TNF and colony-stimulating activity. In addition M-CSF has been reported to stimulate the antibody dependent cellular cytotoxicity of normal human macrophages. GM-CSF and IL-3 treated macrophages kill more effectively when exposed to a second agonist LPS. The addition of an antibody to TNF abolished this killing suggesting that TNF protein secretion is responsible for these effects (Cannistra et al 1987, 1988).

Monocytes are therefore activated by a number of different agonists to produce soluble chemical mediators which have a range of diverse actions involved in the inflammatory process. The activated cells can also be induced to perform a number of functions which can help eradicate invading microorganisms and repair any tissue damage. The ability of monocyte/macrophage products to affect lymphocyte function adds to the diversity of the role of these cells in inflammation and the immune response. It also highlights the fact that the actions of all these cells, including neutrophils, are intimately linked through the secretion of soluble chemical mediators.

Monocytes also express all three members of the CD11/CD18 family and the main function of these proteins again appears to be adherence to the endothelium. Monocytes like neutrophils, can be prevented from binding to HUVEC by blocking the actions of these molecules using specific monoclonal antibodies. Monocytes also express the LAM-1 molecule and expression can be down-regulated by GM-CSF, PMA or plastic adherence (Griffin et al 1990). As with neutrophils this down-regulation occurs as CD11b expression is increased.

Monocytes behave in much the same way as neutrophils with respect to chemotaxis, including the chemotactic agents to which they respond, through polarization of the cell and redistribution of receptors. IFNgamma and LPS have been shown to promote the ability of macrophages to interact with the

basement membrane glycoproteins laminin and type IV collagen. This may be a requisite step for diapedesis (Shaw and Mercurio 1989). The ability of macrophages to secrete elastase and collagenase (Werb and Gordon 1975b,a) at this time may allow for penetration into the tissues. Once reaching the tissues these cells will be induced to secrete cytokines in response to an appropriate stimulus. They are able to affect neutrophil function through the secretion of these inflammatory mediators, either by the promotion of the inflammatory process through cytokines such as TNF or inhibition of PMN responses as a result of secreting inhibitory factors such as prostaglandin E_2 (PGE_2).

1.4. Cytokines

Cytokines are soluble chemical mediators secreted from a number of cells involved in the inflammatory response. The pro-inflammatory cytokines appear to be synthesized primarily in association with disease states or during host perturbation. These pro-inflammatory cytokines are very potent molecules which trigger a variety of cell responses in vitro and in vivo. They act in cell to cell communication and after secretion can exert their actions on adjacent or distant cells. There is a certain amount of overlap in the biological activities of cytokines designated as growth factors and those thought of as the pro-inflammatory cytokines. For example some growth factors such as GM-CSF and granulocyte colony-stimulating factor (G-

CSF), in addition to promoting cell growth in haemopoietic cells, have also been shown to affect the biological activity of fully mature neutrophils (Nathan 1989). In addition pro-inflammatory cytokines have also been shown to stimulate T and B lymphocytes and activate haemopoietic cells (Cannistra and Griffin 1988).

Attention has focussed on the pro-inflammatory cytokines because of their role in the pathogenesis of acute and chronic diseases. TNF and IL-1, in particular, have been studied in disease states such as local and systemic infection, septic shock, degenerative arthritis and autoimmune diseases. The circulating levels of TNF correlate with disease activity in patients with sepsis (Casey et al 1990). In addition the deleterious effects of gram-negative sepsis can be abrogated by passive immunisation with anti-TNF. This cytokine therefore plays a very important role in acute inflammation.

1.4.1. Tumour Necrosis Factor Alpha

TNF, as the name suggests, was first discovered because of its tumour killing activity. The reduction in the size of patients' tumours appeared to be associated with bacterial infections. William Coley (1893) first demonstrated success in treating cancer patients by infecting them with live bacteria. This work and studies in other laboratories demonstrated that injection of live or killed strains of gram-negative bacteria could cause

haemorrhagic necrosis of tumours. The active component secreted by the bacteria was identified by Shear et al (1943) as lipopolysaccharide. However, it was not the LPS that was causing the necrosis of the tumours but some other factor which was secreted as a result of LPS stimulation. Serum taken from animals treated with either bacillus Calmette-Guerin or LPS provided a source from which this soluble factor, now known as TNF, could be purified. Several groups working on TNF showed that activated macrophages were the source of this cytokine. Technological advances allowed for the cloning of the gene encoding this protein and identification of its amino acid sequence, whilst more recently, X-ray crystallography has revealed the tertiary structure (Jones et al 1990).

TNF is a protein consisting of 157 amino acids with a molecular weight of 17kD, which forms one sub-unit of the TNF molecule. In its bioactive form TNF sub-units associate to from a wedge-like trimer. This trimer consists of two anti-parallel beta sheets, the N-terminus is highly flexible while the C-terminus is embedded in the back beta sheet and forms an integral part of this secondary structural unit. In this biologically active form the evidence points to a receptor site which involves the interface between two sub-units near the base of the TNF trimer (Jones et al 1990).

TNF elicits a wide range of reactions in the body in addition to its tumour necrosing activity. It appears to be crucial in

inflammation and immunity and is secreted early on in the induction of these processes. Gifford and Flick (1987) showed that TNF appeared almost immediately upon LPS injection into animal models. It reached a maximum in the circulation after 1½-2 hours and was undetectable by 4-6 hours. In the same study they show that *in vitro* TNF was shown to be synthesized for 8-12 hours by macrophages in culture with LPS. Once released from monocytes/macrophages TNF is capable of exerting a number of effects, including a profound action on leucocytes and endothelial cell adhesion molecule expression.

TNF can induce neutrophil adhesion *in vitro* by two mechanisms, one a neutrophil dependent mechanism and the other by acting on the endothelial cells. This induction of neutrophil adherence to endothelial monolayers occurs very rapidly through a process that is independent of both RNA and protein synthesis (Gamble *et al* 1985). The endothelium dependent induction of neutrophil adherence by TNF takes longer and is dependent on both RNA and protein synthesis. The CD11/CD18 complex on the neutrophil membrane is important for both the neutrophil dependent and endothelium dependent induction of adherence by TNF. Patients which have a congenital absence of this complex have neutrophils that cannot be induced by TNF to adhere.

TNF appears to exert other effects upon endothelial cells other than inducing the expression of integrin molecules. *In vitro* investigations have shown that this cytokine increased the

permeability of endothelial cells (Brett et al 1989). This alteration in normal barrier function of the endothelium may accelerate the movement of cells and solutes from the circulation and into the tissues.

In addition to its effects on neutrophil adherence TNF modulates neutrophil responses. There is evidence to suggest that TNF increases neutrophil phagocytosis since in the presence of recombinant human TNF, neutrophils have been shown to have increased uptake of fluoresceinated latex beads (Shalaby et al 1985). In addition, TNF has also been shown to prime neutrophils for increased respiratory burst activity when the cells are subsequently exposed to a second agonist. Klebanoff et al (1986) showed that recombinant human TNF alone was a weak direct stimulus of the neutrophil respiratory burst and degranulation. However, an increase in hydrogen peroxide production and lysozyme release was observed in cells preincubated with TNF and then exposed to unopsonised zymosan. Other second agonists, such as FMLP and phorbol esters, also initiated a marked increase in neutrophil responses when the cells were exposed to TNF. With respect to FMLP stimulation TNF is thought to affect the affinity and not the number of cell surface receptors (Atkinson et al 1988). However, other workers have suggested that TNF may also prime neutrophils by inducing secondary degranulation which leads to increased numbers of receptors at the cell surface. More recently TNF has been shown to prime for PMN responses to FMLP through enhancing

phosphatidic acid following the activation of phospholipase D (Bauldry et al 1991).

The ability of TNF to directly stimulate the neutrophil respiratory burst remains contentious. Klebanoff et al (1986) noted that TNF did cause a small but significant increase in hydrogen peroxide production. This finding has been confirmed by Yuo et al (1988), who showed TNF to act as a weak stimulus that did not require either membrane depolarization or Ca^{2+} mobilization for the TNF receptor mediated activation. Other workers insist that TNF is not capable of initiating respiratory burst activity (Nathan 1987) or secondary degranulation (Richter et al 1989) unless the neutrophils are adhered to a biological surface.

TNF initiates these responses by binding to specific receptors on the neutrophil surface. Studies with ^{125}I-TNF have shown that TNF binding at $4^{o}C$ occurs rapidly and is maximal by 20 minutes (Pichyangkul et al 1987). These receptors are reported to be of a single high affinity type. The method of signal transduction by which TNF elicits responses in cells once it has bound to its receptor is unknown. It does not involve intracellular changes in Ca^{2+} (Yuo et al 1988), nor does it appear to involve a pertussis toxin sensitive G-protein or PKC (Berkow and Dodson 1988). However, there is no doubt that recombinant human TNF is capable of profoundly modulating neutrophil responses to inflammatory stimuli with respect to

adhesion, phagocytosis and respiratory burst activity. Some of these PMN responses to TNF can be further enhanced by pre-incubating cells with IFNgamma. Larrick et al (1987) suggested that TNF could induce respiratory burst activity and that this effect was augmented by IFNgamma. More recently Diamond et al (1991) demonstrated that TNF and IFNgamma had differing effects on PMN fungicidal responses to Candida albicans. In this case IFNgamma did not prime cells for increased fungicidal activity in the presence of TNF.

Neutrophil priming has also been demonstrated ex vivo where PMN from subcutaneous animal lesions showed enhanced responses when stimulated by FMLP, PMA and opsonised zymosan (Paty et al 1988).

In addition to its effects upon endothelial cells and neutrophils, TNF has been shown to affect lymphocyte function. Resting lymphocytes lack cellular receptors for TNF (Kull et al 1985), however, human peripheral T lymphocytes can be activated to express TNF receptors (Scheurich et al 1986). In addition, the treatment of IL-2 dependent T cell lines with high doses of TNF results in both the enhanced proliferation of these cells and increased IFNgamma production. Immunofluorescence analyses have shown TNF to be capable of increasing the expression of major histocompatibilty antigens (MHC) as well as increased IL-2 receptor expression on these cells. Several lines of evidence also indicate that TNF is capable of inducing the production of

IL-1 in various cell types. Nawroth et al (1986) showed that incubation of HUVEC with TNF resulted in the release of IL-1. Dinarello et al (1986) found that culture supernatants of human mononuclear cells incubated with TNF contained secreted IL-1. The importance of TNF in the induction of other cytokines has also been demonstrated in animal models, where induction of IL-1 and IL-6 in lethal bacteraemia were reduced in the presence of circulating antibodies to TNF (Fong et al 1989).

Although TNF has profound effects on a number of functions associated with acute inflammation, other pro-inflammatory cytokines are produced in response to the same stimuli and also exert overlapping effects.

1.4.2. Interleukin-1

IL-1 was first described as an endogenous pyrogen derived from leucocytes and was linked to infective/inflammatory processes (Atkins and Wood 1955). Since its discovery, like TNF, advances in technology have allowed its amino acid sequence and structure to be determined. IL-1 has a molecular weight of 17kD and exists in two forms, IL-1alpha and IL-1beta, which have 159 and 153 amino acids respectively. The alpha form of the molecule is membrane bound while the beta form is secreted upon stimulation with agonists such as LPS.

Il-1beta (IL-1) is secreted from a number of cell types. In addition to monocytes and macrophages, Langerhans cells, natural killer cells, fibroblasts, epithelial cells and others have been shown capable of synthesizing this protein in response to a suitable stimulus. Not only is there a wide range of cells that secrete this cytokine, but there is a huge diversity in the number of target cells that respond to IL-1. IL-1 exerts its effects through a specific 80-82kD receptor (Singer et al 1988, Urdal et al 1988).

TNF and IL-1 are secreted with a similar time course from mononuclear cells (MNC) in response to LPS stimulation (Burchett et al, 1988). The effects of IL-1 on neutrophil function are unclear due to differing reports. The previously reported chemotactic properties appear to be due to NAP-1/IL-8 rather than IL-1. IL-1 has been reported to induce neutrophil chemotaxis (Cybulsky et al 1986) and to prime respiratory burst activity in response to a second agonist (Dularay et al 1990), although IL-1 alone did not directly stimulate respiratory burst activity. Georgilis et al (1987) showed that IL-1 had no effect on intracellular calcium ions or functional responses of the cells, including superoxide production, degranulation, phagocytosis or chemotaxis.

Although the actions of IL-1 on PMN response remains unclear, this cytokine plays an important role in the acute inflammatory response due to its ability to induce the expression of

adhesion molecules on endothelial cells (Bevilacqua et al 1985, Pohlman et al 1986). IL-1 can also act as a stimulus for the production of other cytokines, for example the release of IL-2 from T lymphocytes (Durum et al 1984, Goeken and Staggs 1987).

1.4.3. Neutrophil Activating Peptide-1/Interleukin-8

The pro-inflammatory peptide NAP-1/IL-8 was first isolated from LPS stimulated MNC by Yoshimura et al (1987). It was characterised and the cDNA cloned by Matsushima et al (1988). The amino acid sequence revealed it to be a low molecular weight peptide of 72 amino acids with a molecular weight of 6kD. Other groups working on this factor also identified a low molecular weight peptide that co-eluted with IL-1 and was chemotactic for PMN (Peveri et al 1988, Schroder et al 1987, Van Damme et al 1988). It appeared that the bioactivity of PMN chemotaxis which had been attributed to IL-1 was in fact due to this new peptide. Further investigations by a number of groups have suggested that NAP-1/IL-8 is also capable of enhancing the binding activity of CD11b/CD18 (Detmers et al 1989), inducing degranulation, initiating intracellular calcium transients and stimulating PMN respiratory burst activity (Thelen et al 1988). The ability of recombinant human NAP-1/IL-8 to directly stimulate superoxide anion production remains a contentious issue, with some evidence for stimulation of oxygen radicals (Thelen et al 1988) while other data fail to support this finding (Djeu et al 1990).

As NAP-1/IL-8 induces chemotaxis of neutrophils and not monocytes, it has been suggested that this peptide may play a role in the specificity of leucocyte infiltrates in acute inflammation. This contrasts with the activity of other chemoattractants such as C5a and FMLP which induce infiltration of both types of myeloid cells. Leonard et al (1990) showed that NAP-1/IL-8 favoured chemotaxis by neutrophils > basophils > lymphocytes, with eosinophils failing to respond. In addition NAP-1/IL-8 has been shown to induce the release of histamine from basophils that have previously been primed with IL-3 (Dahinden et al 1989).

Although originally isolated from LPS stimulated MNC, this peptide has also been shown to be secreted by endothelial cells (Schroder and Christophers 1989), human dermal fibroblasts (Mielke et al 1990), keratinocytes (Larsen et al 1989) and synovial cells (DeMarco et al 1991). There is also a suggestion that stimulated human peripheral blood neutrophils are capable of synthesizing and releasing NAP-1/IL-8 (Bazzoni et al 1991).

In addition to the in vitro investigations of this peptide, sensitive ELISAs have allowed for the detection of this pro-inflammatory cytokine in biological fluids. In patients with rheumatoid arthritis there appears to be a correlation between the severity of inflammation and the concentrations of immunoreactive peptide assayed in both synovial fluids and serum (Matsushima and Oppenheim 1989). Also, high levels of

NAP-1/IL-8 are associated with psoriatic scales and correlate with the degree of PMN infiltration (Schroder and Christophers 1986). In vitro studies on MNC isolated from the synovial fluid of rheumatoid arthritis patients, show increased production of NAP-1/IL-8 when compared to cells from control individuals (Seitz et al 1991). NAP-1/IL-8 has been detected in the circulation of human subjects receiving endotoxin infusions, whose levels peaked 2 hours after infusion of endotoxin and fell to baseline levels by 5 hours (Martich et al 1991). In vivo studies using primates have shown that NAP-1/IL-8 could be measured in the circulation in septic shock, endotoxaemia and after IL-1 administration, with NAP-1/IL-8 levels peaking later than those of TNF and IL-1, but with the same time course as IL-6 (Van Zee et al 1991). This suggests that NAP-1/IL-8 may be important in the early stages of gram-negative infection due to its potent neutrophil chemotactic activity and ability to activate a number of cell responses. It is possible that this cytokine may be associated with exacerbation of the inflammatory response in a number of disease states that involve neutrophil activation.

1.5. PMN Priming by Cytokines

As already mentioned, a number of factors are capable of modulating PMN function, in particular cytokines. TNF and GM-CSF have been shown to enhance neutrophil function in response to FMLP by exerting effects upon the FMLP receptor. TNF has

been shown to enhance respiratory burst activity in response to FMLP by modulating the affinity and not the number of receptors. TNF induced a change in the cell surface expression of these receptors from a heterogeneous population of high and low affinity, to a homogeneous population of low affinity. The exact mechanisms by which these receptor populations change are not fully understood.

GM-CSF has also been shown to prime neutrophil responses to FMLP by increasing the number of responsive cells within a population (Fletcher and Gasson 1988). This enhancement of a population of responsive cells has also been associated with changes in FMLP receptor affinity. Weisbart et al (1986) suggested that GM-CSF after a short preincubation (5-15 minutes) causes a rapid increase in the number of high affinity FMLP receptors. A more prolonged incubation was accompanied by a change to low affinity receptors. They suggested that high affinity receptors were associated with enhanced chemotactic activity, while low affinity receptor expression correlated with increased respiratory burst activity. This suggests that sequential changes in receptor number and affinity induced by GM-CSF may enhance different physiological responses. English et al (1988) found that the GM-CSF effect on neutrophil oxidative responsiveness was induced in a temperature dependent manner, and was not removed by washing. They reported that GM-CSF primed cells to FMLP by upregulation of cell surface receptors and oxidative responsiveness to FMLP. This

upregulation occurred as a result of increased receptor expression, these data therefore differ from those of others which report that increased responsiveness is due to changes in affinity of the FMLP receptor. Jaswon et al (1990) showed that in whole blood, GM-CSF treatment caused an increase in the number of responding neutrophils and also an increase in total respiratory burst activity when subsequently stimulated with FMLP. GM-CSF is also capable of increasing the magnitude of intracellular calcium transients when cells are stimulated with FMLP (Naccache et al 1988). However, these data may not be sufficient to completely explain the potentiation of neutrophil responses by GM-CSF.

Although TNF and GM-CSF do not appear to have a major direct effect on the functional responses of neutrophils, there is now a wide body of evidence to show that they can profoundly affect the responsiveness of these cells in response to a second agonist.

1.6. PMN Priming by Lipopolysaccharide

Mononuclear phagocytes respond to LPS by secreting cytokines and can also be induced to increase their superoxide production in response to this stimulus. Guthrie et al (1984) showed that isolated human neutrophils could also respond to LPS. They demonstrated that cells were primed for increased respiratory burst activity to a second stimulus, after an initial

preincubation with LPS. LPS pretreatment of neutrophils does not appear to prime by altering the kinetic parameters of the respiratory burst NADPH-oxidase, nor does the priming appear to be associated with a pertussis toxin sensitive G-protein. However, LPS does induce changes in intracellular calcium ion concentration after stimulation with FMLP (Forehand et al 1989). This would suggest that LPS primes cells much in the same way as GM-CSF by enhancing stimulus-induced cellular activity through modifying a calcium-dependent step in signal transduction.

Aida and Pabst (1991) showed that LPS priming of neutrophils to FMLP stimulation was reduced in adherent cells, while cells maintained in suspension showed enhanced superoxide anion release after stimulation with the second agonist. However, LPS was capable of directly stimulating the respiratory burst in adherent cells. Therefore triggering and priming appeared to be reciprocally regulated with the former being a CD11b/CD18 dependent mechanism, while priming occurred in a CD11b/CD18 independent fashion. The role of CD14 also appears to be important in LPS priming of neutrophils with the secondary granules being suggested as an intracellular store for this molecule (Wright et al 1991). Neutrophils appear to be more effectively primed by LPS in the presence of plasma (Aida and Pabst 1990). Removal of plasma from the reaction mixture resulted in reduced priming of the cells and restoring the plasma after washing did not restore the priming activity. This

enhancement of responses to LPS was not due to TNF but appeared to be a result of having some other factor present in the plasma. This would therefore appear to confirm the role of an LBP, which allows cells to respond to LPS once the LPS-LBP complex has formed (Wright et al 1991).

All of these investigations indicate how important it is, when working with neutrophils, to maintain endotoxin free conditions whenever possible, as very low concentrations of contaminating endotoxin can upregulate cellular responsiveness. Therefore, since neutrophil function can be modulated by small amounts of LPS the preparative methods used for the isolation of cells can profoundly affect their function if trace concentrations of LPS are present (Haslett et al 1985).

1.7. Effect of Pentoxifylline on PMN Function

As already mentioned the initiation of an acute inflammatory response leads to the production of superoxide and other radicals produced by activated PMN. These toxic products of respiratory burst activity may be involved in causing tissue damage in a number of inflammatory conditions. PMN have been implicated in tissue damage following myocardial infarction (Schmid-Schonbein and Engler 1986). These cells are also believed to cause damage in a number of inflammatory conditions one of the most extensively studied being adult respiratory distress syndrome (Tracey et al 1989). A drug which suppresses

PMN responses to physiological stimuli *in vivo* is potentially important. Such stimuli include C5a des arg and formyl peptides derived from bacterial cell walls.

Pentoxifylline (PTOX) is a methylxanthine usually prescribed for patients with peripheral or cerebral arterial insufficiency but is also effective in healing varicose ulcers, a condition in which PMN mediated tissue damage may be important (Nash *et al* 1988). In patients with cerebrovascular disorders, open studies with PTOX, 600-1200 mg/day, have shown marked overall improvement in about 85% of patients.

PTOX is rapidly and extensively absorbed from the gastrointestinal tract in addition to being rapidly metabolised, systemically peak concentrations of the parent drug and its main metabolite (MET V) being reached at 1.05-1.8 hours respectively. Pharmacokinetic studies show that following administration of a 400mg sustained release dose, peak plasma concentrations of PTOX and metabolites of 300 to 343ng ml^{-1} were reached at 3.3 and 3.2 hours respectively (Ward and Clissold 1987). Absolute bioactivity has been calculated at approximately 20% for this form of the drug. However, recovery of the metabolite in the urine accounted for almost the complete dose of the drug, suggesting that extensive enterohepatic recycling occurs. PTOX does not appear to bind significantly to plasma proteins with the distribution being uniform throughout the tissues. There is no apparent

accumulation of methylxanthines with repeated doses. Therefore it is important to determine whether effects *in vivo* are due to the parent drug or to one or more of the resulting metabolites.

Metabolism of the drug is primarily by reduction to for metabolite 1 (BL-194) and then by oxidation to form metabolites 4 and 5 (MET IV and V). Several other metabolites have been detected but their exact metabolic pathways have still not been fully elucidated. Rapid excretion into the tissues occurs, predominantly as MET V and to a lesser extent as MET IV. Urinary recovery of these metabolites accounts for about 45% and 5% of the dose respectively. Despite the fact that neither the parent drug nor each of the methylxanthine metabolites are maintained in the circulation or in the tissues, therapeutic trials have shown that constant dosing does lead to improvement in symptoms of perivascular disease.

PTOX has been demonstrated *in vitro* to exert a number of effects upon leucocyte function. It has been demonstrated to increase the filterability of whole blood through its effects on leucocytes and increasing the deformability of blood cells (Schmalzer and Chien 1984, Nees and Schonharting 1988). This effect is thought to be due to decreased actin polymerisation in cells exposed to the drug. Currie *et al* (1990) showed that PTOX exerted a dose and time dependent decrease in the proportion of F-actin in leucocytes, while still permitting the proportional increase in F-actin that resulted from stimulation

with chemotactic peptides. In addition they showed that PTOX reduced CR3 (CD11b) expression, primary degranulation and superoxide anion production. Slater et al (1988) showed that PTOX inhibits C5a des arg stimulated secondary degranulation.

With receptor mediated stimuli, such as C5a des arg and FMLP, the receptor-ligand complex couples with a G-protein and ultimately results in reduced adenylate cyclase activity. Adenylate cyclase catalyses the conversion of ATP to cAMP and increased levels of cAMP can arise through activation of this enzyme or via the inhibition of phosphodiesterase (PDE) which catalyses the hydrolysis of cAMP to 5'AMP. Adenosine and adrenalin inhibit PMN responses by increasing cAMP (Bazzoni et al 1991), although the effects of these two agents have not been confirmed to be via their action on adenylate cyclase, prostaglandins, catecholamines and histamines are potent inhibitors of neutrophil degranulation through stimulation of adenylate cyclase (Ignarro and Columbo 1973, Marone et al 1980). Methylxanthines exert similar inhibitory effects by acting as PDE inhibitors. This again results in increased intracellular concentrations of cAMP, the result being inhibition of a number of cell functions which are dependent on this second messenger pathway.

While PTOX has been shown to exert a number of inhibitory effects on PMN function in vitro many of the studies are carried out using mM (ug ml^{-1}) concentrations. These

concentrations of the drug are not achievable *in vivo*. Investigation of the kinetics of intravenous and oral PTOX administration indicate that with repeated dosing, with either method resulted in minimal cumulation of PTOX in the plasma (Beerman *et al* 1985). It is therefore important to determine whether PTOX is capable of demonstrating these properties at physiological concentrations. Currie *et al* (1991) measured neutrophil elastase/alpha 1 proteinase inhibitor complex (E/alpha) in patients with claudication receiving PTOX. The results indicate that after two months treatment there was a reduction in neutrophil function as indicated by the measurement of plasma E/alpha. However, PTOX is rapidly metabolised into a number of other methylxanthines (Beerman *et al* 1985), which are all potential PDE inhibitors.

As well as its direct effects on PMN function PTOX has also been shown to act on MNC. Animal models have shown this methylxanthine to be capable of increasing the ability of mice to survive LPS induced sepsis (Schade 1989). In addition, normal healthy human adults have received endotoxin and administration of PTOX was shown to reduce circulating levels of TNF (Zabel *et al* 1989). While subjects treated with PTOX in addition to LPS showed reduced TNF, they still presented with symptoms associated with bacteraemia. This lack of relief from clinical symptoms suggested that the production of IL-1 was not being inhibited. *In vitro* investigations have confirmed these findings and postulated that PTOX acts to selectively inhibit

TNF secretion from LPS stimulated MNC with no effect on secreted levels of IL-1 (Endres et al 1991). As PTOX is acting on PDE to cause increases in cAMP and inhibition of TNF production, IL-1 is thought to be unaffected as a result of its production being modulated by a cGMP dependent pathway. Substances that increase the intracellular levels of cGMP act to inhibit IL-1 production but have no effect upon TNF.

More recently these effects of PTOX on cytokine production have been associated with decreased replication of the human immunodeficiency virus type 1 in vitro (Fazely et al 1991). Increased TNF levels have been associated in cachectic patients with the acquired immunodeficiency syndrome. As TNF decreases the therapeutic efficacy of zidovudine (AZT), it would appear that PTOX, by acting to inhibit TNF production reduces replication of the virus, leading to another possible therapeutic use of the drug.

With respect to PMN, as TNF can prime for increased respiratory burst activity PTOX may potentially act at two levels; directly on the PMN and/or via the inhibition of TNF production from activated MNC. Because of the important implications of this drug and its possible uses it remains important to show effectiveness in vitro at physiological concentrations and also confirm the role of the metabolites in the actions of this drug.

1.8. Project Aims

The aim of this project was to study the effects of MNC products on PMN function, and in so doing compare the effects of pro-inflammatory agents secreted by cells *in vitro* with the recombinant human cytokine preparations available. Certain aspects of PMN function have been investigated which include:-

1) the expression of adhesion molecules that are required for margination and migration of cells.

2) secondary degranulation has also been examined using Lf as a secondary granule marker.

3) the modulation of respiratory burst activity using lucigenin-enhanced chemiluminescence.

As Lf has been implicated as a negative regulator of cytokine production and as Lf is released from neutrophils in response to TNF stimulation (Richter *et al* 1989), the role of Lf in the modulation of MNC cytokine production has been investigated in an attempt to determine its physiological significance.

While the activation of MNC and PMN normally appears to be tightly controlled there are certain conditions where excessive neutrophil activation leads to tissue damage. The role of the drug PTOX has been investigated for its ability to regulate PMN function *in vivo* and *in vitro*.

This study therefore aims to identify factors that can upregulate PMN responses and also try to elucidate how negative regulators may be involved in switching off the inflammatory response once the injurious agent has been removed.

CHAPTER TWO: MATERIALS AND METHODS

2.1. Isolation of Leucocytes

Venous blood was obtained from normal healthy volunteers into EDTA-dipotassium at a final concentration of 3mM. The blood was used immediately after venesection. The PMN and MNC were obtained using a rapid single step technique. Three to five mls of blood was layered onto 3 mls of a Ficoll-Hypaque solution of 1.114g ml^{-1} density (Mono-Poly Resolving Medium: Flow Laboratories). After centrifugation at 400g for 40 minutes at 22°C, the leucocytes resolved into two bands; MNC forming a band at the interface and PMN collected in a second band below the MNC. During centrifugation the erythrocytes sedimented to the bottom of the tube. The platelet-rich plasma was discarded and the MNC collected and washed twice in RPMI-1640 medium (Northumbria Biologicals). This process routinely produced a MNC fraction of greater than 98% viability, as determined by trypan blue exclusion, with a purity of around 97%. The MNC collected contained an average of 30% monocytes as determined by 'Diff-Quick' (Merz and Dade) stained cytospin preparations. These MNC were then used either for culture with LPS or for setting up mixed lymphocyte cultures (MLC).

The PMN were collected from the second band and washed in sterile PBS pH 7.2, and after centrifugation at 400g for 10 minutes at 4°C, the cells were resuspended at a concentration

of 1 x 10^7 ml^{-1} again in sterile PBS pH 7.2. Cell viability was greater than 99% as determined by trypan blue exclusion, with a purity greater than 96% as determined by Wright-Giemsa staining of cytospin preparations. These preparations contained eosinophils at < 2% and basophils at < 1%, the neutrophils were not further purified from these cells. The PMN were kept on ice at 4°C until required.

2.2. Mononuclear cell cultures

In order to determine cytokine production from MNC these cells were cultured with 1ug ml^{-1} of LPS, E.Coli 0111:B4 (Sigma Chemical Co.). Separate donor MNC were cultured at 3 x 10^6 ml^{-1} in complete medium. This medium comprised RPMI-1640 medium supplemented with 10% (v/v) heat inactivated foetal calf serum (FCS), 2mM L-glutamine, 100Uml^{-1} penicillin and 100ug ml^{-1} streptomycin (all Flow Laboratories). These cells were incubated in 1ml volumes in 12 well tissue culture plates (Costar) at 37°C + 5% CO_2 with 95% humidity for the required time (see results chapter 3). At the end of the culture period the conditioned media (MNCM) were collected and the non-adherent cells spun down at 400g for 10 minutes. The supernatants were collected and filtered through 0.2um sterile filters (Sartorius). The cell free MNCM were then stored at -70°C until required for measurement of cytokines or for PMN priming experiments.

2.3. Mixed Lymphocyte cultures

MNC from three individual donors were suspended at 1.1 x 10^6 ml^{-1} in complete medium with the addition of 10^{-6}M indomethacin (Sigma Chemical Co.). Equal volumes of each donor suspension were mixed together and 180ul added to triplicate wells in a 96 well tissue culture plate (Costar). 50%-Fe saturated Lf was added where required at the appropriate concentrations at the beginning of the culture period. In some experiments wells were set up with the addition of a rabbit polyclonal antibody to human Lf (Dakopatts) at a dilution of 1:500. In control wells the same concentration of preimmune rabbit serum (Dakopatts) was used. Both preparations were dialysed against PBS pH 7.4 for 24 hours to remove sodium azide from the preparations. The MLC were then cultured for 5, 24, 48 and 72 hours at 37°C + 5% CO_2 with 95% humidity. At the end of each culture period cell proliferation was determined by measuring tritiated thymidine (Amersham UK) incorporation following a 6 hour pulse. The samples were then counted for three minutes each in a Packard Tri Carb series 4000 scintillation counter with window settings on 2-19MeV. The means of triplicates were taken to represent the proliferation for a particular sample.

Duplicate plates were also set up for determining cytokine production; after incubation the plates were spun down at 400g for 10 minutes and the cell free supernatants harvested and stored at -70°C until required for assay.

2.3.1 Lactoferrin

Human breast milk Lf (approximately 98% pure from Sigma Chemical Co.) was diluted in 0.2M Tris/HCl buffer at pH 7.3. Iron was added to give 50% saturation of available binding sites. This was achieved according to the method of Gutteridge et al (1981). Briefly, Lf was diluted in half the volume of Tris/HCl buffer (containing 10mM $NaHCO_3$) needed to give the required concentration of Lf. Ferrous ammonium sulphate was prepared in freshly distilled water collected into glass. Based on the calculation that 1.4ug of Fe gives 100% saturation of 1mg Lf, 49ug per 10mg of Lf was required to give 50% saturation. Equal volumes of the two solutions were mixed and incubated together for 30 minutes at 37°C. The protein was dialysed against Tris/HCl for 24 hours at 4°C. Successful 50% Fe-saturation was determined by detection of an absorbance peak at 460nm using a Gilford 260 spectrophotometer

2.3.2 Cytokine Assays

2.3.2.1. Tumour necrosis factor

Bioactive TNF was measured using the mouse fibroblast L929 assay according to the method of Matthews and Neale (1987). Briefly, MNCM were diluted 1:200 in the complete medium required for growth of the fibroblasts, containing 2ug ml^{-1} of Actinomycin-D (Sigma Chemical Co.). Recombinant human TNF

(kindly supplied by Dr A Meager at NIBSC) was diluted in the same medium, with the standard curve used ranging from 0.156U ml^{-1} TNF to 1.2 U ml^{-1} using serial doubling dilutions. 75ul of either TNF or samples were added to triplicate wells of a 96 flat-bottomed well tissue culture plate (Costar) containing monolayers of the L929 cells. For each sample control wells were set up with the addition of anti-TNF-alpha and anti-TNF-beta (both Genzyme), at a dilution of 1:100 to ensure that any cytotoxicity observed was due to TNF-alpha in the samples. The cells were incubated for 18 hours at 37°C + 5% CO_2 + 95% humidity, after this time the culture medium was removed and 250ul of 5% (v/v) formaldehyde (BDH) in PBS pH 7.2 was added to each well for 5 minutes. The formaldehyde solution was removed and the plates washed thoroughly in running tap water. 250ul of 1% aqueous crystal violet (Sigma Chemical Co.) was then added to each well for 5 minutes. The plate was washed thoroughly in running tap water. Any excess water was removed and the plates were left at room temperature to dry thoroughly. The crystal violet dye was solubilised with 33% (v/v) glacial acetic acid (BDH) and the plate was mixed thoroughly on a plate mixer before reading on a Kontron SLT 210 plate reader at 590nm.

A reduction in the absorbance reading correlated with increasing concentrations of TNF. The absorbance reading for each sample was read from a curve of TNF concentration plotted against % cell cytotoxicity, calculated as a percentage of control cells not exposed to TNF. This allowed for the

determination of the number of bioactive units TNF in each sample to be calculated.

Immunoreactive TNF was assayed using a modification of the ELISA method of Meager et al (1987). This sensitive ELISA involved adding 50ul, to each well of a 96 well plate (Dynatech), of a polyclonal rabbit-anti-human TNF-alpha antibody (Genzyme) at a dilution of 1:1000 in 50mM sodium carbonate/bicarbonate coating buffer pH 9.5. After an overnight incubation at 4°C the coating antibody was removed and 50ul of 2% (w/v) BSA in PBS pH7.4 added to each well to block any non-specific binding sites. After incubation at 37°C for 1 hour the plate was washed thoroughly (4 x 3 minutes) in 0.9% NaCl containing 0.05% (v/v) Tween 20 (BDH). Recombinant human TNF and samples (1:5) were diluted in PBS pH 7.4 containing 0.5% (w/v) BSA and 0.05% (v/v) Tween 20. The concentrations of TNF standards used were from 0.156U ml^{-1} to 10U ml^{-1} with serial doubling dilutions. After incubation overnight at 4°C the plates were washed as described previously. 50ul of mouse-anti-human TNF-alpha monoclonal antibody (A kind gift from Dr A Meager at NIBSC) at a dilution of 1:2000, in sample diluent buffer, was added to each well and incubated for 2 hours at room temperature (RT). The plates were washed again and 50ul of biotinylated rabbit-anti-mouse IgG_1 (Amersham UK) at a dilution of 1:4000 was added to each well. The plates were incubated for 1 hour at 37°C. After washing 50ul of streptavidin-biotin conjugate at a dilution of 1:4000 was added to each well and

incubated for 1 hour at 37°C. The plates were washed as before with a final rinse in distilled water. Finally 50ul of colour reagent and substrate, 10mg o-phenyldiamine (OPD) substrate in 10 mls of citrate buffer pH 5.0 + 0.006% (v/v) hydrogen peroxide, were added to each well and the plate incubated in the dark at RT for 30 minutes. The reaction was stopped with 1M H_2SO_4 and the plate read on a Kontron SLT 210 plate reader at 492nm with a 620nm reference filter.

2.3.2.2. Interleukin-1

Immunoreactive IL-1 in culture supernatants was determined using an R&D systems ELISA kit (British Biotechnology: lot number 90 46 056). Test samples were diluted 1:4 in PBS pH 7.4 and 50ul of each sample added to duplicated wells. The plates were read on a Kontron SLT 210 plate reader at 450nm with 540nm reference filter.

2.3.2.3. GM-CSF

Immunoreactive GM-CSF was measured by ELISA, the method used was the same as for the TNF ELISA but substituting GM-CSF antiserum at 1:500 dilution for the capture antibody. Samples were diluted 1:4 in PBS pH 7.4 containing 0.5% (w/v) BSA and 0.05% (v/v) Tween 20. Recombinant human GM-CSF standard (Glaxo) was also diluted in this buffer and 50ul of standard and test samples were added to each well in triplicate.

Monoclonal anti-GM-CSF was added to each well at a dilution of 1:1000. The rest of the procedure was the same as for the TNF ELISA (section 2.3.2.1).

2.4. Lucigenin-Enhanced Chemiluminescence

Lucigenin-enhanced chemiluminescence (LUCL) was used to determine superoxide anion production from PMN stimulated with either FMLP or zymosan-activated serum (ZAS). 50ul of PMN at a concentration of 1 x 10^7 ml^{-1} was added to cuvettes containing 50ul of 2.5 x 10^{-5}M lucigenin (Sigma Chemical Co), 350ul of PBS pH 7.2 containing 0.9mM $CaCl_2$ and 0.7mM $MgCl_2$ + 0.1% (w/v) low endotoxin BSA (Sigma Chemical Co.) (PBS Ca/Mg/BSA) and 50ul of stimulus or control PBS, to give a final concentration of PMN at 1 x $10^6 ml^{-1}$. LUCL was monitored using a Bio-Orbit 1251 luminometer with phagocytosis assay software package. The amount of 'light' emitted, measured in mV, after addition of the stimulus was monitored over a 30 minute period. Each sample was run in duplicate and the results were expressed as the integral CL at 28 minutes, taking the means of the two samples to represent the final result.

In order to confirm the measurement of superoxide anions experiments were set up with the addition of 1000U ml^{-1} of superoxide dismutase (Sigma Chemical Co.).

2.4.1. Zymosan-Activated Serum

Zymosan-activated serum (ZAS), as a source of C5a des arg was prepared according to the method of Fernandez et al (1978). Briefly, venous blood was taken from 12 normal healthy volunteers, the blood was allowed to clot in serum tubes for 1 hour at RT. The clotted blood was then centrifuged at 1000g for 15 minutes and the supernatant collected from each tube. The serum from all the donors was then pooled together and incubated for 1 hour at 37°C in the presence of 1mg ml^{-1} zymosan-A (Sigma Chemical Co.). At the end of the incubation period the zymosan particles were spun down at 1000g for 15 minutes and the supernatants collected. This supernatant was filtered twice through sterile 0.2um filters in order to remove any remaining zymosan particles. The ZAS was then aliquoted and stored at -70°C until required.

For use in chemiluminescence experiments 50ul of ZAS was added to luminometer cuvettes to give a final concentration of ZAS at 10% by volume.

2.4.2. FMLP

FMLP supplied by Sigma Chemical Co. was diluted initially in DMSO (BDH) and then made up with sterile PBS pH 7.2 to give a final stock concentration of FMLP at 10^{-3}M, the final concentration of DMSO in the stock solution was 0.02% (v/v) .

For use in LUCL this stock was diluted further to give a final concentration in the cuvette of 10^{-6}M. Each time a new batch of FMLP was reconstituted a dose response curve, with respect to LUCL, was carried out to ensure use of the optimal concentration in these experiments.

2.5. Measurement of Lactoferrin

Lf was measured in supernatants from stimulated PMN using a sensitive ELISA technique. PMN at 1 x 10^6 ml^{-1} in PBS Ca/Mg/BSA were incubated for the required time (see results chapter 4) on a rotating wheel at 37°C. The PMN were centrifuged at 400g for 10 minutes and the cell free supernatants were collected and stored at -20°C until required for assay.

The ELISA method involved adding 200ul of rabbit-anti-human Lf (Dakopatts) at a dilution of 1:1000 in 50mM sodium carbonate/bicarbonate coating buffer pH 9.5, to each well of a 96 well microtitre plate (Dynatech). After an overnight incubation at 4°C the plates were rinsed twice with PBS pH 7.4. Blocking buffer, 2% (w/v) BSA in PBS pH 7.4, was added in a 200ul volume to each well to block any unfilled binding sites; the plate was incubated at room temperature for 30 minutes. The plate was washed (4 x 4 minutes) in PBS pH 7.4 containing 0.01% (v/v) Tween 20. Lf standards were prepared from a stock solution of 10^{-6}M Lf to give a range of standards from 0.4 to 0.05 nmoles. Lf and test samples were diluted in PBS pH 7.4

containing 0.5% (w/v) BSA and 0.05% (v/v) Tween 20. 50ul of standards and samples (diluted 1:10) were added to each well and incubated on a continuous shaker for two hours at RT. The plates were washed as previously described and 50ul of biotinylated-anti Lf (Dakopatts) at a dilution of 1:1000, was added to each well. The plates were incubated for 1½ hours at RT, with continuous shaking. After washing, 50ul of a 1:1000 dilution of avidin-peroxidase conjugate in borate saline buffer pH 8.6 was added to each well and incubated with continuous shaking for 1 hour at RT. The plates were washed with borate saline pH 8.6 containing 0.01% (v/v) Tween 20 (4 x 4 minutes). 5-aminosalicylic acid colour substrate at 1mg ml^{-1} in 0.2M phosphate buffer plus 0.01% (v/v) hydrogen peroxide in a 100ul volume was added to each well and incubated with continuous shaking for 40 minutes at RT. The reaction was stopped with the addition of 50ul of 3M NaOH to each well. After mixing the plate was read immediately at 455.5nm with a 620nm reference filter using a Kontron SLT 210 plate reader.

2.6. Adhesion Protein Expression

The cell surface expression of adhesion molecules on PMN was determined by flow cytometry using an indirect immunofluorescence technique. PMN at 1 x 10^6 ml^{-1} in PBS/Ca/Mg/BSA were incubated at 37°C, in the presence or absence of stimulus. At the end of the required incubation period the cells were centrifuged at 400g for 10 minutes and

washed twice in cold sterile PBS pH 7.2. Mouse monoclonal antibodies to CD11a, CD11b, CD11c and CD18 (Dakopatts) were added at a final dilution of 1:200. The cells were incubated at 4°C for 30 minutes. The PMN were centrifuged and washed, as previously described, and then incubated with a rabbit-anti-mouse-FITC conjugated polyclonal IgG_1 (F_{ab}) (Dakopatts) at a final dilution of 1:30 for 20 minutes at 4°C. The cells were again centrifuged and washed before being analysed by a Becton and Dickinson FACS analyser. Any contaminating erythrocytes were gated out and cytometer settings were established using unstimulated control preparations.

A change in the expression of these molecules with stimulation was calculated as the percentage shift in mean fluorescence over readings for unstimulated controls.

2.7. Sephadex Gel Filtration

In order to try and partially purify some of the proteins and peptides secreted by LPS stimulated MNC, MNCM was filtered over Sephadex G-75 and Sephadex G-50 columns. The Sephadex (Pharmacia) was swollen and packed using sterile PBS pH 7.2, and was autoclaved before packing. Once layered onto the columns fractions were eluted with sterile PBS pH 7.2. 2ml fractions were collected from G-75 columns and 3ml fractions from G-50 columns using a Gilson model 202 fraction collector and the UV absorbance for each fraction at 280nm was monitored

using a Gilford 260 spectrophotometer. All fractions with a UV absorbance were stored at -70°C for determining their effects on PMN respiratory burst activity.

2.7.1. Protein Determination

Fractions eluted from both types of columns were tested for the presence of proteins using the method of Lowry et al (1951). Briefly, 100ul of each sample was taken and added to a plastic tube containing 1ml of the following solution; 2% anhydrous Na_2CO_3 in 0.1M NaOH, 0.5% $CuSO_4.5H_2O$ and 1% sodium potassium tartrate in a ratio of 48:1:1. 1M NaOH is added to bring the pH to 7.0. Each tube was mixed well and left at RT for 10 minutes. 100ul of Folin's reagent, previously diluted 1:1 with distilled H_2O, was added. The tubes were mixed well and left at RT for 30 minutes. After this time the samples were diluted with 2mls of distilled H_2O and absorbance read on a Gilford 260 spectrophotometer at 660nm.

2.8. Intracellular Free Calcium Concentration

Changes in the concentration of calcium ions (Ca^{2+}) was measured using fura-2-am (the acetoxymethyl ester of fura-2) fluorescence according to a method outlined by Kuroki et al (1989). PMN were isolated from circulating peripheral blood, as previously described. To ensure that any fluorescence changes were due to PMN, the erythrocytes were lysed by hypotonic

lysis. This involved a brief (15 seconds) incubation with 0.2% NaCl, followed by addition of an equal volume of 1.6% NaCl. The cells were then centrifuged at 400g for 10 minutes and resuspended at 1 x 10^7 ml^{-1} in Hepes buffer containing 135mM NaCl, 5mM KCl, 5mM glucose and 20mM Hepes (pH 7.4). PMN were then incubated in 1uM fura-2-am (Sigma Chemical Co.) at 37°C in the dark on a rotating wheel for 30 minutes. PMN were then washed in Hepes buffer and centrifuged at 400g for 10 minutes, this washing procedure was repeated to ensure removal of excess fura-2-am. The cells were then resuspended at 4 x 10^6 ml^{-1} in Hepes buffer and stored on ice until the experiments were performed.

Measurements were taken using a Kontron fluorimeter, the excitation and emission wavelengths were 340nm and 500nm respectively. The fluorimeter was set up against blank cuvettes containing Hepes buffer. Cuvettes containing 2mls of PMN suspension were added to the fluorimeter and maintained at a temperature of 37°C. After a steady baseline was established stimulus was added and changes in fluorescence intensity monitored continuously. Free $[Ca^{2+}]_i$ was calculated using the following equation:-

$$[Ca^{2+}]_i = Kd. \frac{(F - F_{min})}{(F_{max} - F)}$$

Where F is the fluorescence intensity of the dye in the cells, F_{max} is the fluorescence at saturating Ca^{2+} concentrations, which is determined by the addition of 50ul of 4% Triton X100 (Sigma) (final concentration 0.1% by volume). F_{min} is the intensity at zero Ca^{2+} concentration determined by the addition of 50ul of 400nM EGTA (final concentration 10mM). The dissociation constant (Kd) of fura-2-am for Ca^{2+} was assumed to be 224nM at 37°C (Grynkiewicz et al 1985). Any remaining extracellular fura-2-am was not quenched; this may be achieved by the addition of manganese ions which will preferentially bind to the fura-2-am with a greater affinity than calcium but without excitation of the fluorochrome.

2.8.1. Preparation of Fura-2-am

1mg of Fura-2-am (Sigma) was dissolved initially in 1ml of DMSO to give a 1mM stock solution, this was aliquoted into 50ul volumes and stored at -20°C until required. This stock solution was diluted 1:10 in Hepes buffer prior to use and 10ul of this solution was added per 1ml of PMN suspension.

2.9. Endotoxin

All reagents were tested for the presence of endotoxin using the limulus-amoebocyte lysate assay, E-toxate from Sigma Chemical Co.. The use of this assay allowed for detection of endotoxin down to 10pg ml^{-1}. Reagents which had more than 20pg

ml^{-1} of contaminating endotoxin were not used in any of the experiments involving cell function. However, in fractions eluted from the Sephadex columns it was impossible to achieve endotoxin scores below 50pg ml^{-1}, and the possible effects of this level of endotoxin had to be taken into consideration when interpreting results.

2.10. Pentoxifylline

Eight normal healthy volunteers received a 1 x 400mg oral dose of slow release pentoxifylline (PTOX) (Hoechst UK). Blood was taken prior to ingestion and at 1½, 5, 24 and 48 hours post ingestion. At each of these time points venous blood was collected, PMN purified and LUCL monitored in response to ZAS and FMLP stimulation, as previously described (methods 2.4.).

2.11. Statistics

Statistical significance was determined using the Mann-Whitney U non-parametric test. Correlations were determined using Spearman's Rank correlation co-efficient. For both tests a value of $p<0.05$ was considered to be significant.

CHAPTER THREE: RESULTS

3. PMN Respiratory Burst Activity

3.1. Lucigenin-Enhanced Chemiluminescence

Preliminary experiments were carried out in order to determine the optimal concentrations of stimuli to be used in further investigations. The two stimuli used were ZAS and FMLP; PMN were separated from whole blood, as described in methods 2.1. Figure 3 shows an example of the chemiluminescence curves seen for both stimuli; the area under the curve, or integral CL (mV x mins) at 28 minutes was taken to represent overall superoxide anion generation over this time period. Dose response curves were carried out for both stimuli using this measurement. Figure 4 shows the dose response curve for ZAS, with maximal respiratory burst activity seen with 10% ZAS, higher concentrations gave erroneous results probably due to increased protein concentrations. Albumin acts to scavenge superoxide anions (Campbell 1988) and increasing the amount of serum used delayed the rate of increase in LUCL with this stimulus. Therefore, all further experiments were carried out using ZAS at a concentration of 10% by volume.

An FMLP dose response curve was also carried out, the results (shown in figure 4, panel b) indicate that maximal respiratory burst activity occurred at an FMLP concentration of 10^{-5}M to

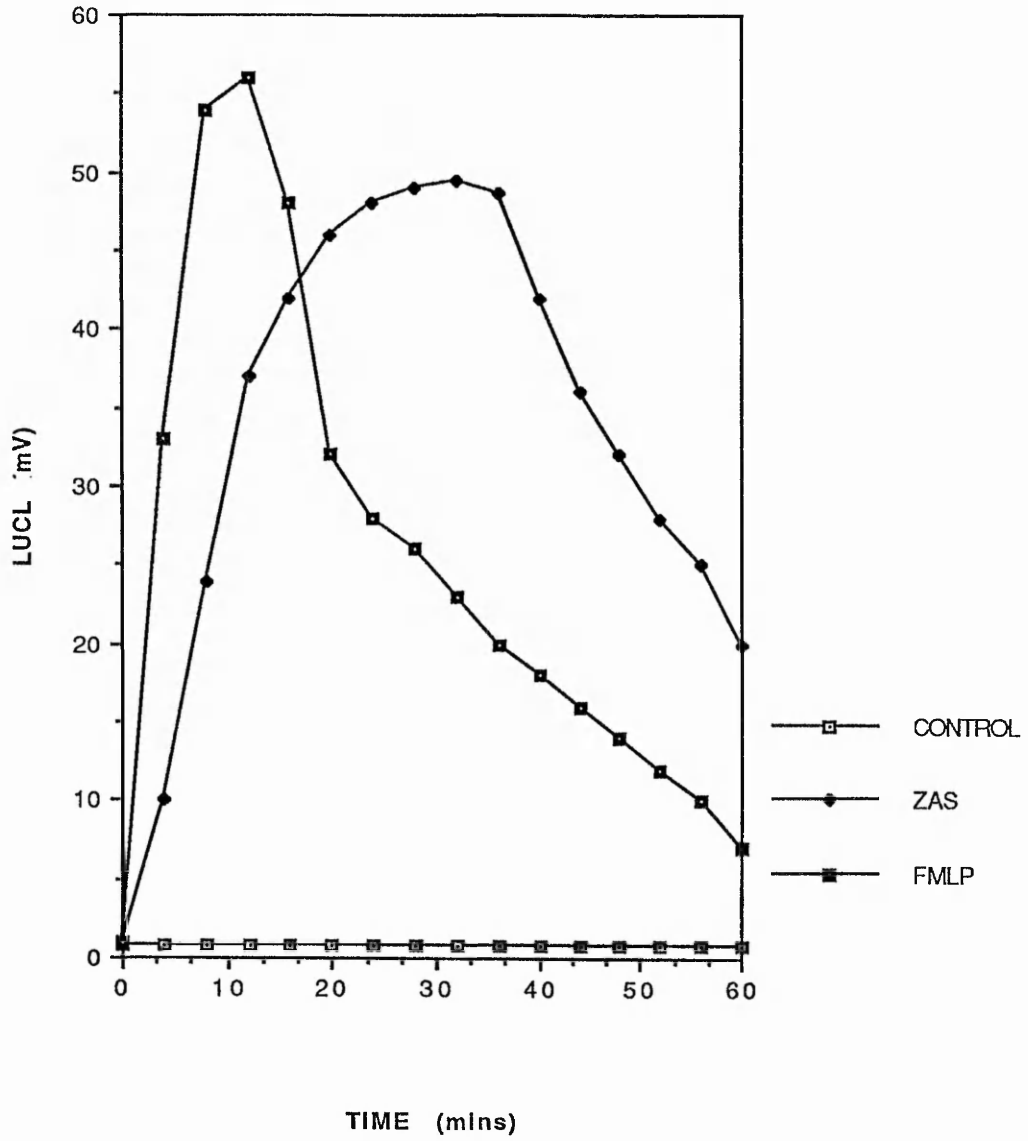

Figure 3: Chemiluminescence curves for PMN responses to ZAS and FMLP stimulation, compared with unstimulated controls. Stimulus was added at time 0 and the cells were monitored continuously for the time shown.

The results are from one representative experiment.

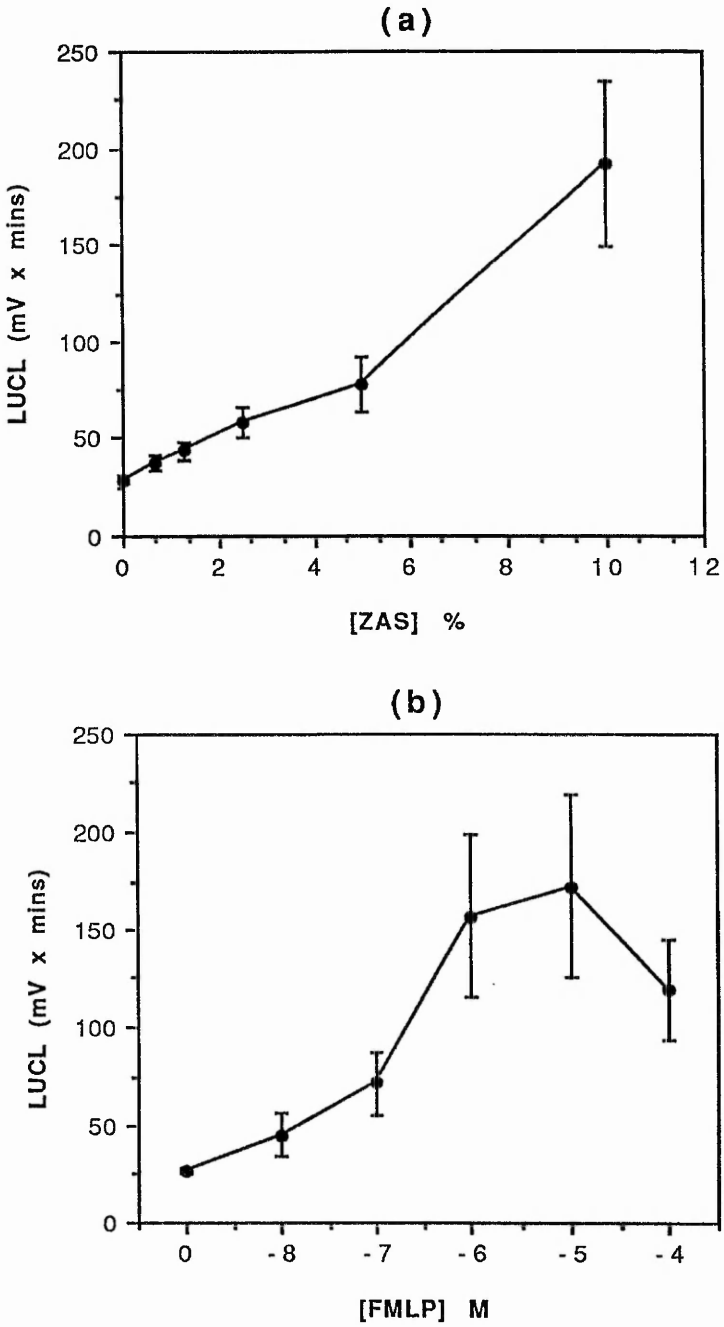

Figure 4: PMN superoxide anion production, as measured by LUCL, in response to increasing concentrations of ZAS (a) and FMLP (b).

The results represent the means of 12 experiments ± SEM, using different individual donor PMN

10^{-6}M. There were no statistically significant differences between these two concentrations and therefore a concentration of 10^{-6}M was used for all subsequent experiments.

Having determined the concentrations of stimuli to be used it was important to establish that the CL curves did represent superoxide anion production, therefore control experiments were carried out in the presence of the enzyme superoxide dismutase (SOD). Figure 5a shows a dose dependent reduction in both the rate of CL and overall amount of superoxide anions detected, when PMN were stimulated with 10^{-6}M FMLP. Further experiments carried out using 1000U ml^{-1} of SOD showed that detectable superoxide anions were substantially reduced in response to both stimuli (figure 5b). When higher concentrations of SOD were used LUCL was not detectable above unstimulated controls. These findings confirmed those of Gyllenhammer (1987), showing that LUCL is a sensitive means of monitoring superoxide anion production from PMN.

3.2. The Effect of MNC products on PMN Respiratory Burst

MNC were initially cultured for 18 hours in the presence of 10ug ml^{-1} LPS according to the method of Sullivan et al (1988) in order to stimulate the secretion of inflammatory cytokines into the surrounding medium. The MNCM generated were tested against PMN for their ability to prime these cells for increased respiratory burst activity when subsequently

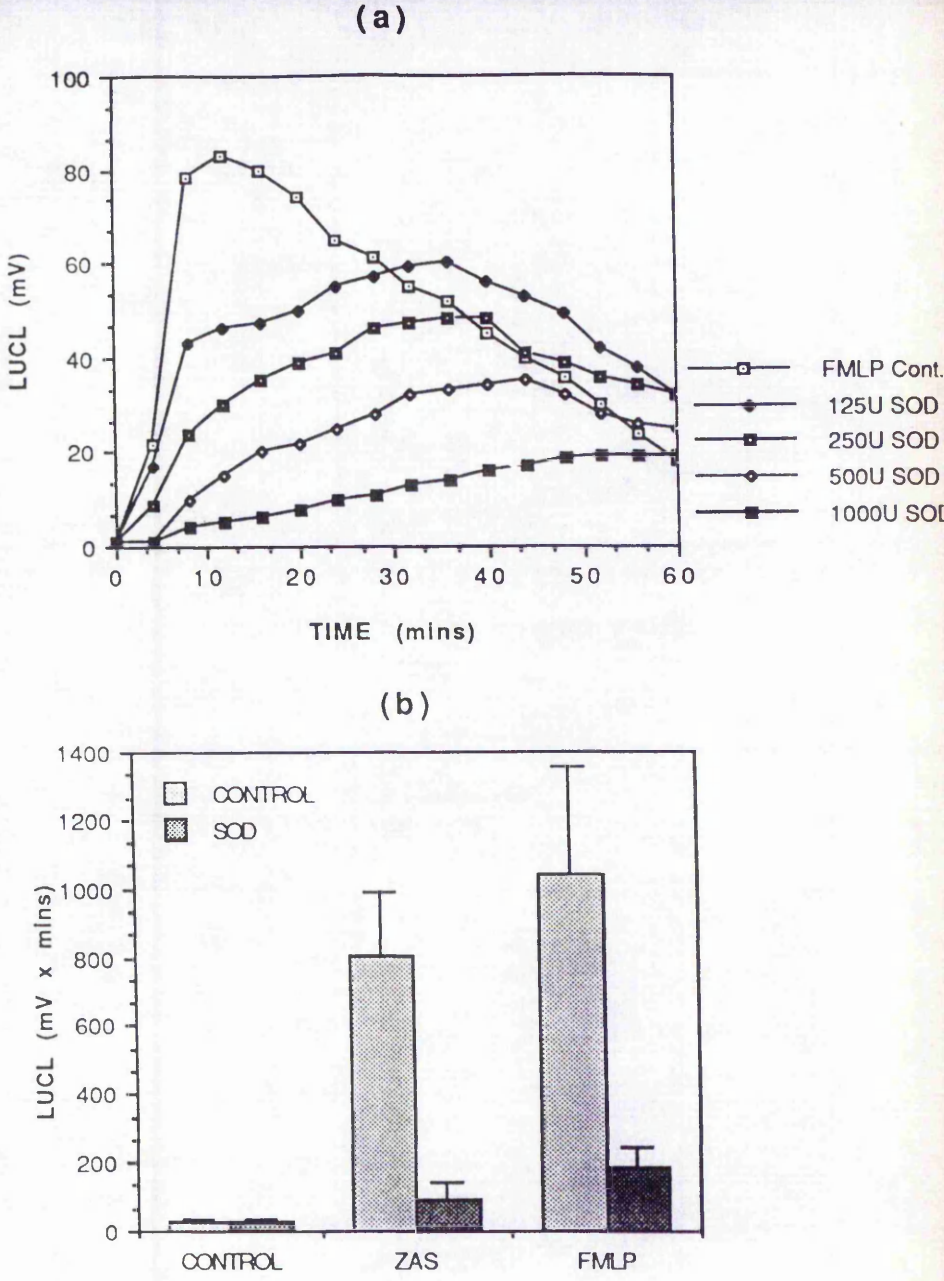

Figure 5: Panel (a) shows the effect of increasing concentrations of SOD on LUCL from FMLP stimulated PMN.
The results are from one representative experiment of six.
Panel (b) shows the effect of 1000U ml^{-1} of SOD on PMN LUCL responses to both ZAS and FMLP stimulation.
The results represent the means of 6 separate experiments $\pm$ SEM.

stimulated with ZAS. The PMN were incubated with MNCM for 15 minutes at either 37°C or 4°C in polypropylene tubes with constant agitation. The PMN suspensions were then transferred into luminometer cuvettes and LUCL was monitored in response to ZAS stimulation. Greater respiratory burst activity was observed in those cells incubated with MNCM at 4°C than those at 37°C (figure 6). This may have been due to the amount of FCS present (10% v/v) in both the controls (complete medium with LPS) and the MNCM. FCS contains activated complement components which may have been stimulating the cells and resulting in a reduction in LUCL at 37°C due to PMN exhibiting tachyphylaxis when exposed to ZAS. Therefore all further MNC cultures were carried out using heat inactivated FCS, the FCS was heated at 56°C for 2 hours to prevent activation of complement. The effectiveness of preincubating at 4°C compared with 37°C may also have been due to loss of cells through adherence at the higher temperature.

High concentrations of albumin have been shown to scavenge superoxide anions (Campbell 1988). Since FCS contains albumin in addition to other proteins, in order to reduce the amount of albumin present when measuring chemiluminescence, further experiments were carried out incorporating a washing step after the initial incubation with MNCM. All subsequent priming experiments were carried out using a 15 minute preincubation with MNCM at 4°C followed by washing in sterile PBS pH 7.2, centrifugation at 400g for 10 minutes and then resuspension of

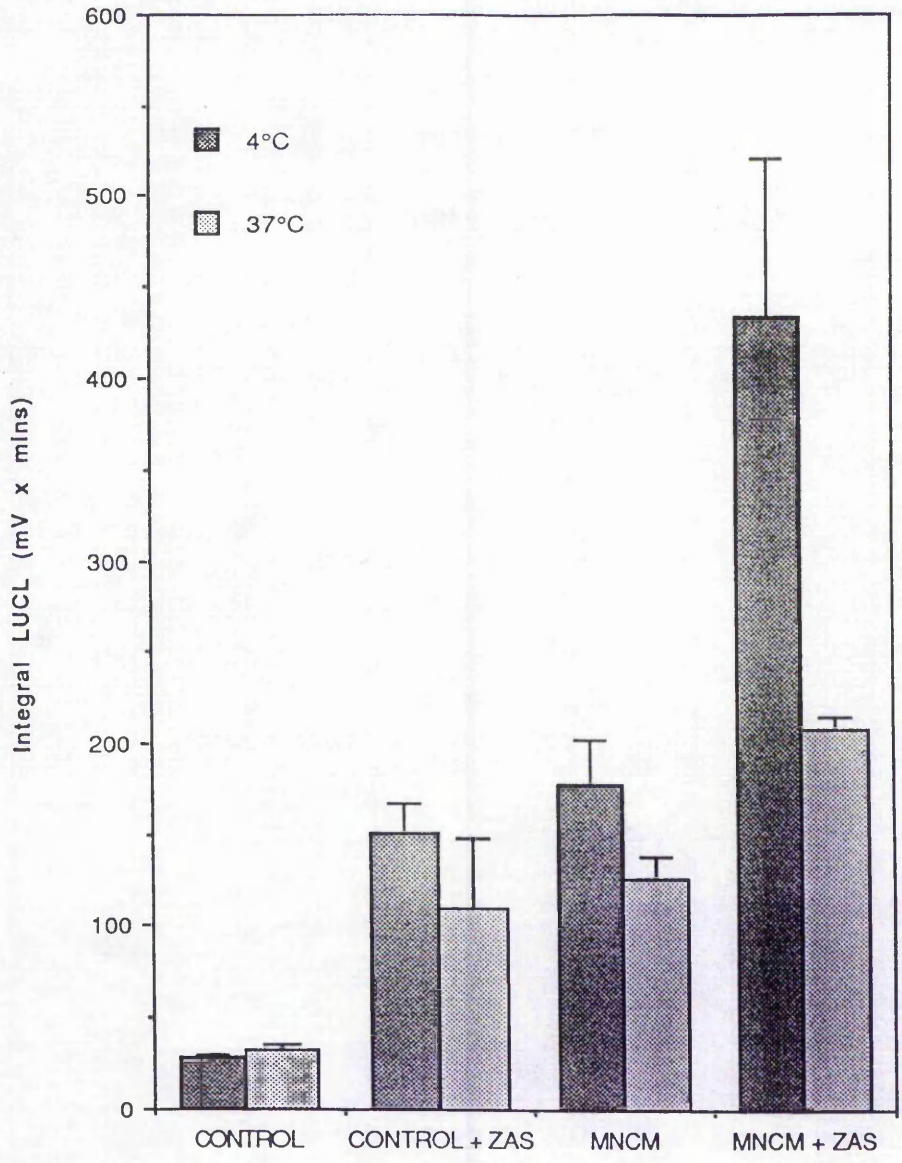

Figure 6: The effect of temperature MNCM priming of PMN to ZAS. MNCM was generated after an 18 hours incubation of MNC with 10ug ml^{-1} LPS. Controls represent complete medium containing the same concentration of LPS.

The results represent the means of 4 experiments ± SEM, using MNCM and PMN from different individual donors.

PMN in RPMI-1640 medium containing 0.1% (w/v) low endotoxin BSA. This meant the albumin content was constant when measuring LUCL, while this low concentration of albumin present prevented PMN aggregation (Campbell et al 1988). The data from these experiments demonstrated that the priming effect was irreversible and could occur after a short pre-incubation at 4°C (figure 7). The enhanced activity seen with cells resuspended in RPMI-BSA was not due to the BSA as there were no significant increases in controls. Under these conditions involving washing and resuspension in RPMI-1640 with 0.1% (w/v) BSA there was a marked enhancement in PMN superoxide anion production. PMN preincubated with MNCM generated after 18 hours in culture with 10ug ml^{-1} LPS showed a 167.21% + 32.97% increase in LUCL over control PMN containing the same concentration of LPS.

3.3. Priming Factor(s) Production

Initial experiments using MNCM were carried out with MNC cultured with 10ug ml^{-1} LPS for 18 hours. The results shown in figure 8 show that with increasing concentrations of LPS there was an increase in priming factor production from MNC which was not associated with the effect of LPS alone on PMN priming. These data show that the greatest enhancement in respiratory burst activity, over controls, was seen with MNCM generated with 10ug ml^{-1} LPS.

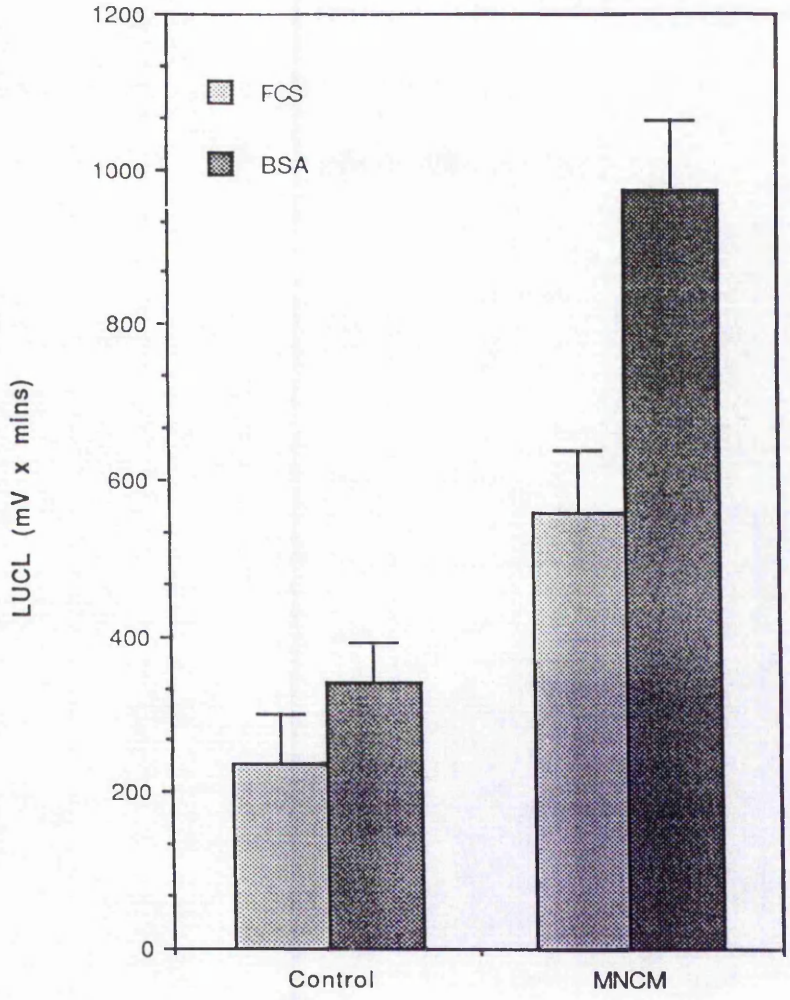

Figure 7: PMN were preincubated for 15 minutes at 4°C in MNCM followed by washing and resuspension in either RPMI-1640 containing 10% FCS v/v ( ) or 0.1% BSA w/v ( ). PMN were then stimulated with ZAS and LUCL monitored continuously.
The results represent the means of 4 separate experiments ± SEM, using different individual donor PMN and MNC.

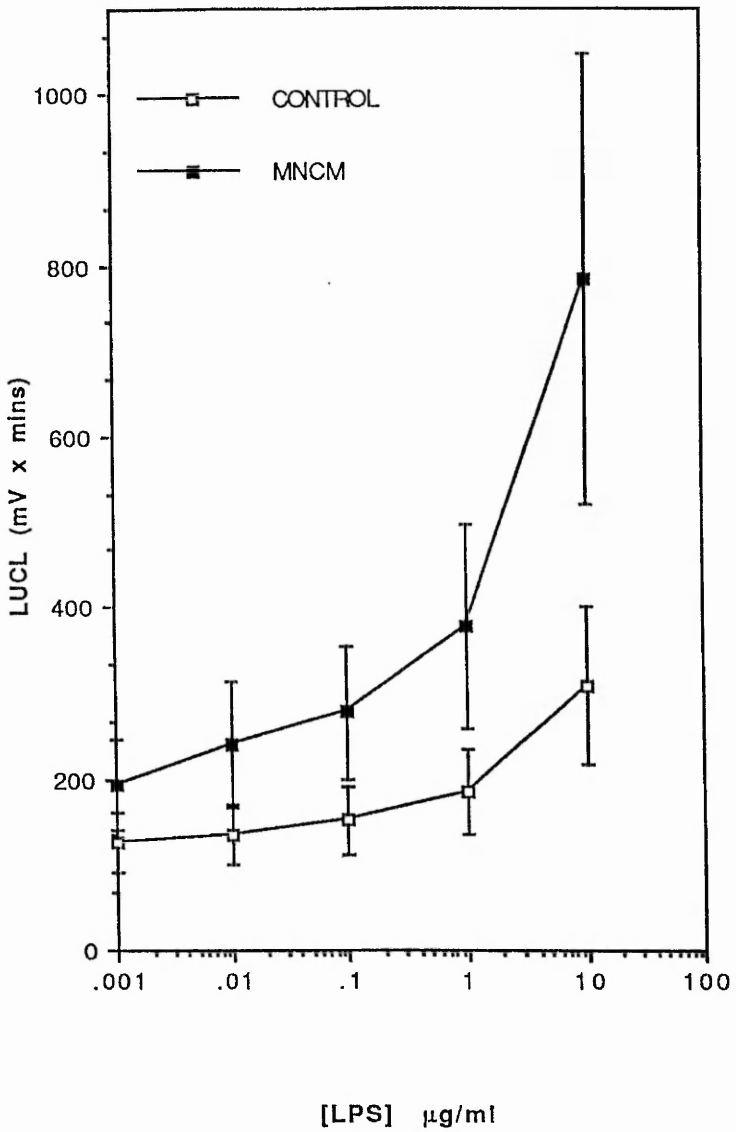

Figure 8: The effect of increasing concentrations of LPS on the priming activity of MNCM in response to the second agonist ZAS. MNC were incubated with these concentrations of LPS for 18 hours, the MNCM generated were then used for priming experiments.

The results represent the means of 12 separate experiments ± SEM, using different donor PMN and MNC.

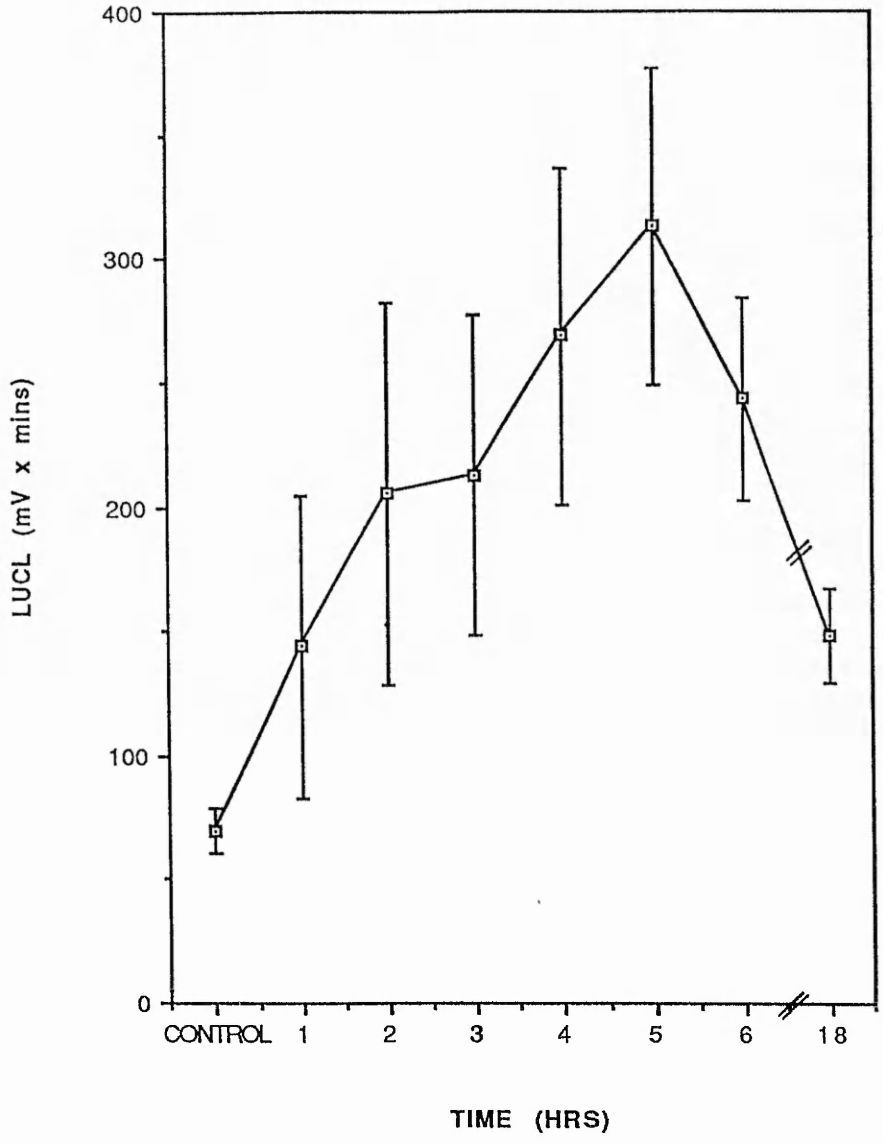

Figure 9: The time course for the production of priming activity from MNC stimulated with 10ug ml^{-1} LPS.
The results represent the means of 12 separate experiments ± SEM using individual donor PMN and MNC.

The time course for the production of priming factor from LPS stimulated MNC was then investigated. Using 10ug ml^{-1} LPS, MNC were cultured for 1 to 18 hours, as shown in figure 9. These data indicated that the factor causing this priming of PMN respiratory burst activity was secreted maximally by stimulated MNC at 4 to 6 hours, and was significantly higher ($p<0.01$) than the priming activity seen with MNCM generated after an 18 hour incubation.

Further LPS dose response curves were carried out using MNC cultured for 5 hours. Under these conditions a concentration of LPS at 1ug ml^{-1} gave maximal priming activity over controls (323.29% $\pm$ 57.94%) (figure 10).

To ensure that the effect of MNCM was due to enhance superoxide anion production, the priming experiments were repeated in the presence of SOD. Figure 11 shows that the priming effect of MNCM to ZAS was reduced in the presence of 1000U ml^{-1} of SOD. The effect of MNCM was dose dependent, since decreasing the concentration of MNCM resulted in a reduction in LUCL. This reduced respiratory burst activity with SOD also showed a dose dependent effect with increasingly dilute preparations of MNCM (figure 11).

Having shown that priming activity in the MNCM occurred maximally after 5 hours, a supply of pooled MNCM was generated to provide a constant source of priming agent for investigating

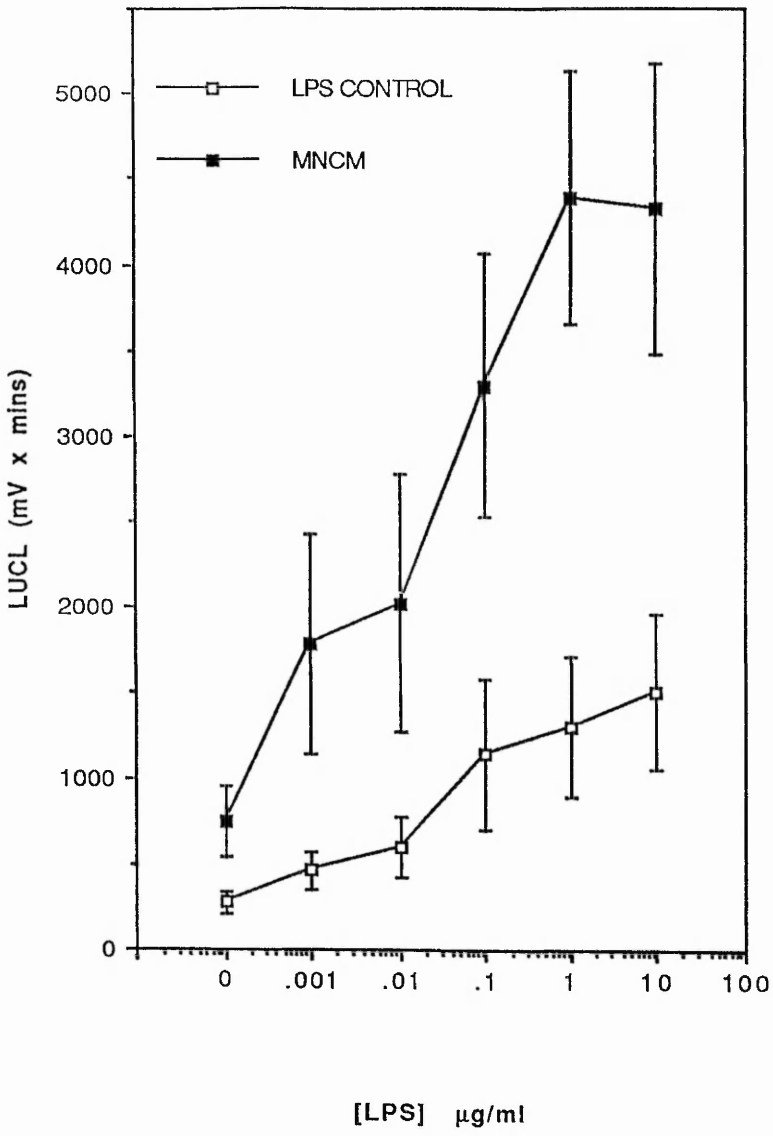

Figure 10: The effect of increasing concentrations of LPS on MNCM priming activity in response to ZAS stimulation. The MNC were stimulated with the above concentrations of LPS for 5 hours.

The results represent the means of 15 separate experiments ± SEM using different donor PMN and MNC.

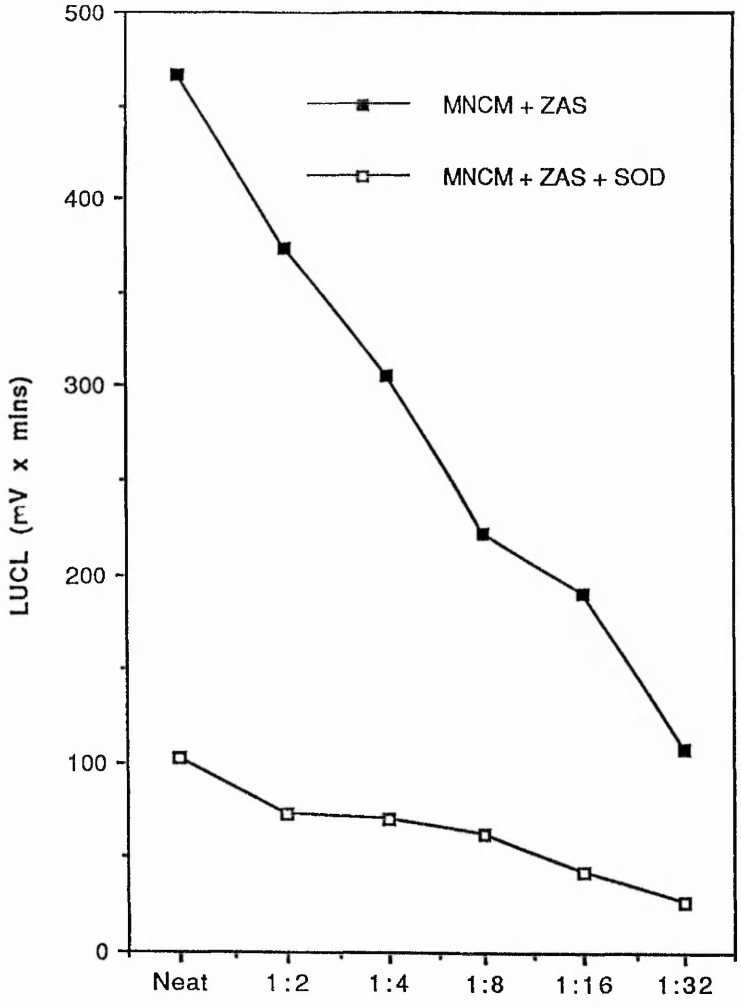

Figure 11: The dose dependent priming effect of MNCM; serial doubling dilutions of MNCM were used in priming experiments using ZAS as the second agonist. Control experiments on the same donor PMN were carried out in the presence of SOD to confirm that enhanced LUCL was due to superoxide anion production.

The results, using pooled MNCM are from one representative experiment of three carried out on separate donor PMN.

the kinetics of PMN priming to ZAS. The generation of pooled MNCM involved culturing MNC from individual donors for 5 hours with 1ug ml^{-1} LPS, the conditioned media were collected as described in the methods section (2.2.) and after filtration, the MNCM from individual donors were pooled together, aliquoted and stored until required.

3.3.1. Kinetics of MNCM Priming of PMN

Pooled MNCM was pre-incubated with PMN for increasing times at 4°C before washing, warming and stimulation with ZAS. Figure 12 shows that PMN could be induced to show enhanced respiratory burst activity as early as 5 minutes after exposure to MNCM. Priming activity was maximal after 15 to 20 minutes, with no further significant increases seen with prolonged pre-incubation times. As the cytokine TNF has been reported to bind maximally to its neutrophil receptor after 20 minutes at 4°C (Pichyangkul et al 1987), it seemed likely that the identity of this priming factor was TNF. In addition work with recombinant human TNF has shown it to be a potent priming agent for neutrophil respiratory burst activity (Klebanoff et al 1986). Furthermore, since the time course for the production of priming activity in MNCM was consistent with this factor being TNF (Burchett et al 1988), we went on to investigate the actions of TNF and the possible priming effects of other cytokines which may be present in MNCM.

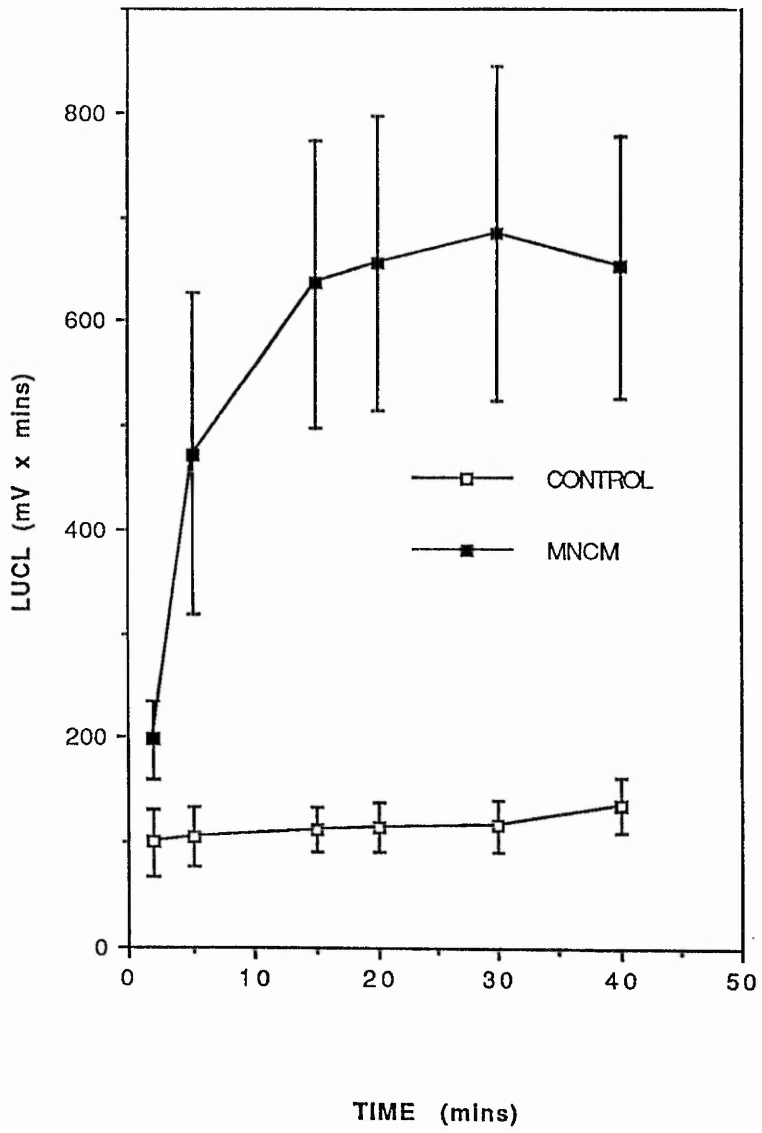

Figure 12: The effect of preincubation times on the priming activity of MNCM on PMN in response to ZAS as the second agonist.

The results represent the means of 8 separate experiments ± SEM, using pooled MNCM on different individual donor PMN.

3.4. Recombinant Human Cytokines

The direct effect of TNF on respiratory burst activity and its PMN priming activity to ZAS were investigated. As with the MNCM priming, PMN were preincubated with increasing concentrations of recombinant human TNF for 15 minutes at 4°C followed by washing and warming to 37°C. PMN were then either stimulated with ZAS or not exposed to a second agonist (controls). Figure 13 shows that TNF alone had only a slight direct effect on PMN superoxide anion production. At concentrations of 100 and 1000U ml^{-1} this recombinant cytokine was more effective, but not consistently, in that not all donor PMN responded. TNF was, however, effective at priming PMN for increased superoxide anion production when subsequently stimulated with ZAS. PMN showed significant enhancement ($p<0.025$) of respiratory burst activity with concentrations of TNF as low as 10U ml^{-1} (514.09% $\pm$ 62.78%). This suggested that TNF secreted from MNC in response to LPS stimulation, may be responsible for the observed biological activity of MNCM. However, it was not possible to exclude a role for other cytokines which may be present in the conditioned media.

Priming experiments were carried out in the same way using recombinant human forms of both IL-1beta and GM-CSF. IL-1beta is secreted from LPS stimulated MNC with a similar time course to TNF (Burchett *et al* 1988). GM-CSF is a 22kD molecule that was first isolated from Mo T lymphoblast cell lines (Wong *et al*

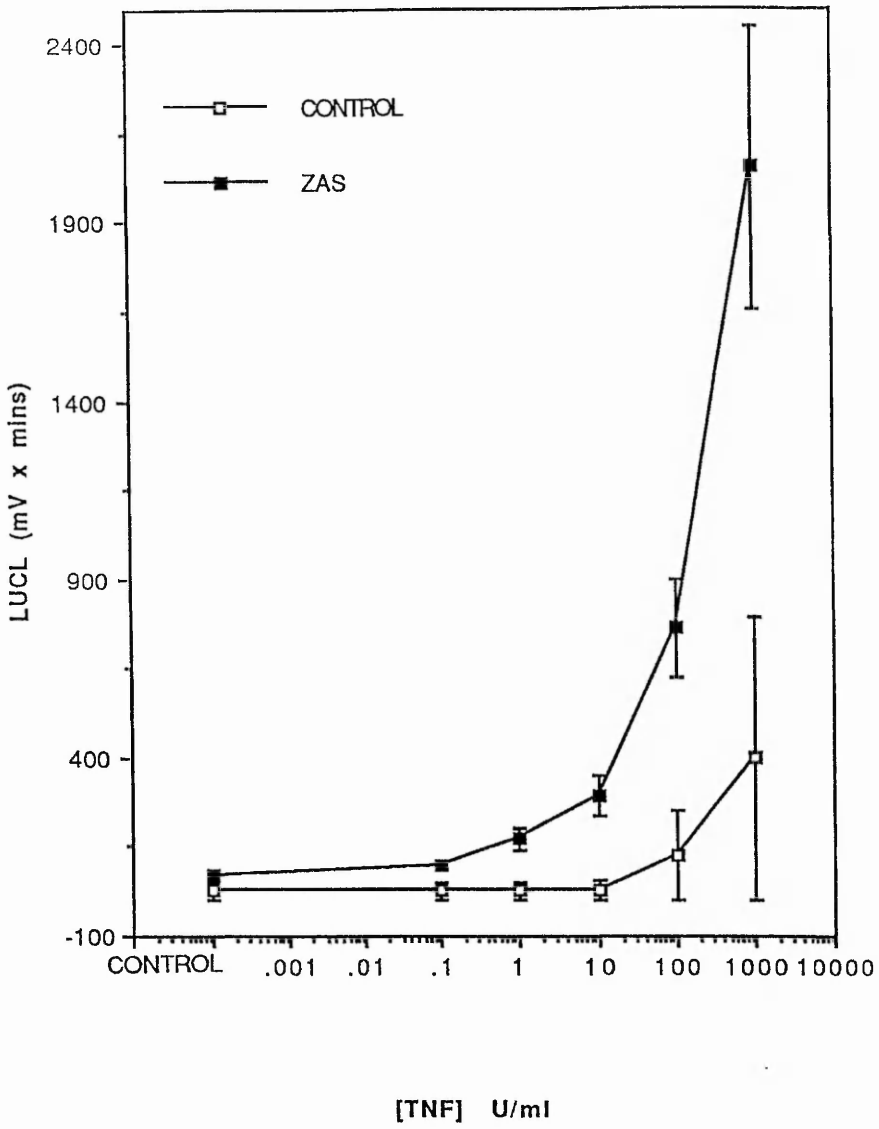

Figure 13: The effect of increasing concentrations of recombinant human TNF on direct stimulation of PMN superoxide anion production, and also priming to ZAS stimulation.
The results represent the means of 6 separate experiments ± SEM.

1985). GM-CSF is secreted from activated T-lymphocytes, monocytes, endothelial cells and fibroblasts (Clark and Kamen 1987), and as GM-CSF has been extensively studied for its ability to prime neutrophil responses (Weisbart et al 1986, McColl et al 1990) this cytokine was also investigated. While TNF was capable of significantly enhancing PMN LUCL neither IL-1 or GM-CSF could reproduce the effects. IL-1beta at concentrations up to 100U ml^{-1} had no effect on PMN responses, figure 14 shows that the results achieved with IL-1beta did not compare with the priming activity of either the LPS control medium or MNCM when tested against the same donor PMN. GM-CSF showed a slight but significant enhancement of respiratory activity after a 60 minutes incubation at 37°C (figure 15). This enhancement did not compare with the effect on PMN observed after incubation in the presence of TNF or MNCM.

3.4.1. The Effect of Anti-Cytokine Antibodies

The pre-incubation experiments at 4°C were carried out using pooled MNCM with the addition of a number of anti-human cytokine antibodies. The antibodies used included those raised against TNF, IL-1beta, GM-CSF and a pre-immune rabbit serum as control. Only the anti-TNF polyclonal was capable of reducing the priming effect of MNCM (figure 16), with none of the other antibodies present in the preincubation step having any effect. However, the effect of MNCM on PMN respiratory burst activity

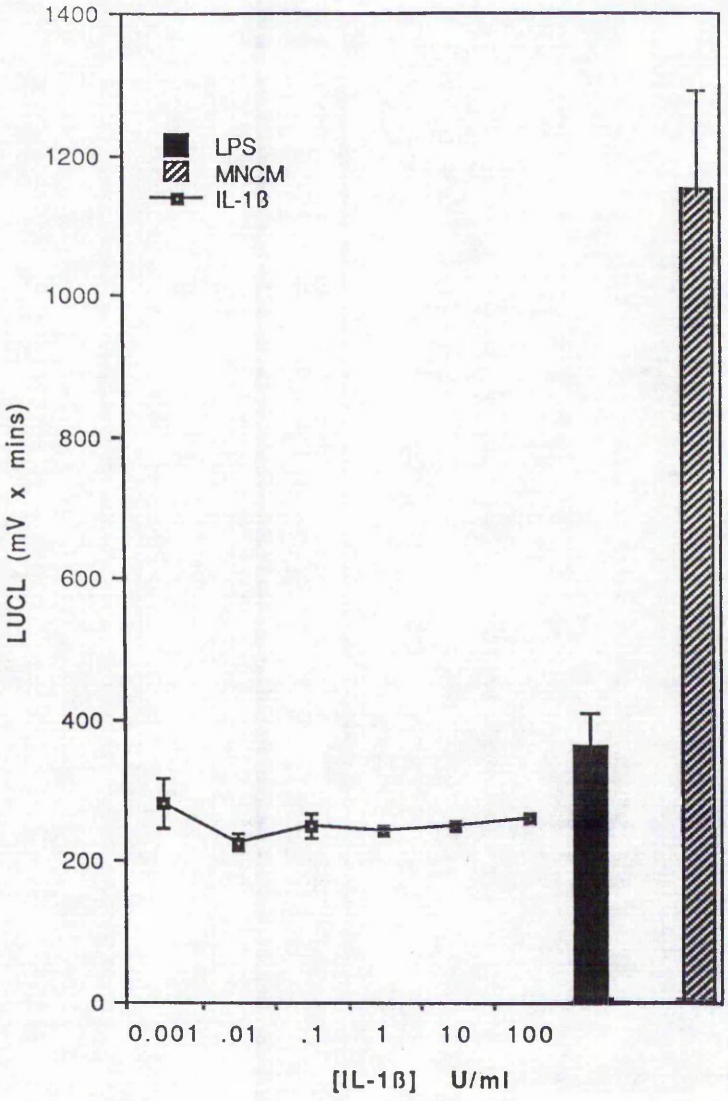

Figure 14: The effect of increasing concentrations of recombinant human IL-1beta on the response of PMN to ZAS stimulation. The effect of this cytokine was compared to that seen with 1ug ml^{-1} LPS (LPS Control) and pooled MNCM, tested against the same donor PMN.

The results represent the means of 6 separate experiments ± SEM.

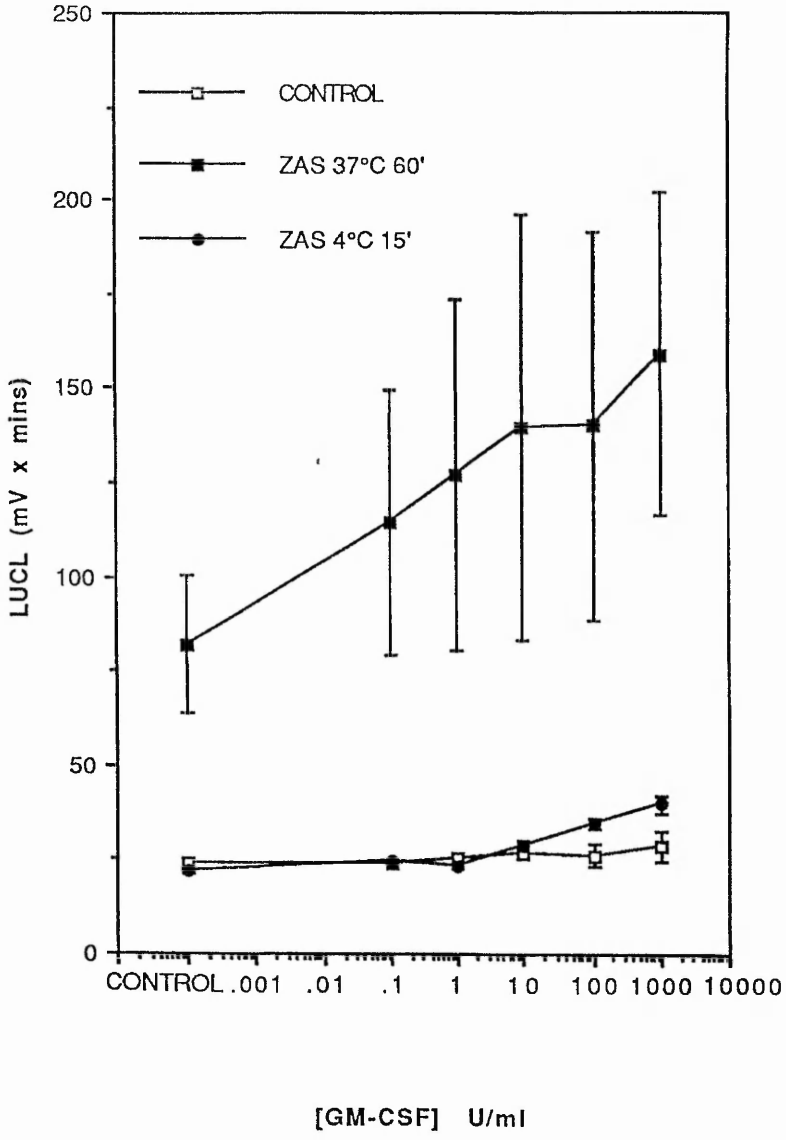

Figure 15: The effect of increasing concentrations of recombinant human GM-CSF on PMN responses to ZAS stimulation. PMN were preincubated with GM-CSF for either 15 minutes at 4°C or 60 minutes at 37°C, before washing and stimulation with ZAS. The results represent the means of 4 separate experiments ± SEM.

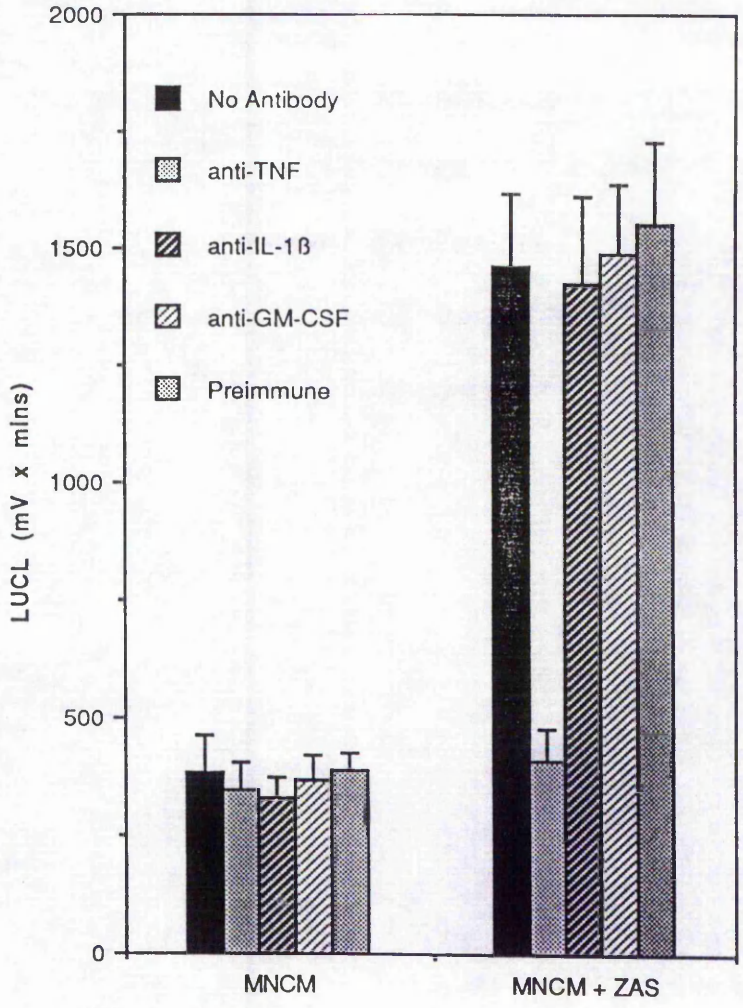

Figure 16: The effect of anti-cytokine antibodies on the direct stimulatory activity of MNCM and the priming effect to ZAS stimulation.

The results represent the means of 8 separate experiments ± SEM using pooled MNCM on different individual donor PMN.

could not be completely abrogated. As figure 16 shows MNCM alone has a direct effect on the stimulation of superoxide anions from PMN. This direct effect did not appear to be explained by TNF since an antibody concentration sufficient to neutralize 1000 bioactive Uml^{-1}, was only effective at blocking priming activity. It therefore seemed possible that there may be some other factor in MNCM capable of directly stimulating PMN respiratory burst activity.

3.4.2. Assay of Cytokines in MNCM

To provide further evidence of a role for TNF as the priming agent in MNCM, we decided to investigate whether there was a correlation between priming activity and the amount of bioactive TNF present in each MNCM. TNF concentrations were determined using the L-929 mouse fibroblast assay. Figure 17 shows a dose response curve for TNF with respect to the cytotoxicity of L-929 cells. MNC cultured with 1ug ml^{-1} LPS for increasing incubation times were used for the priming experiments, with duplicate samples set up for the assay of TNF. The data represented in figure 18 shows there to be a correlation between the levels of TNF and the degree of enhancement in LUCL observed. In addition it was possible to demonstrate that LPS dose response curves showed a dose dependent increase in TNF secretion into the medium, which appeared to correlate with enhanced respiratory burst activity (figure 19).

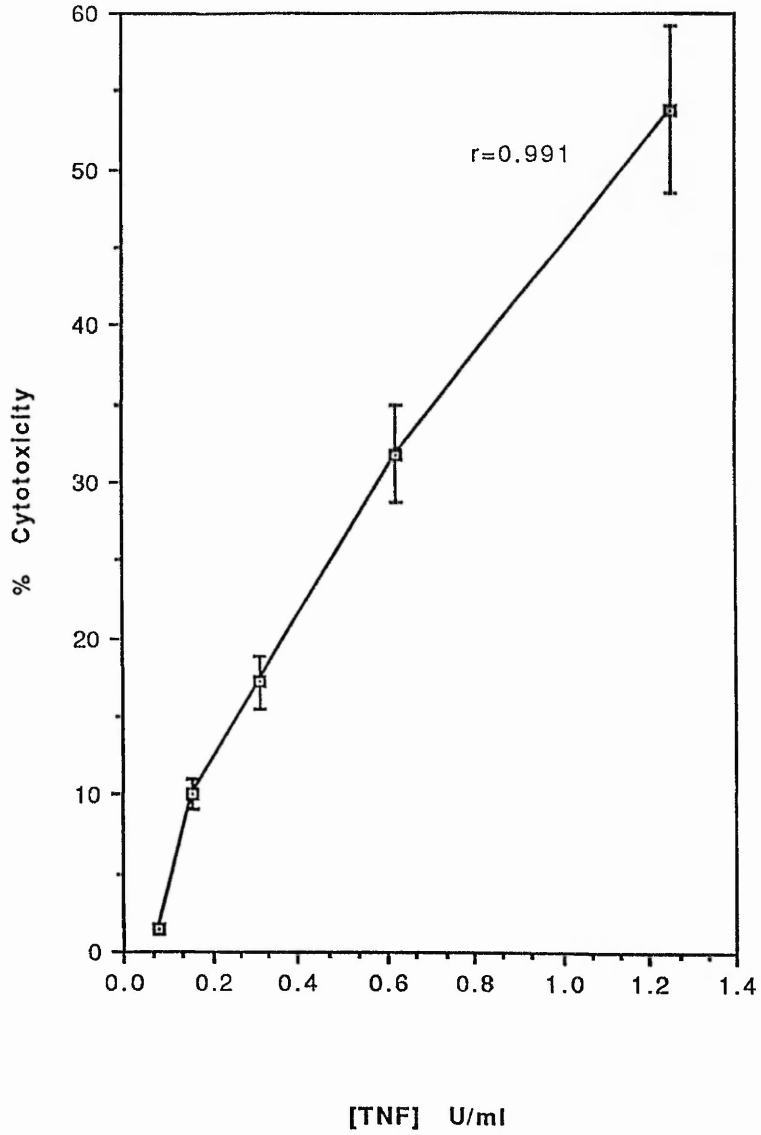

Figure 17: L-929 mouse fibroblast cytotoxicity assay; increasing concentrations of TNF cause increased cytotoxicity of cells calculated as a percentage of control cells incubated in the absence of TNF.

The results represent the means of 12 separate standard curves ± SEM.

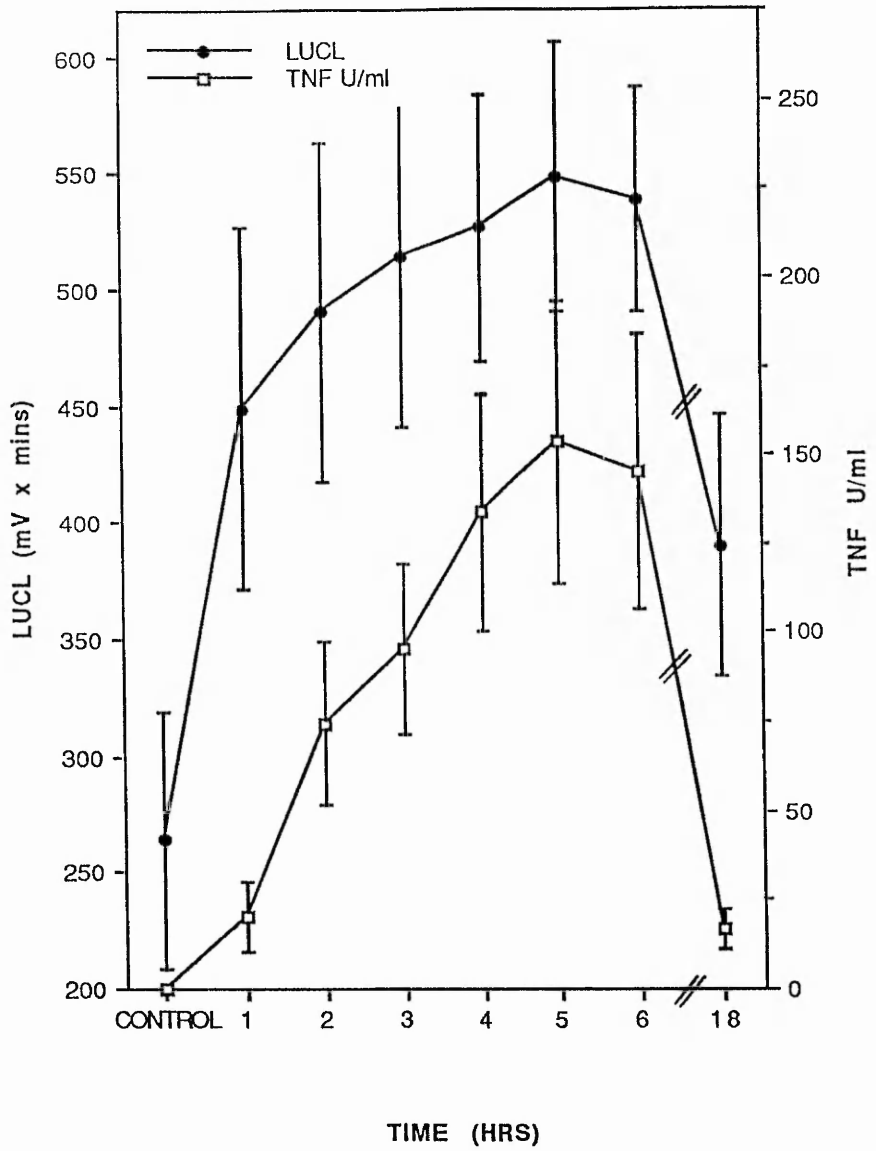

Figure 18: The effect of increasing MNC incubation times with 1ug ml^{-1} LPS stimulation on both priming factor production in MNCM (LUCL) and TNF secreted into the medium (TNF).

The results represent the means of 8 separate experiments ± SEM, using individual donor MNC and tested against different donor PMN.

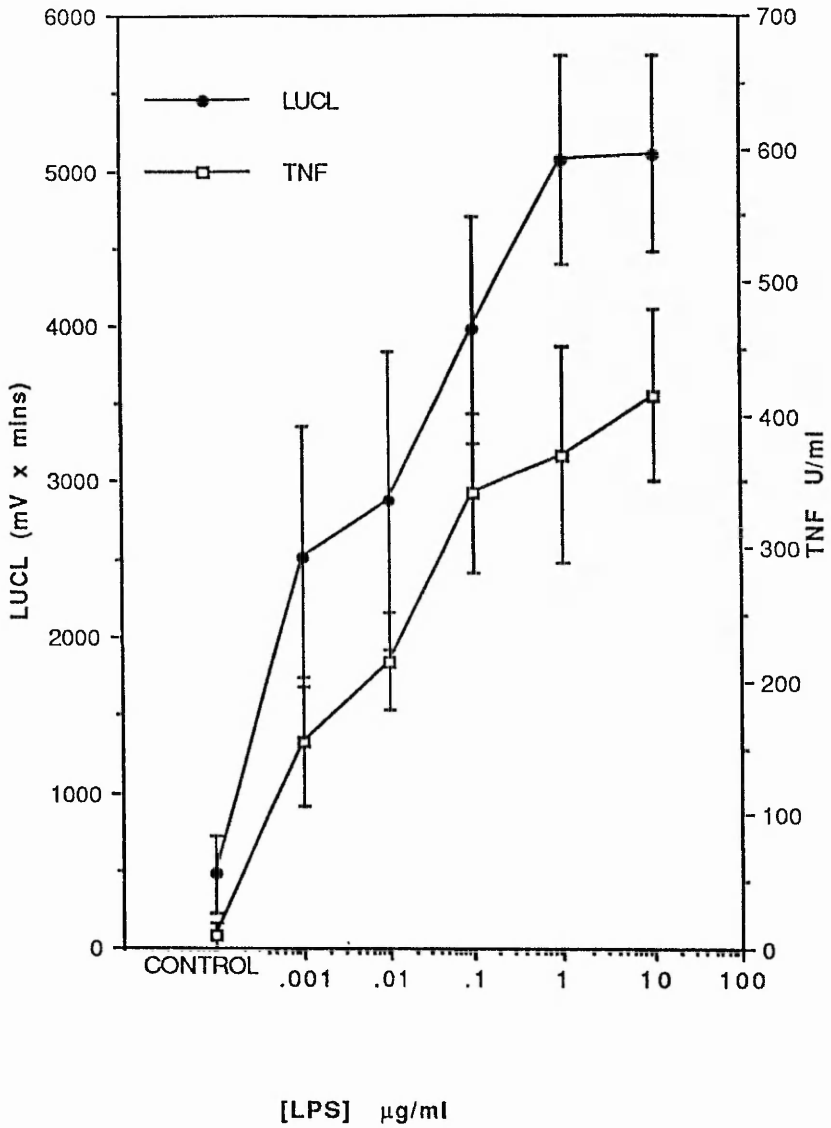

Figure 19: The effect of increasing LPS concentrations on MNCM priming activity to ZAS stimulation (LUCL) and the amount of bioactive TNF secreted into the medium (TNF).

The results represent the means of 8 separate experiments ± SEM, using different individual donor MNC and tested against different donor PMN.

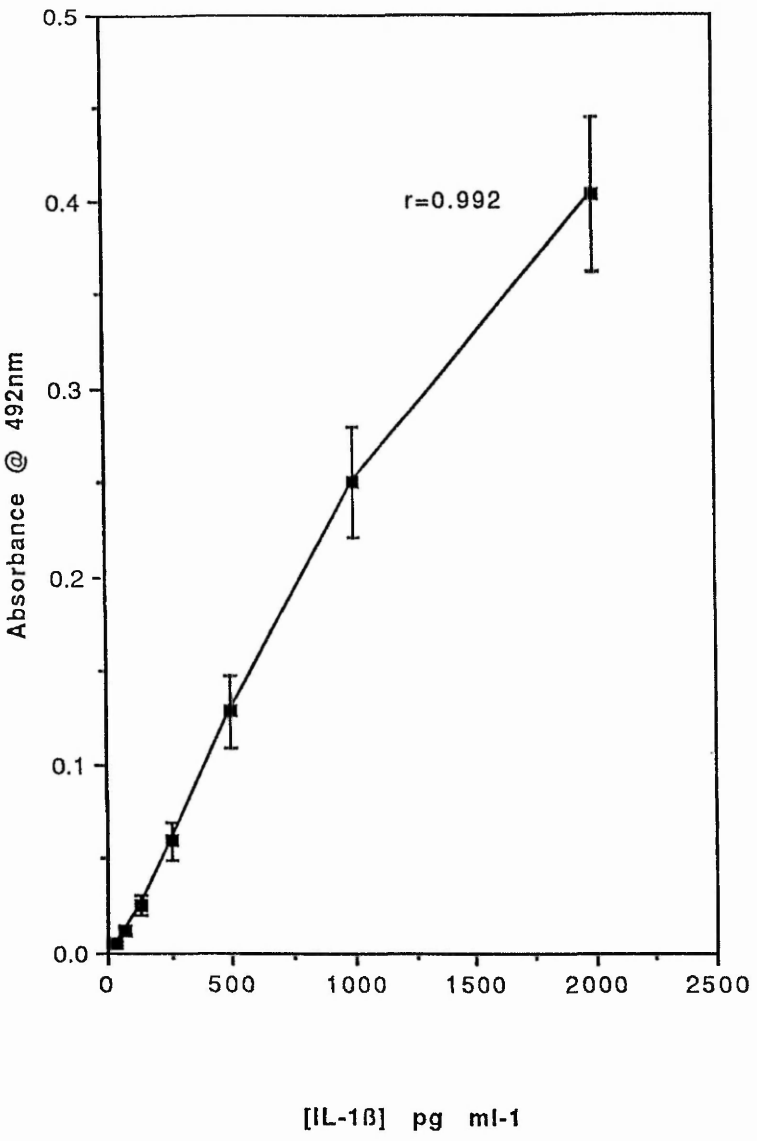

Figure 20: IL-1beta ELISA; increasing concentrations of IL-1beta standard correlated with increased absorbance

The results shown are from 6 individual standard curves ± SEM.

As IL-1beta is released in response to LPS from MNC with a similar time course to TNF, IL-1beta was also measured in these MNCM. Immunoreactive IL-1beta was determined using an ELISA kit and a typical standard curve for this assay is shown in figure 20. Although IL-1beta was measured in these conditioned media (figure 20), the evidence from the previous experiments suggested that IL-1beta was not responsible for the observed effects on PMN; the recombinant human cytokine could not reproduce the effects and the anti-IL-1beta antibody could not reduce the priming activity of MNCM. Also, whereas maximal TNF production and priming activity were observed at between 4 and 6 hours, the amount of IL-1beta assayed in the conditioned media continued to increase up to 24 hours. The levels of this cytokine did not correlate with the time course for increases in priming activity.

Immunoreactive GM-CSF in the conditioned media was assayed (for assay standard curve see figure 22) and although present in MNCM when MNC were cultured for 48 hours, it could not be detected in the 5 hour MNCM being used for the priming experiments (figure 23).

3.4.3. Effect of MNCM and TNF on PMN Responses to ZAS and FMLP Stimulation

To give an indication of the effects of 100U ml^{-1} TNF and MNCM on the population of cell surface receptors to C5a des arg and

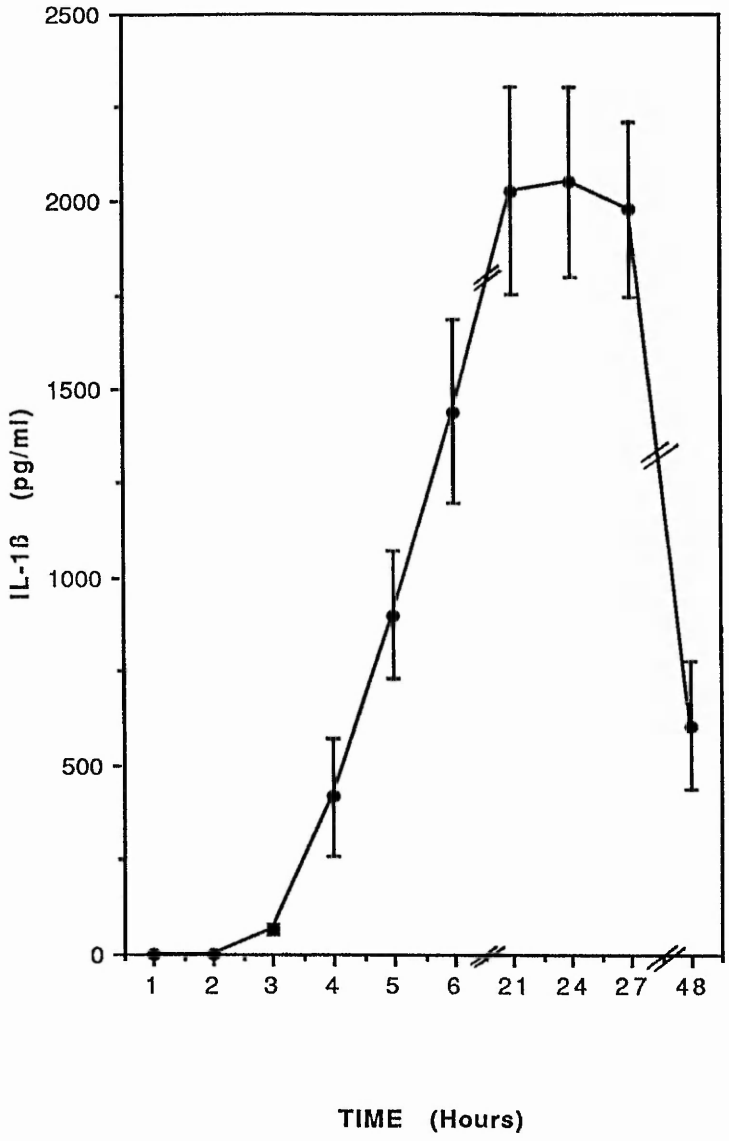

Figure 21: The time course for production of IL-1beta from MNC stimulated with 1ug ml^{-1} LPS. MNCM were collected at the time points shown and immunoreactive IL-1beta was measured by ELISA. The results represent the means of experiments on 3 different donor MNC $\pm$ SEM.

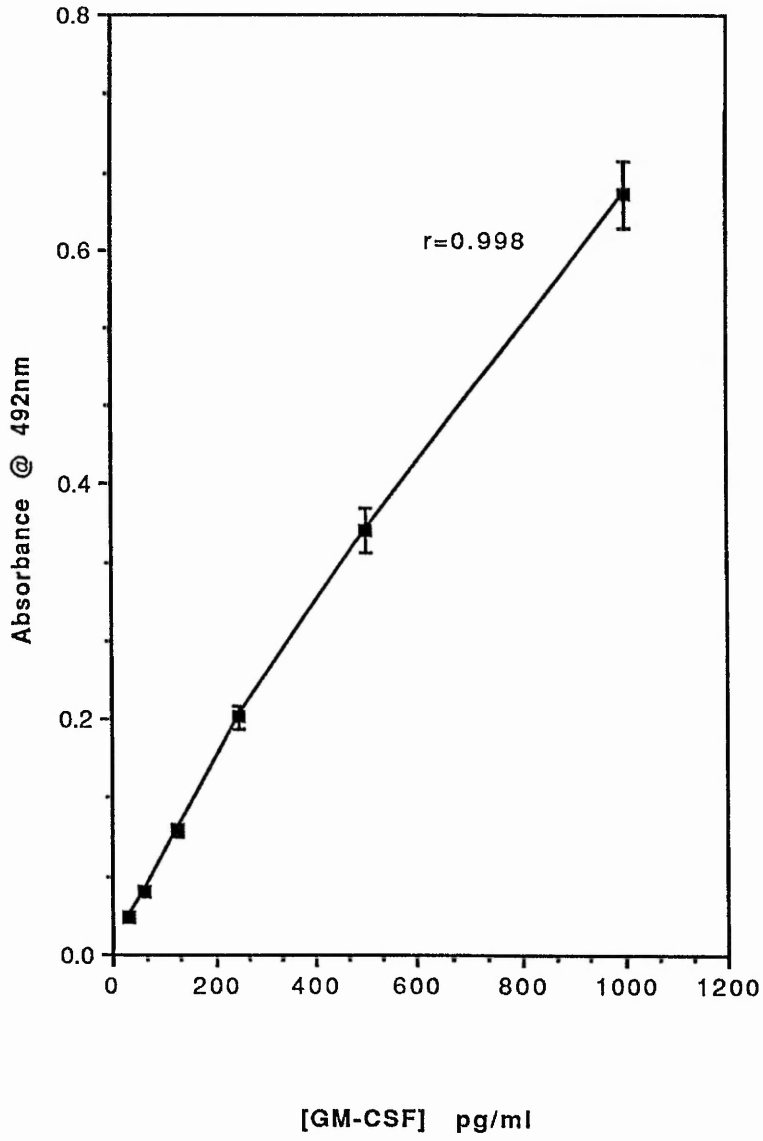

Figure 22: GM-CSF ELISA standard curve; increasing concentrations of GM-CSF correlated with increased absorbance. The results represent the means of 6 separate experiments ± SEM.

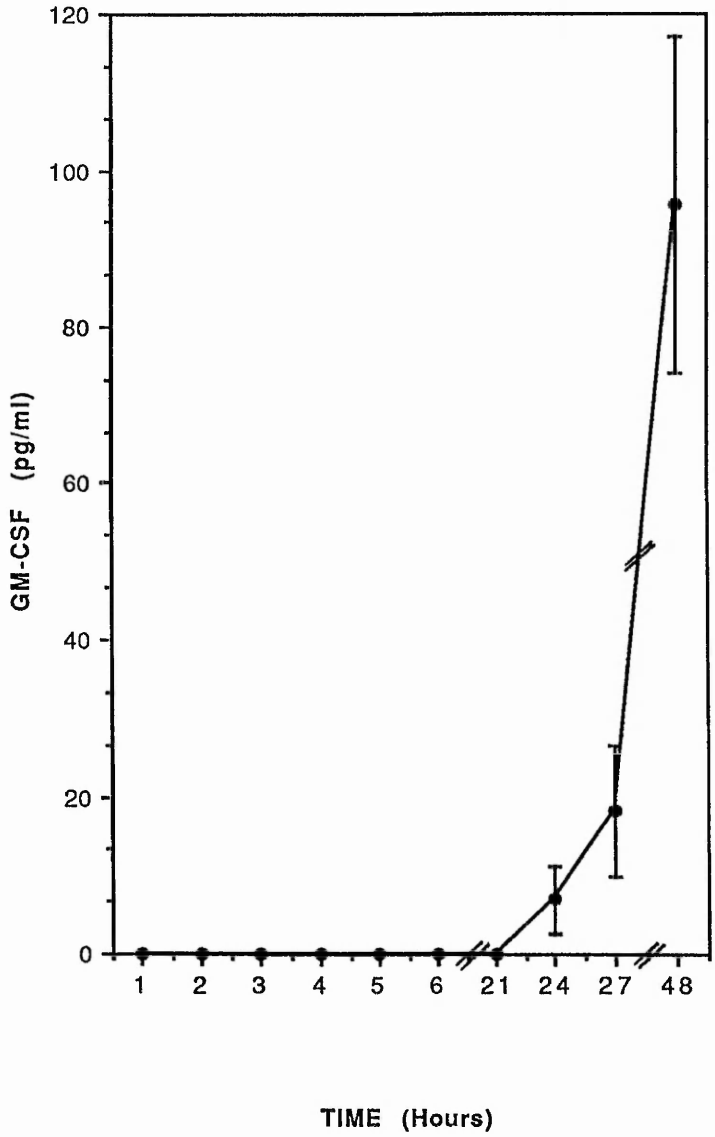

Figure 23: The time course for the production of immunoreactive GM-CSF from MNC stimulated with 1ug ml^{-1} LPS. MNCM were collected at the time points shown and GM-CSF was measured by ELISA.

The results represent the means of 3 separate experiments $\pm$ SEM on individual donor MNC.

FMLP, priming was carried out with PMN stimulated with increasing concentrations of both ZAS and FMLP. TNF is reported to prime neutrophils by affecting the affinity and not the number of FMLP receptors (Atkinson et al 1988). Figure 24 shows that while both TNF (a) and MNCM (b) were capable of inducing amarked enhancement in PMN LUCL there was no shift in the concentration of FMLP eliciting maximal response. Had the affinity of receptors to FMLP been changed it would be expected that incubation with TNF and MNCM would cause the cells to respond maximally at lower concentrations of the second agonist. Similar experiments were carried out using ZAS as the second stimulus (figure 25) and, although the curves do not run exactly parallel there is no marked change in the pattern of cellular responsiveness with increasing concentrations of ZAS. However, there is no doubt that both TNF and MNCM cause dramatic increases in the amount of superoxide anion production when cells were subsequently stimulated with a second agonist.

3.4.4. Direct Stimulatory Activity of MNCM

As shown in figure 26 MNCM was capable of directly stimulating PMN respiratory burst activity as well as priming to ZAS and FMLP. The direct stimulatory activity was greater than that seen with either ZAS or FMLP and could not be explained by TNF or any of the other cytokines tested. Recent publications suggested that a newly identified inflammatory peptide

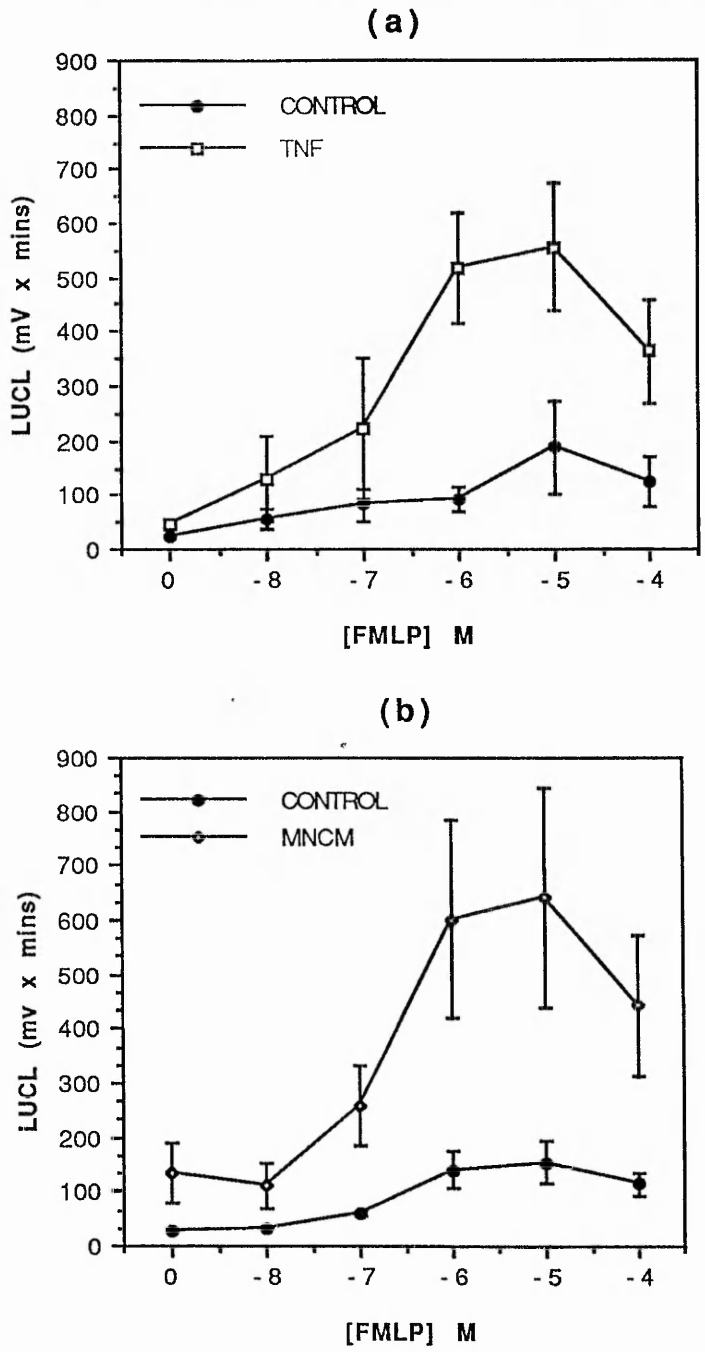

Figure 24: The effect of 100U ml^{-1} of recombinant human TNF (a) and MNCM (b) on LUCL in the presence of increasing concentrations of FMLP. Control medium (control) contained 1ug ml^{-1} LPS, which was also used to dilute the TNF.

The results represent the means of 6 separate experiments $\pm$ SEM, using different individual donor PMN.

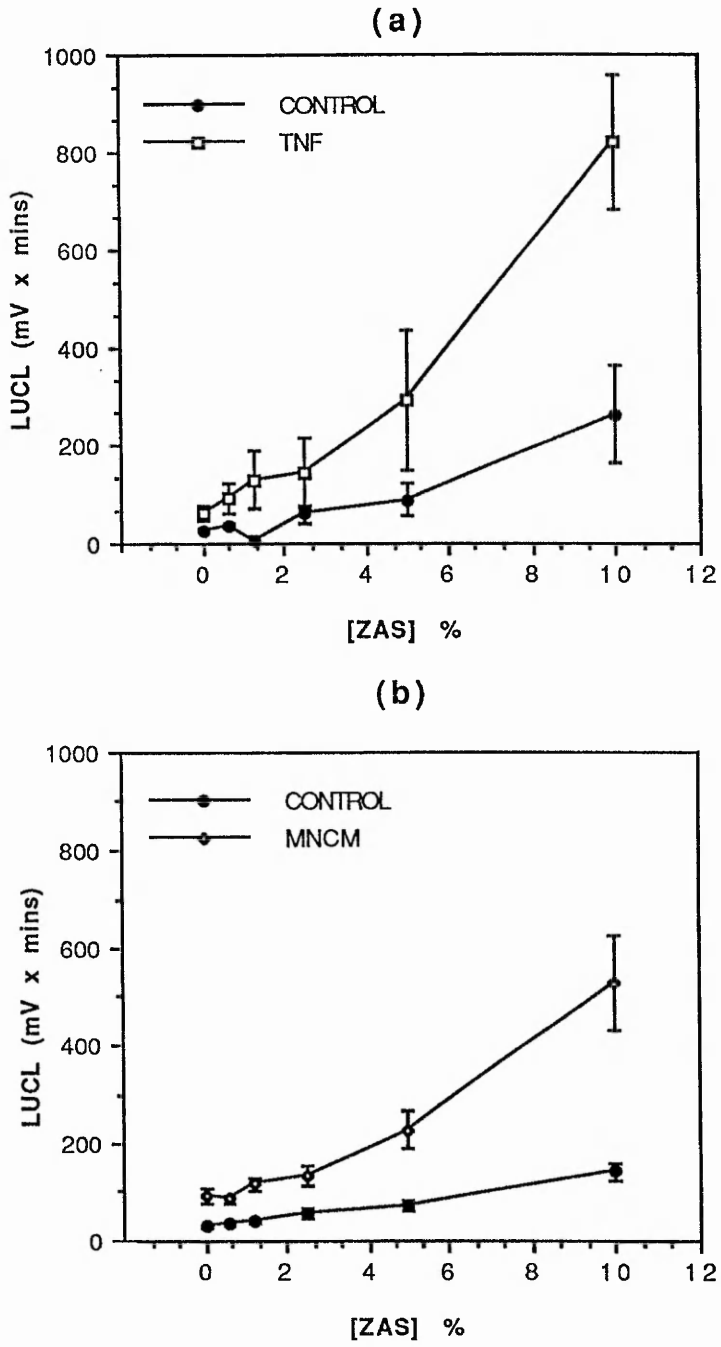

Figure 25: The effect of 100U ml^{-1} recombinant human TNF (a) and MNCM (b) on PMN LUCL in response to increasing concentrations of ZAS. Control medium (control) contained 1ug ml^{-1} LPS and this complete medium was used to dilute the TNF. The results represent the means of 6 separate experiments $\pm$ SEM

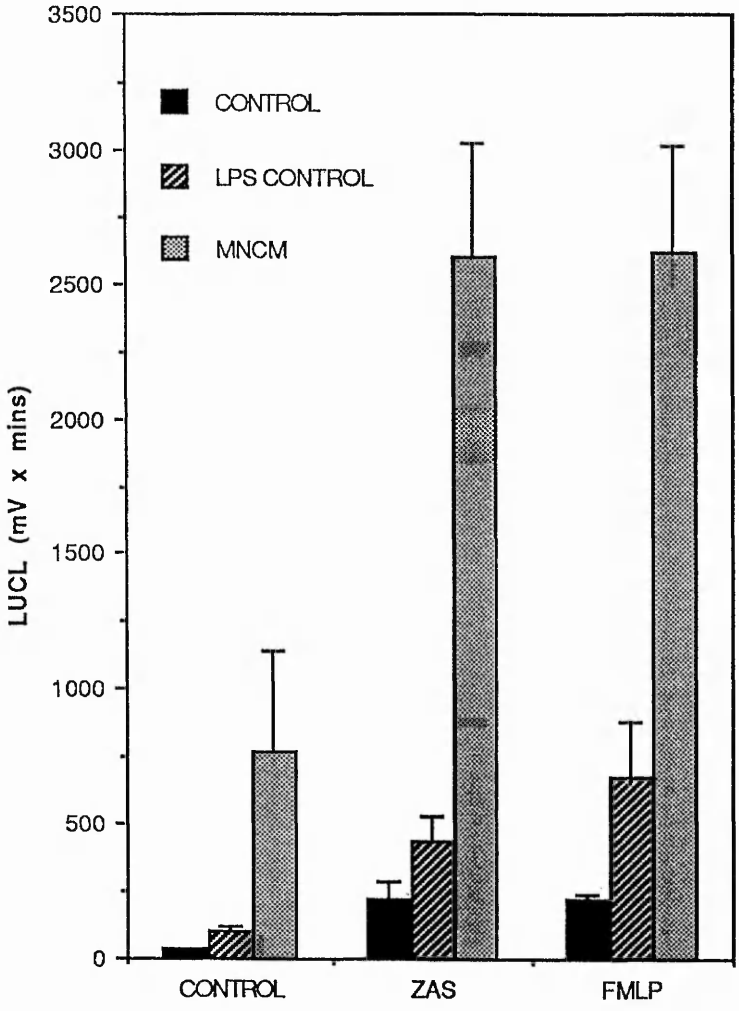

Figure 26: A comparison between the effects of control medium, control medium containing 1ug ml^{-1} LPS (LPS control) and MNCM on direct stimulatory activity of the PMN respiratory burst and priming to ZAS and FMLP.

The results represent the means of 4 separate experiments ± SEM using pooled MNCM and different individual donor PMN.

(NAP-1/IL-8) was capable of directly stimulating respiratory burst activity in human neutrophils (Thelen et al 1988). The effect of recombinant human NAP-1/IL-8 was therefore investigated for its ability to stimulate PMN superoxide anion production. Figure 27 shows that three different sources of NAP-1/IL-8 were investigated, the first a monocyte derived 72 amino acid form (1), the second a 77 amino acid endothelial cell form (2) and the third was another monocyte derived form (3). It appeared that neither of the first two preparations were capable of stimulating an increase in measured chemiluminescence. However, the third preparation (3) did induce a dose dependent increase in superoxide anion production; figure 28 shows that this effect was significant (compared with controls) at concentrations upwards of 20ng ml^{-1}. The induction of respiratory burst activity was much less than that seen with ZAS, FMLP or MNCM alone (figure 28).

Experiments with MNCM were repeated in the presence of anti-NAP-1/IL-8, anti-TNF or preimmune control serum. Again the PMN were preincubated with MNCM in the presence of relevant antibody for 15 minutes at 4°C before washing with cold sterile PBS pH 7.4 and then warming to 37°C in the luminometer. Unlike the priming experiments, no further stimulus was added. Figure 29 shows that there was a significant reduction in the amount of superoxide anions released in the presence of the anti-NAP-1/IL-8. However, the direct action of MNCM could not be completely abrogated using the anti-serum. Increasing the

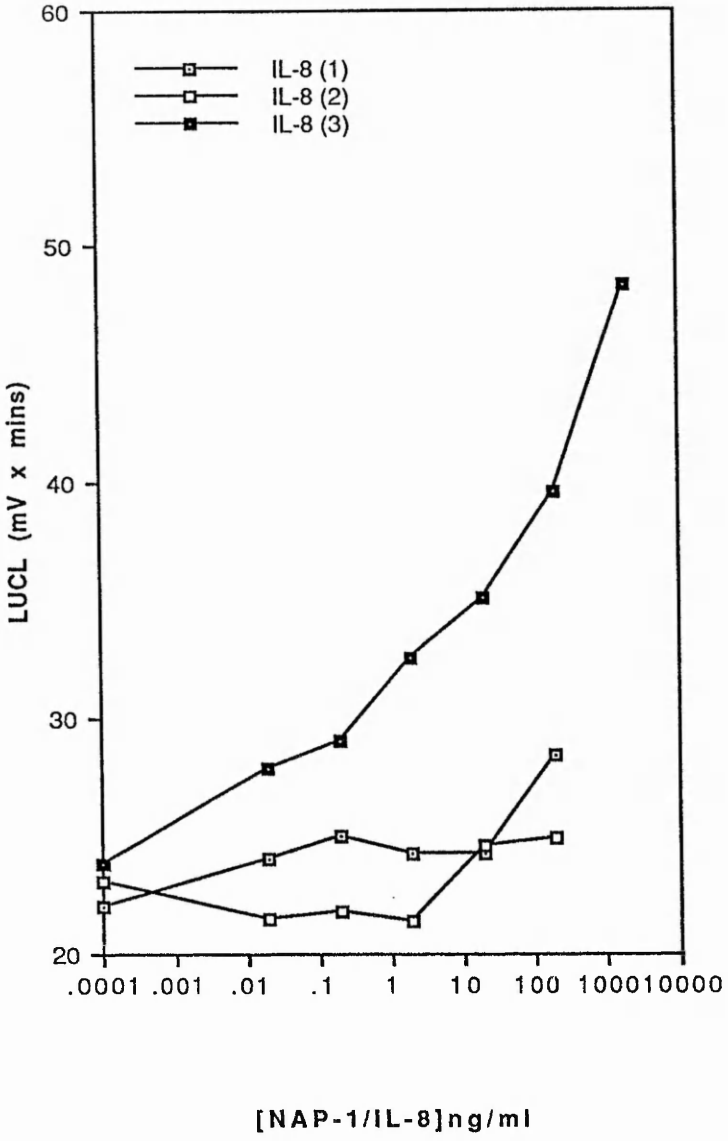

Figure 27: The effect of three different recombinant human NAP-1/IL-8 on direct stimulation of PMN respiratory burst activity, as measured by LUCL. PMN were incubated with increasing concentrations of the cytokine for 15 minutes at 4°C prior to washing and warming to 37°C. LUCL was monitored continuously in the absence of any further stimulation.
The results represent the means of 8 separate experiments on different individual donor PMN

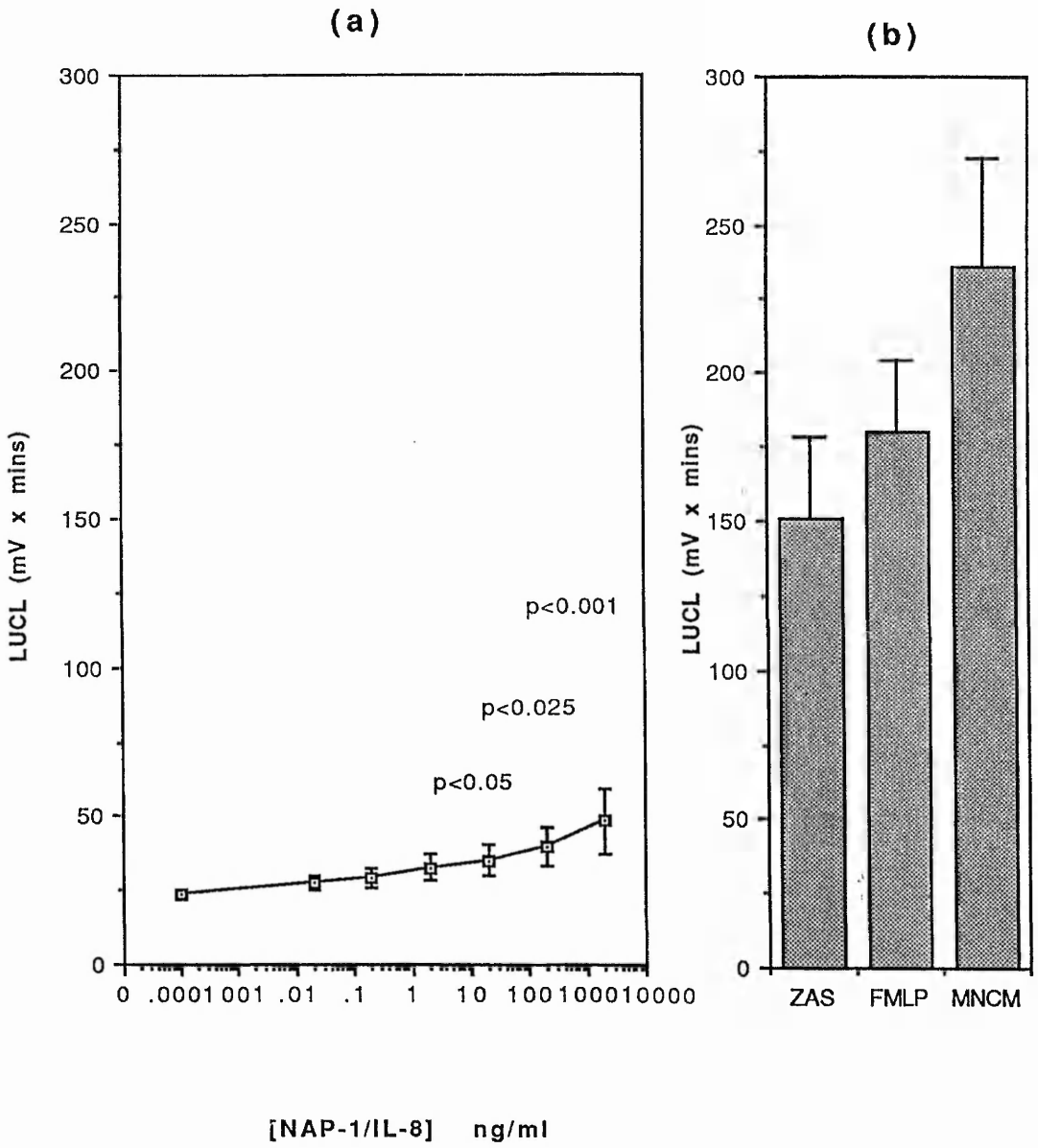

Figure 28: A comparison between the effect of (a) increasing concentrations of recombinant human NAP-1/IL-8 (preparation 3) and (b) the amount of superoxide anion production seen with ZAS, FMLP and MNCM stimulation. No preincubation steps were used in these experiments, cells were stimulated with each of the agonists at 37°C in the luminometer.

The results represent the means of 8 separate experiments ± SEM with different individual donor PMN.

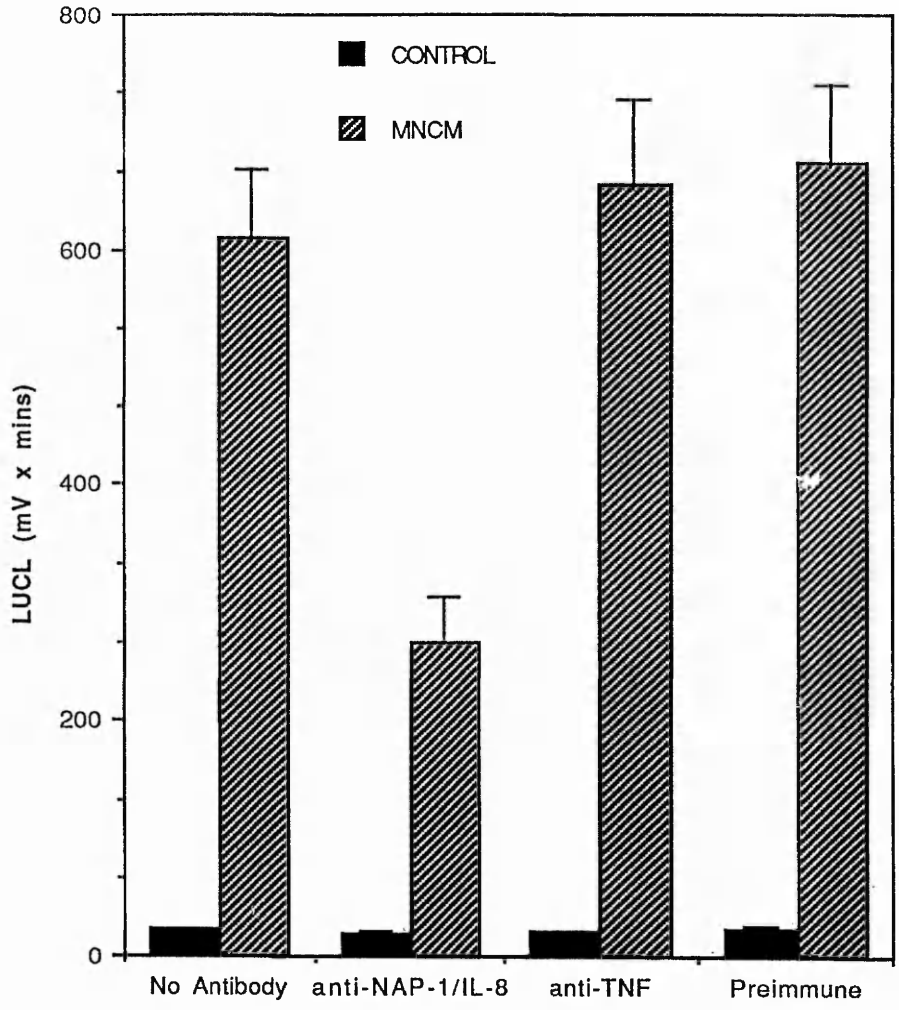

Figure 29: The effect of anti-cytokine antibodies and control preimmune serum on the direct stimulatory activity of MNCM. Controls represent PMN incubated in control medium containing 1ug ml^{-1} LPS. PMN were preincubated with MNCM or control medium plus antibodies (all at a 1:200 dilution) for 15 minutes at $4^{o}C$ prior to washing, warming and monitoring of LUCL.
The results represent the means of 4 experiments $\pm$ SEM, using pooled MNCM and different individual donor PMN.

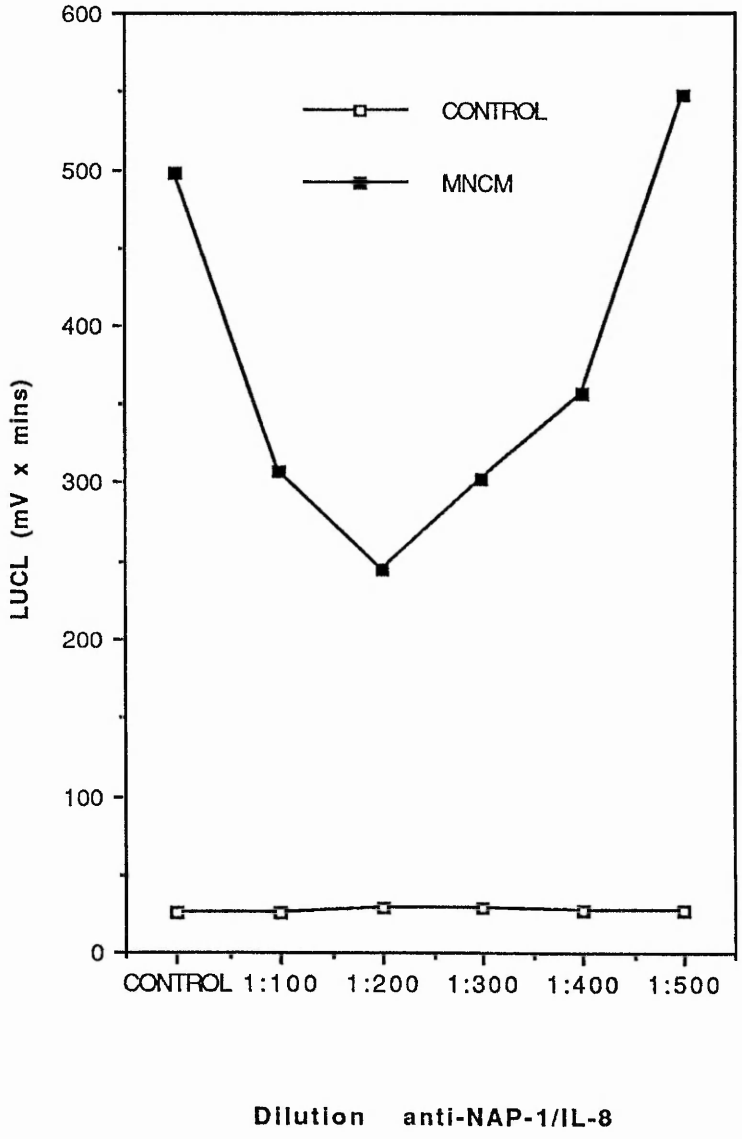

Figure 30: The effect of decreasing concentrations of NAP-1/IL-8 antiserum on the direct stimulatory activity of MNCM. The controls represent preimmune antiserum diluted in the same manner, in complete medium plus 1ug ml^{-1} LPS.
The results are from one representative experiment of three carried out using pooled MNCM on different donor PMN.

concentration of antiserum did reduce the direct effect of MNCM on respiratory burst activity, while the antibody itself had no direct effect on PMN superoxide anion production. When increasing concentrations of the antibody were used in the MNCM preincubation a dose dependent effect could be observed as shown in figure 30.

3.4.5 The Effect of NAP-1/IL-8

Since it may have been possible that NAP-1/IL-8 was acting synergistically with other cytokines to directly stimulate PMN respiratory burst activity, experiments were performed using combinations of recombinant human cytokines. However, there was no apparent synergistic effect of NAP-1/IL-8 on the respiratory burst with any of the cytokines, either alone or in combination (figure 31). When these experiments were performed with the additional step of ZAS stimulation, the priming effect was again due to TNF (figure 32) with no contribution from the other cytokines used.

Adding increasing concentrations of NAP-1/IL-8 to pooled MNCM did not show any apparent synergy. As shown in figure 33, in the presence of MNCM a dose dependent effect of NAP-1/IL-8 was observed, however the effect of this cytokine was proportionally the same as that seen in controls. When increasing concentrations of NAP-1/IL-8 were added to 100U ml^{-1}

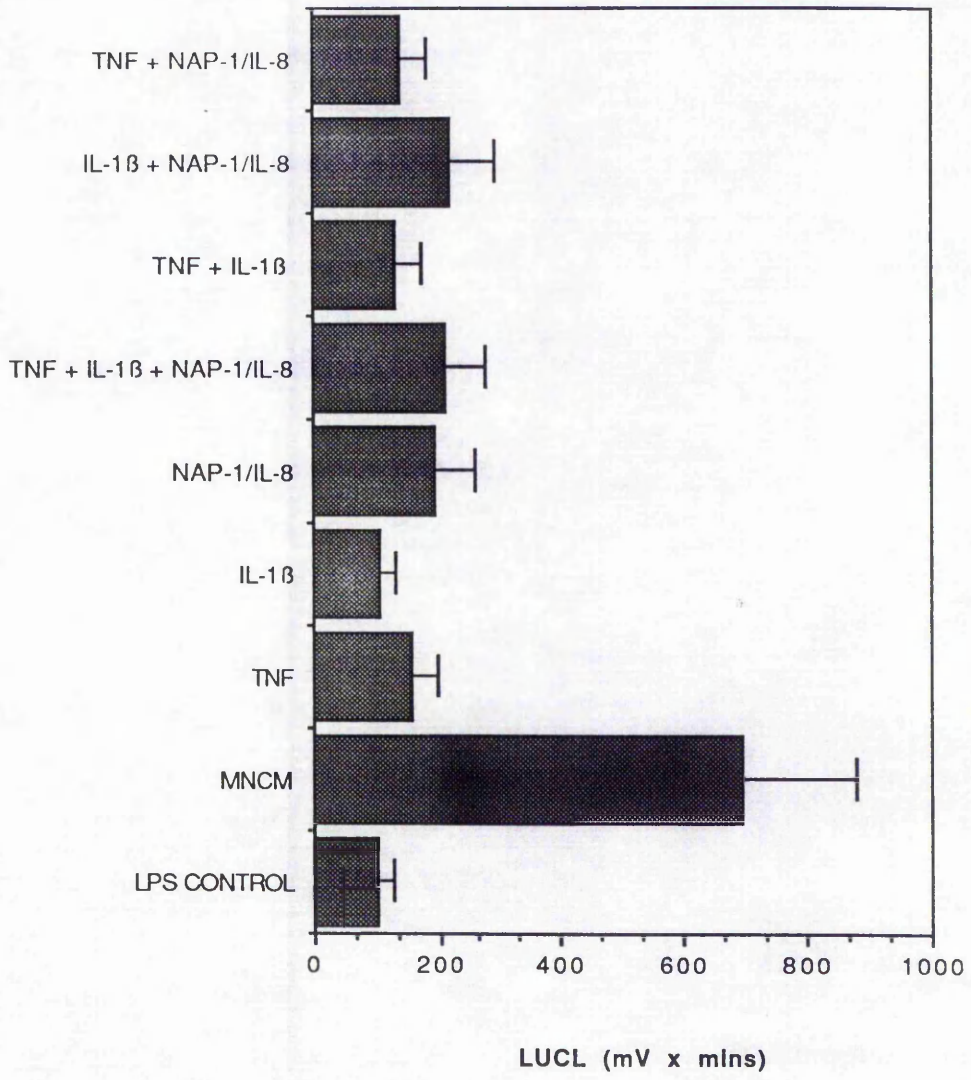

Figure 31: A comparison between the direct stimulatory activity of MNCM and LPS control with various concentrations (TNF at 100U ml^{-1}, IL-1 at 100U ml^{-1} and NAP-1/IL-8 at 2ug ml^{-1}) of recombinant human cytokines (as shown). All cytokines were dissolved in complete medium containing 1ug ml^{-1} LPS.
The results represent the means of 6 separate experiments $\pm$ SEM on individual donor PMN

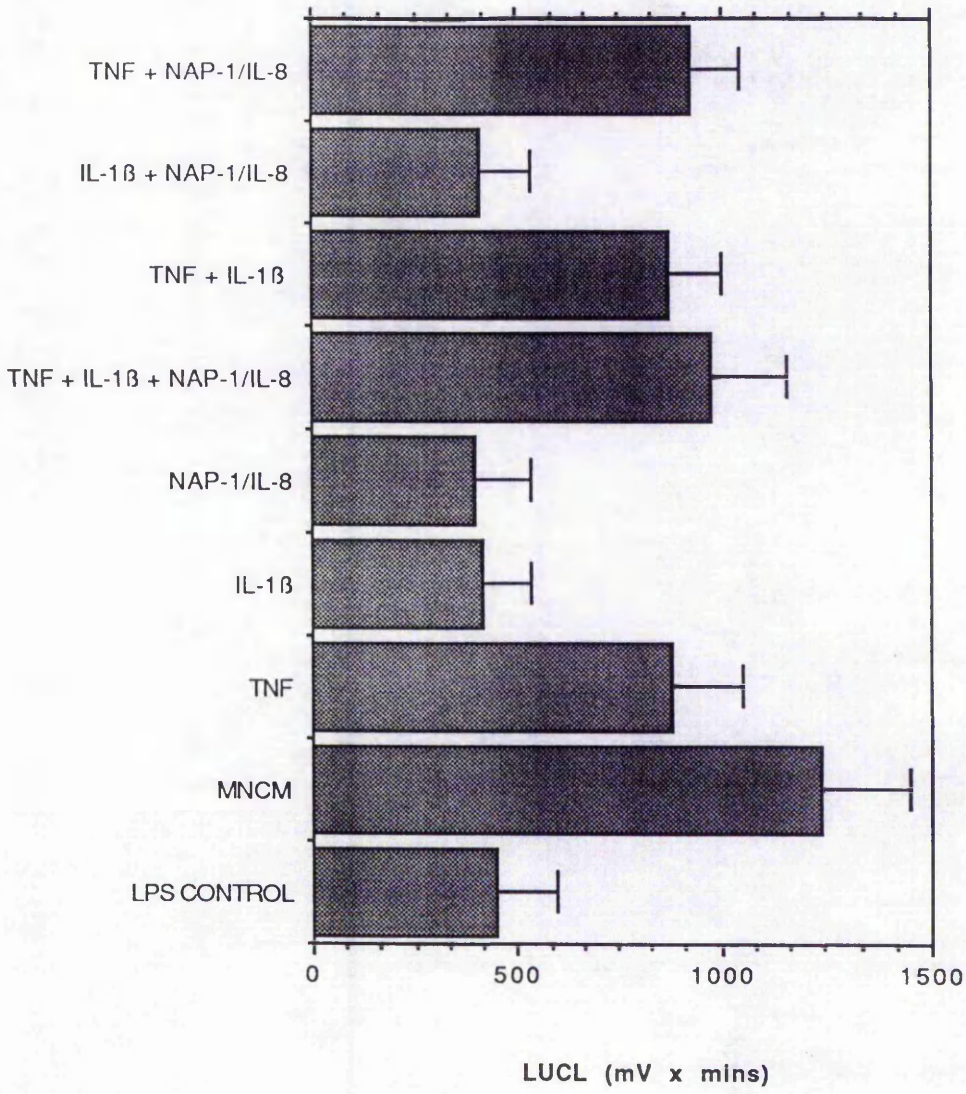

Figure 32: A comparison between different combinations of recombinant human cytokines (as indicated, for concentrations see figure 31) with the priming activity of LPS control medium and MNCM on PMN LUCL in response to ZAS stimulation. All cytokine preparations were diluted in complete medium containing 1ug ml^{-1} LPS.

The results represent the means of 6 separate experiments ± SEM.

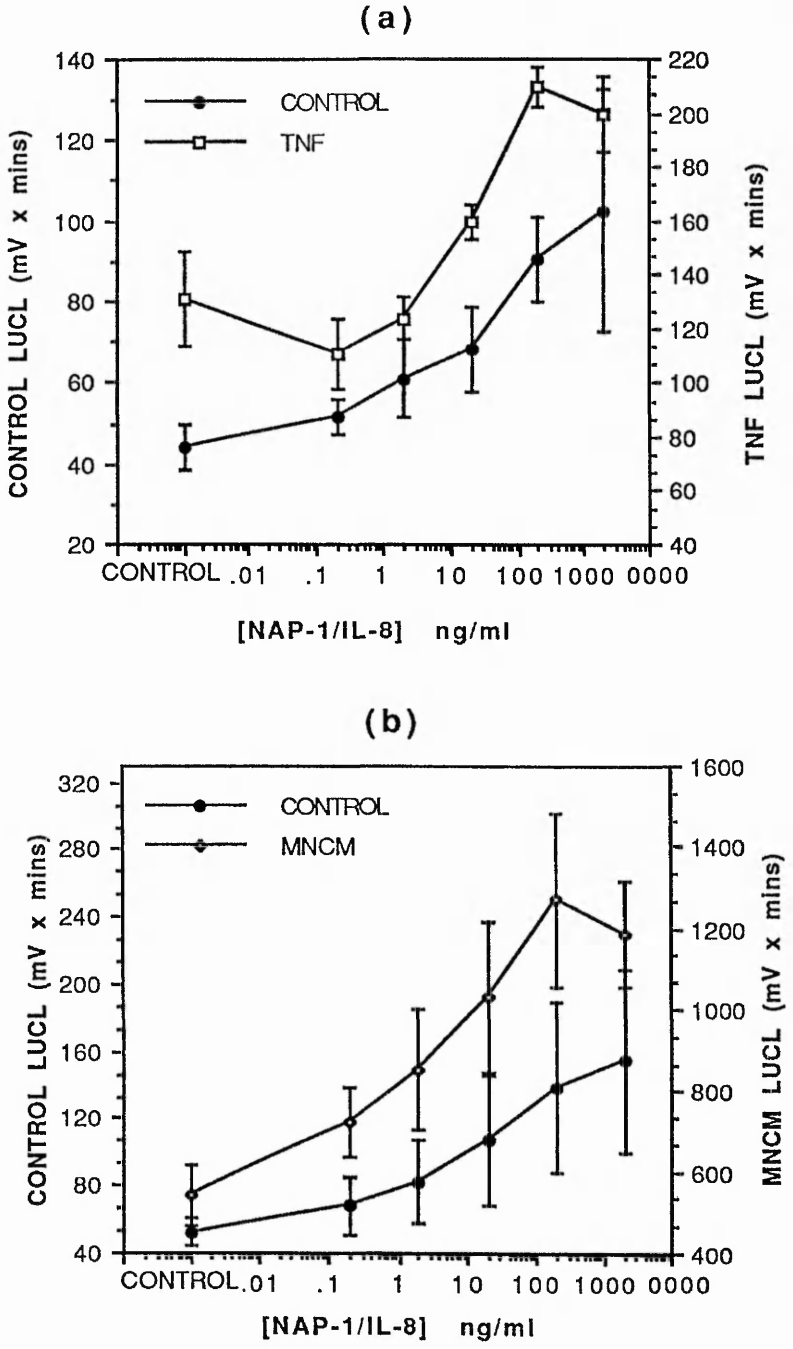

Figure 33: The effect of increasing concentrations of recombinant human NAP-1/IL-8 direct stimulatory activity in the presence of 100U ml^{-1} TNF (a) and MNCM (b).

The results represent the means of 6 separate experiments $\pm$ SEM on individual donor PMN.

TNF there was no synergy observed with respect to PMN superoxide anion production.

Due to the differences observed with the NAP-1/IL-8 preparations, an attempt was made to confirm bioactivity using a method other than superoxide anion production. NAP-1/IL-8 has been reported to stimulate increases in intracellular calcium concentrations (Peveri et al 1988, Thelen et al 1988), therefore we went on to investigate the ability of each preparation to induce calcium transients.

3.4.6. Effect of Recombinant Human NAP-1/IL-8 on Intracellular Calcium

Changes in intracellular calcium concentrations were determined fluorimetrically using fura-2-am. Figure 34 shows that all three preparations were capable of inducing calcium transients. However, the potency of each preparation varied greatly when tested at the same concentrations against the same donor PMN. From table 2 it can be seen that both forms 2 and 3 produced a dose dependent increase in the maximum intracellular Ca^{2+} concentration obtained. Form 1 was only able to stimulate a small increase at the highest concentration employed. These results also demonstrate that the potency of the various forms of NAP-1/IL-8 vary considerably, with an order of potency, form 3> form 2> form 1. This disparity in the ability of each form to stimulate increases in intracellular Ca^{2+} may also explain

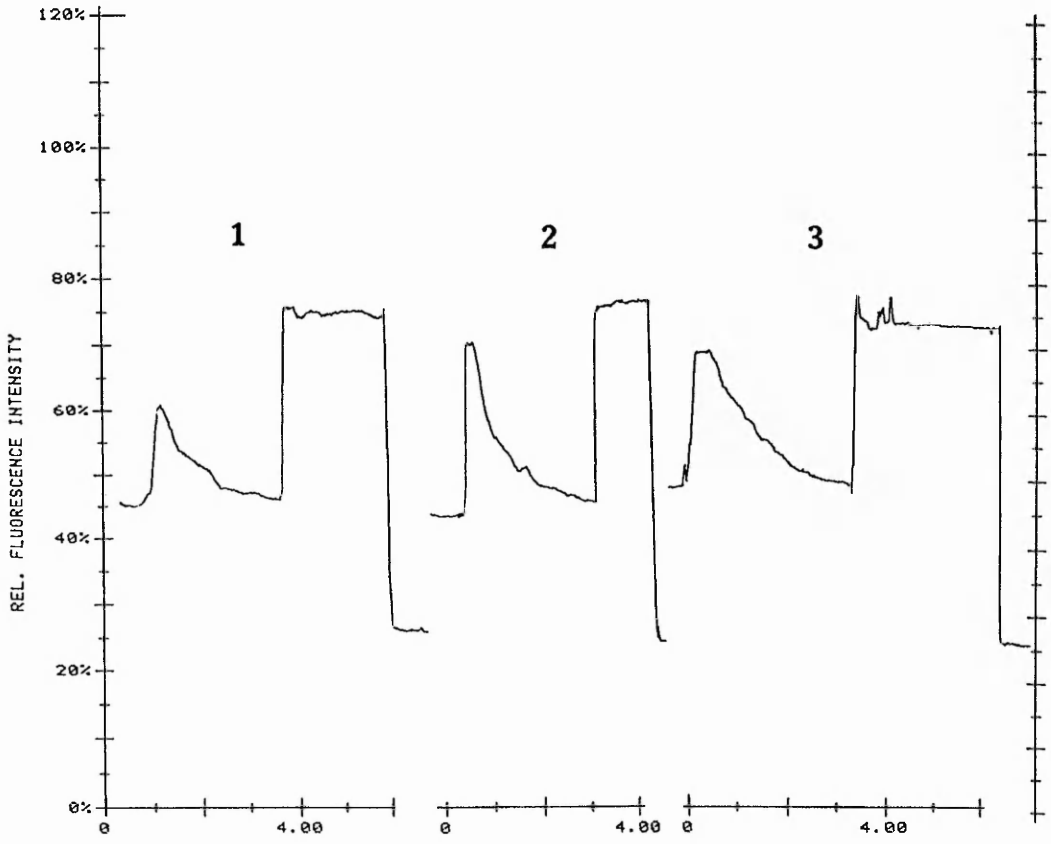

Figure 34: A comparison between the three different preparations of recombinant human NAP-1/IL-8 at 20ng ml^{-1} with respect to the induction of intracellular calcium transients as determined by Fura-2-am fluorimetry.

The results are from one representative experiment of three carried out on different donor PMN.

Table 2: Effect of NAP-1/IL-8 on Intracellular Calcium Concentration

Sample	[IL-8] ng ml^{-1}	Subject Number 1	2	3	$[Ca^{2+}]$ nM x ± SEM
10^{-6}M FMLP		971	1095	2150	1405 ± 374
(1)	20	655	576	BG	410 ± 206
	2	BG	BG	BG	BG
	0.2	BG	BG	BG	BG
(2)	20	1020	1312	1602	1311 ± 168
	2	709	946	BG	552 ± 284
	0.2	BG	BG	BG	BG
(3)	20	728	2464	5488	2893 ± 1391
	2	655	2352	1792	1600 ± 499
	0.2	592	1312	1712	1205 ± 328

BG = Background Ca^{2+} concentration = 174.3nM ± 10.1nM (mean of 11 ± SEM). Any extracellular fura-2-am was not quenched, and therefore may contribute to the results.

the observed effect on superoxide anion production, form 3 was the only preparation to induce respiratory burst activity.

3.5. Identification of Low molecular weight Factor(s) in MNCM by Sephadex Gel Filtration

3.5.1. Sephadex G-75

In order to try and identify the factor in MNCM which was directly stimulating PMN respiratory burst activity MNCM were separated by Sephadex gel filtration. Initial experiments were carried out with Sephadex G-75, which retains and separates samples with a molecular weight of less than 75kD. Fractions which eluted from this column that contained protein, as determined by the Lowry test (methods 2.7.1.), or exhibited UV absorbance at 280nm, were used in the PMN priming and direct stimulation experiments. Figure 35 shows that two peaks were eluted, the first was positive in the Lowry while the second peak was negative, but did exhibit UV absorbance and had a molecular weight of less than 12.3kD (cytochrome c was used as the low molecular weight marker). Sample fractions were taken from each of these peaks and used in the direct stimulation and priming of PMN to ZAS. Fraction 30 and 34 (Peak I: 60mls and 68mls elution volume) demonstrated priming activity to ZAS but no direct stimulatory activity when compared to the native MNCM. Peak I had a molecular weight of around 54kD, consistent with it being the trimeric, bioactive form of TNF (51kD).

An ELISA was used to measure TNF in fractions eluting from the column (a TNF standard curve is shown in figure 36), immunoreactive TNF was present in fraction 30 (12.9U ml^{-1}) and fraction 34 (8.7U ml^{-1}), again consistent with TNF being responsible for priming activity. TNF in the intact MNCM was assayed at 143.2U ml^{-1}. Of other selected fractions tested, no immunoreactive TNF could be detected.

The second peak (II), did not show PMN priming activity to ZAS stimulation, but did directly stimulate PMN respiratory burst activity as seen with selected fractions 80 and 99 (elution volumes 160 and 198 respectively). As anti-NAP-1/IL-8 antiserum partially inhibited the direct stimulatory activity of MNCM, the experiments on direct stimulatory activity of fractions and MNCM were repeated in the presence of this antiserum. As shown in figure 37 a reduction in chemiluminescence was seen with the low molecular weight fraction 99 (198 mls). As with the MNCM complete abrogation could not be achieved. Another peak of activity was seen with fraction 80 (160 mls), superoxide anion production stimulated by this fraction was unaffected by the presence of anti-NAP-1/IL-8 antiserum (figure 37).

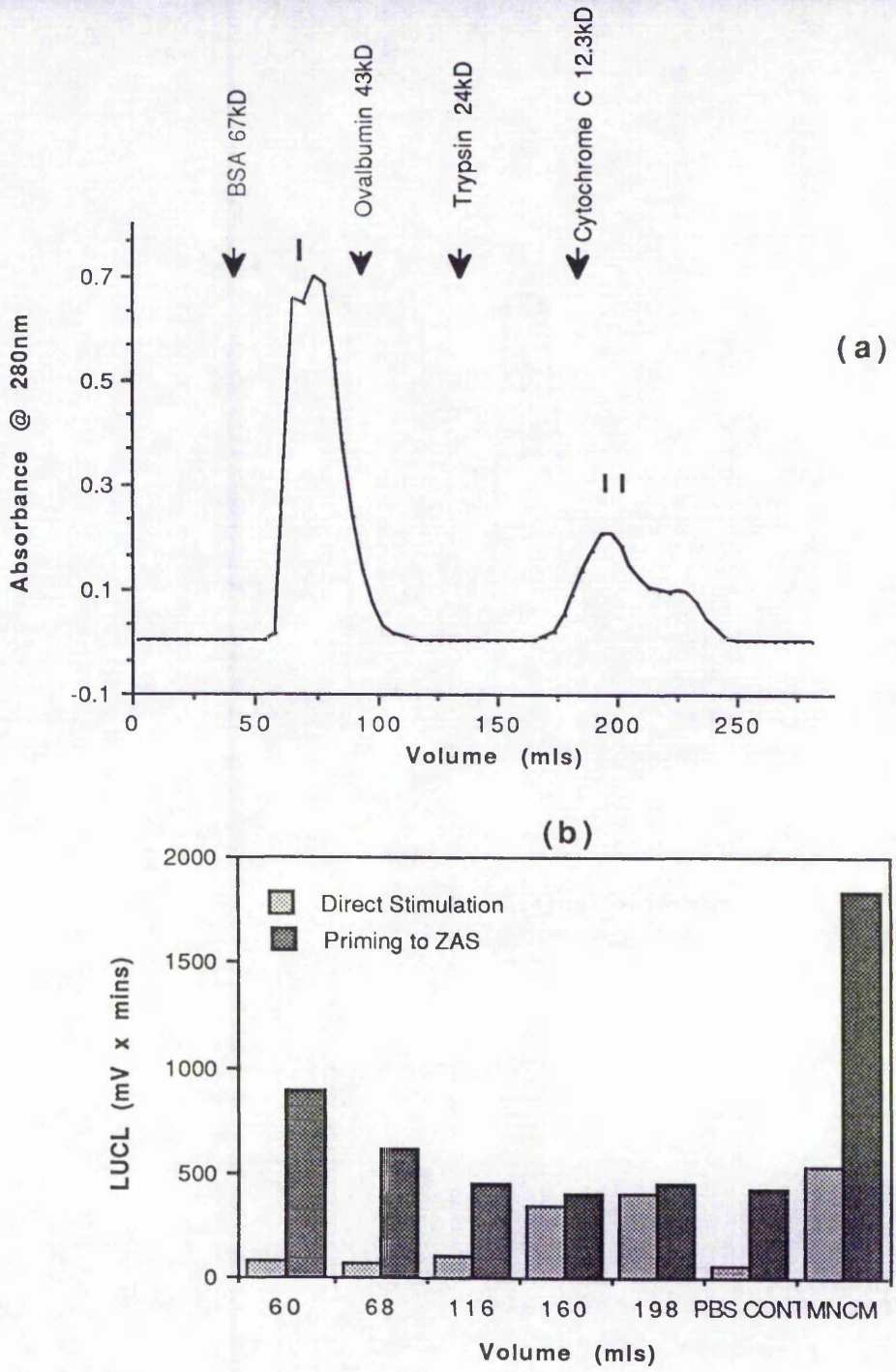

Figure 35: Sephadex G-75 filtration of an MNCM from one individual MNC donor. Absorbance was read at 280nm (a) for determination of fractions containing proteins and peptides. Panel (b) shows the effect of chosen fractions on direct stimulation of PMN LUCL and priming activity in response to ZAS stimulation. The activity of these fractions were compared to a PBS control and the original MNCM on one PMN donor.

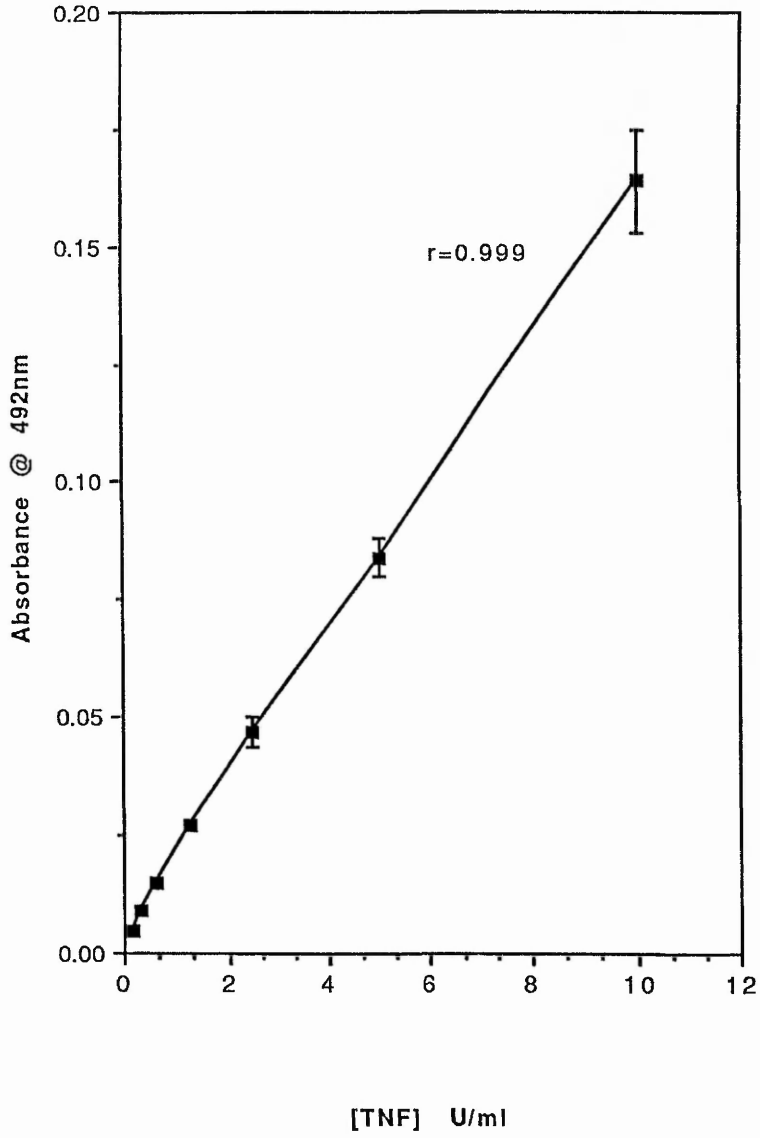

Figure 36: TNF ELISA standard curve; increasing concentrations of TNF correlated with increased absorbance.
The results represent the means of 12 separate experiments ± SEM.

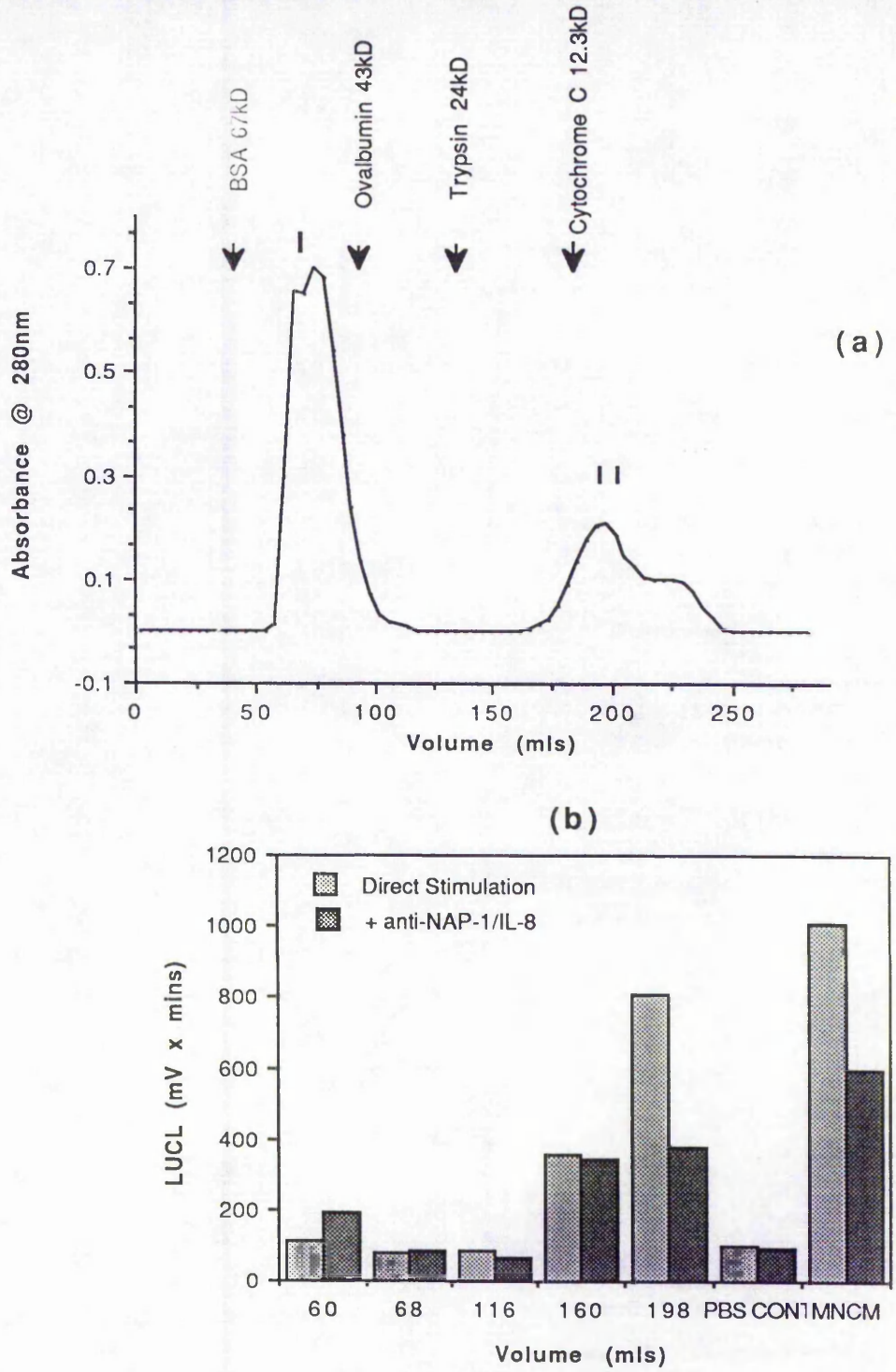

Figure 37: Sephadex G75 filtration of MNCM. Panel (b) represents the direct stimulatory activity of the fractions, PBS control and MNCM (▒) on PMN LUCL. (■) represents the same experiment carried out in the presence of NAP-1/IL-8 antiserum. The results are from one MNCM tested against one donor PMN and is representative of four similar experiments.

3.5.2. Sephadex G-50

To try and further separate out the lower molecular weight molecules MNCM was passed through a Sephadex G-50 column to retain and separate molecules with a molecular weight of 50kD. Substances with a molecular weight higher than 50kD came off the column in the void volume. The fractions were eluted from the column at 4°C to slow diffusion and thereby allow for a greater degree of separation due to the slower flow rate of PBS through the column. Figure 38 shows the elution profile of MNCM separated by Sephadex G-50. Priming of PMN in response to ZAS stimulation was seen in fraction 54 (162mls elution volume) which corresponded to the void volume of the column. This was again consistent with the priming activity being due to TNF, as the biologically active form of TNF is a trimer with a molecular weight of 51kD. Immunoreactive TNF was assayed in this fraction at 6.24U ml^{-1}, TNF was assayed at 164.9U ml^{-1} in complete MNCM. None of the other fractions tested were positive for immunoreactive TNF. The lower molecular weight fractions (145 and 189; 435 and 567mls respectively) with molecular weights less than 12.3kD were capable of directly stimulating PMN superoxide anion generation. Again the activity of these fractions was reduced (43.84% $\pm$ 12.19%), but not completely abrogated, in the presence of the antiserum to NAP-1/IL-8.

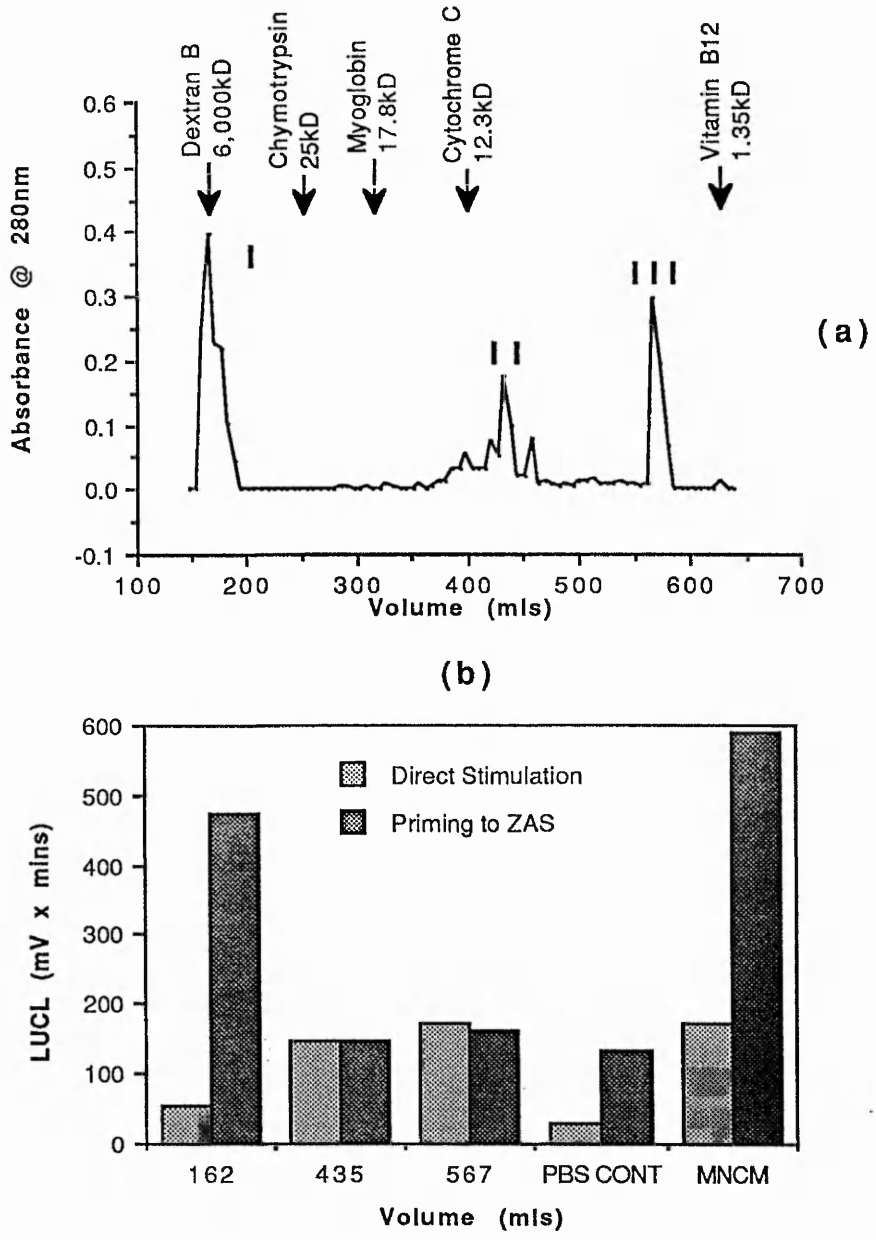

Figure 38: Sephadex G50 filtration of MNCM. The elution profile (a) with absorbance readings taken at 280nm. Panel (b) shows the direct stimulatory activity of individual fractions compared with PBS and MNCM (▒). The priming effect of each of these fractions was also determined in response to ZAS stimulation (■).

The results are from one experiment representative of five.

3.6. Discussion

Preliminary experiments using LUCL showed it to be an efficient and reliable method for measuring the generation of superoxide anions from PMN stimulated with either ZAS or FMLP. ZAS is a physiological stimulus as the active component is C5a des arg (Fernandez et al 1978). The production of C5a des arg occurs in vivo after activation of the complement cascade in response to an inflammatory stimulus. It was therefore an appropriate stimulus to use with respect to attempting to elucidate PMN response to physiological stimuli in vitro. In addition to the expression of receptors for C5a on the surface of neutrophils, receptors specific for formyl peptides have also been demonstrated, and extensively studied. FMLP has been shown to be secreted by certain strains of gram-negative bacteria and is therefore also thought to be a physiologically relevant stimulus. Therefore the effect of FMLP on PMN superoxide anion generation was also investigated. The results from the initial experiments confirmed the findings of others (Goetztl and Austen 1974, Wymann et al 1987), that both of these agonists, in addition to being potent chemotaxins, also have a profound effect upon the induction of PMN respiratory burst activity.

The activity of neutrophils have been shown to be enhanced when exposed to certain MNC products, in the form of recombinant human cytokines, including TNF (Atkinson et al 1988), IL-1 (Dularay et al 1990) and GM-CSF (McColl et al 1990). Instead of

looking at recombinant human cytokines which are highly purified and often go through severe lyophilisation procedures, with the addition of various disaccharides and proteins, we chose to look at the effect of conditioned media harvested from MNC stimulated with LPS. The results showed that MNC cultured under these conditions were capable of secreting a factor which could induce a marked increase in superoxide anion production when the cells were subsequently exposed to a second agonist, ZAS This priming occurred when PMN were incubated with MNCM for 15 minutes at 4°C, washed and then warmed to 37°C prior to stimulation with the second agonist. Experiments looking at the effect of changing the preincubation times showed that the factor bound irreversibly to the cells, as early as 5 minutes with no further enhancement seen after 15-30 minutes.

Investigations were made into the time course for the production of this priming factor. Priming activity was seen as early as 1-2 hours after LPS stimulation of MNC. Burchett et al (1988) investigated the production of both TNF and IL-1 from LPS stimulated macrophages. They showed that the induction of the TNF occurred rapidly after stimulation with LPS with secretion of the bioactive protein occurring soon after. Induction of mRNA expression was maximal at 3-5 hours and by 18 hours had dropped back to the level of unstimulated cells. IL-1 mRNA expression showed a similar pattern with respect to onset of induction, although secretion of the bioactive gene product was increased over a longer time period. Animal models have

also shown that TNF is present in the serum soon after injection of LPS (Gifford and Flick 1987), suggesting that this cytokine is one of the earliest to be produced in response to an infective agent. These and other investigations working with recombinant human TNF *in vitro* suggested that the probable nature of the priming factor was TNF.

Pichyangkul *et al* (1987) had shown that TNF was capable of binding to its receptor on the surface of neutrophils very rapidly and that this binding was maximal after 20 minutes. This binding of TNF to its receptor was irreversible, in that it could not be removed by washing the cells. This was again consistent with the priming factor in 5 hour MNCM being TNF, since enhancement of PMN respiratory burst activity could be observed after only a 5 minutes incubation at 4°C and this effect could not be removed by washing the MNCM from the LUCL reaction mixture.

Although the evidence was strongly in favour of TNF being solely responsible for the priming activity observed, IL-1 has also been reported to enhance neutrophil responses to a second agonist (Dularay *et al* 1990). To eliminate a role for IL-1 we were able to show that recombinant human IL-1 was not capable of reproducing the priming activity of MNCM after a 15 minutes pre-incubation at 4°C followed by washing, warming and stimulation. Furthermore although IL-1 was produced from the MNC with a similar time course to TNF, the priming activity in

MNCM was reduced at 18 hours even though IL-1 secretion continued to increase up to 27 hours in culture.

GM-CSF has also been extensively studied for its ability to prime neutrophils. This enhancement may occur either as a result of upregulating the oxidative responsiveness of the cells (English et al 1988), or the affinity and number of FMLP receptors (Weisbart et al 1986). The events that lead to increased responsiveness of these cells may be the result of a combination of the two (Naccache et al 1988). Priming by GM-CSF also involves increasing the proportion of responsive cells within a population (Fletcher and Gasson 1988). Other investigations have shown that the priming activity of GM-CSF was temperature- and time-dependent (English et al 1988). Therefore it was unlikely that the priming activity in MNCM resulted from the presence of GM-CSF. Using recombinant human GM-CSF showed that no priming activity could be seen when cells were preincubated for 15 minutes at 4°C, however enhancement of PMN LUCL in response to ZAS was seen after a 60 minutes preincubation at 37°C. In addition immunoreactive GM-CSF was not detectable in the 5 hour MNCM used for priming. The only recombinant cytokine tested that was capable of reproducing the priming effects of MNCM was TNF.

The priming effect of MNCM could be significantly reduced when preincubations were carried out in the presence of an anti-TNF polyclonal antibody. None of the other antibody treatments used

had any effect at reducing the priming activity observed. Taken together these results demonstrated that the priming activity of MNCM was due to TNF.

LPS has also been shown to be capable of priming neutrophils for enhanced respiratory burst activity (Guthrie et al 1984, Aida and Pabst 1991). The LPS dose response curves showed that LPS was capable of enhancing PMN superoxide anion production in a dose dependent fashion in response to ZAS stimulation. However, when looking at the length of preincubation time, LPS showed slightly different kinetics from MNCM. While the effect of MNCM was maximal by 15-30 minutes, LPS priming showed the most marked effect after 40 minutes incubation. This effect of LPS was also irreversible in that this slight enhancement could still be observed after washing. There is evidence to suggest that part of the C3bi receptor recognizes and binds LPS (Wright et al 1989), this would explain the ability of LPS to bind irreversibly at $4^{o}C$. LPS has also been shown to bind to cells as part of a complex with a LBP that binds to the CD14 molecule of neutrophils (Wright et al 1991). In the complete medium used to dilute the LPS there was also 10% foetal calf serum which may act as a source of LBP as indicated by Aida and Pabst (1990). This may therefore be the mechanism by which LPS causes irreversible priming under the experimental conditions.

The sensitivity of chemiluminescence demands the use of highly pure reagents and a pure cell preparation. The preparation and

storage of cells, together with conditions for studying their chemiluminescence have marked effects on the time course and the magnitude of the response. As LPS can prime PMN responses, it was important to use buffers and reagents prepared aseptically. These reagents were continually checked for the presence of contaminating endotoxin using the LAL assay. All preparations used contained less than 20pg ml^{-1} of LPS which is far less than the amounts of LPS that were added to the complete medium to induce induction of cytokine secretion, and therefore less than the amount of LPS present in PMN priming experiments.

The method by which TNF is capable of priming cells is unknown, although it appears to act by modulating cell surface receptors. It has been shown to regulate the affinity but not the number of FMLP receptors (Atkinson et al 1988). If TNF was affecting the affinity of the receptors for C5a and FMLP then it would be expected that dose response curves for these two agonists would shift. In both cases lower concentrations of each agonist would be required to give maximal superoxide anion generation. Repeating the dose response curves for ZAS and FMLP in the presence of MNCM and TNF did not reveal any shift to the left. Therefore, MNCM and TNF may prime via some other mechanism which could involve the more efficient recruitment of components of the oxidase system. As activation of the oxidase is dependent upon a number of different factors it is possible that factors such as TNF enhance the respiratory burst by

controlling the number of responsive cells within a population. With ZAS and FMLP activation of the oxidase depends on the generation of intracellular secondary messengers. The concentrations of these secondary messengers may have to reach a critical concentration at the necessary location inside a cell to switch on the oxidase (Campbell et al 1988). This may explain the priming effects observed since the amount and distribution of IP_3, calcium or DAG in the cell will be determined by receptor occupancy, internal buffering and degradation.

A recent study by McColl and colleagues (1990) suggests that priming of PMN by both TNF and GM-CSF involved regulation at a post-cell surface receptor level and that this modulation occurs independently of a modulation in the expression of receptors at the cell surface. If the efficiency of either the second messenger system or the oxidase is increased during priming, this may explain the increased superoxide anion production observed at the same concentration of second agonist. TNF has been shown to increase the activity of phospholipase D, resulting in increased phosphatidic acid (PA) production when cells were subsequently stimulated with FMLP (Bauldry et al 1991). They showed there to be a direct correlation between PA formation and superoxide production. Recruitment of a larger number of responsive cells may also increase the magnitude of the response and rate of increase in

superoxide anion production, but would not necessarily affect the optimal dose of the second agonist required.

While this priming effect could be reduced by blocking the TNF with a suitable antibody, MNCM was capable of directly stimulating the respiratory burst and this activity could not be blocked with any of the antibodies used. It is a subject of great conjecture as to whether TNF is capable of directly inducing respiratory burst activity in neutrophils. Our data suggest that TNF can only induce PMN superoxide anion production at high concentrations, and not in all of the PMN donors tested. The direct stimulatory activity of MNCM was consistent from donor to donor and may indicate that another factor, or factors, secreted from LPS stimulated MNC is capable of stimulating PMN directly. IL-1, although present in the MNCM, does not appear to affect either the intracellular calcium or the functional responses of PMN (Georgilis et al 1987). Using recombinant human IL-1, we were not able to reproduce this effect suggesting that IL-1 was not responsible for the direct stimulatory activity. Recently a peptide secreted from LPS stimulated MNC was identified as a potent PMN chemotaxin. NAP-1/IL-8 has also been demonstrated to have respiratory burst activity (Thelen et al 1988), although again this is a matter of debate. We could not reproduce the direct stimulatory activity of MNCM using two different sources of NAP-1/IL-8, a third form of recombinant human NAP-1/IL-8 did

elicit a dose dependent response, however, concentrations as high as 2ug ml^{-1} were not as effective as MNCM alone.

Separating MNCM using Sephadex gel filtration indicated that the factor in MNCM that caused direct stimulation was a low molecular weight peptide. In addition the use of an anti-NAP-1/IL-8 antiserum significantly ($p<0.025$) reduced the amount of LUCL observed in response to stimulation with MNCM. The direct effect, as with the priming, was irreversible with the PMN being washed after the preincubation step. This would suggest that this factor was also capable of binding to its PMN cell surface receptor in the cold, and after washing and warming to 37°C could exert its effect upon the respiratory burst. NAP-1/Il-8 receptors have been demonstrated on the surface of neutrophils (Samanta et al 1990) and these investigations indicated that NAP-1/IL-8 can bind avidly to the cell after a short incubation period. Therefore it may be possible that NAP-1/IL-8 in MNCM is directly inducing respiratory burst activity. Of the three forms of recombinant NAP-1/IL-8 investigated 1 and 2 (figure 27) were lyophilised preparations whereas number 3 was the highly purified peptide in Tris buffer. It is possible that the additional lyophilisation step may in some way have affected the bioactivity of this peptide. The inability to reproduce these observations using recombinant human forms of this peptide may be due to the lack of this particular bioactivity in these preparations. It is possible that some other factor which co-elutes with NAP-1/IL-8 on Sephadex gel

filtration is responsible for this activity, as the bioactivity of the low molecular weight fractions could not be completely abrogated using the antiserum. Selecting different low molecular weight fractions showed one where bioactivity was reduced in the presence of anti-NAP-1/IL-8, while a second was unaffected in the presence of the antibody. It could also be possible that native NAP-1/IL-8 from MNC may differ in some way from the recombinant human forms being supplied.

Bioactivity of each of the recombinant preparations was confirmed by measuring changes in intracellular calcium. All three preparations were capable of inducing intracellular calcium transients, however the potency of each preparation varied. It has been shown that there is a requirement for a certain threshold concentration of calcium before NADPH oxidase activity occurs (Hallett et al 1990). Therefore it may have been possible that preparations 1 and 2 were not inducing sufficient increases in intracellular calcium. However, the highest concentration of preparation 2 induced a calcium transient similar to that seen with preparation 3, but where as the latter induced superoxide production the second preparation did not. Therefore, it is possible that there is some other requirement in addition to increasing intracellular calcium that is required for induction of respiratory burst activity in response to NAP-1/IL-8 stimulation.

Adding NAP-1/IL-8 in increasing concentrations to MNCM was carried out to investigate the possibility of synergy between this peptide and any other PMN activating substances. Using the NAP-1/IL-8 preparations in conjunction with other recombinant cytokines did not show any synergistic activity. On the assumption that all cytokines were biologically active NAP-1/IL-8 in MNCM may be acting synergistically with other low molecular weight peptides that may be present. Other peptides secreted from LPS stimulated MNC include NAP-2 (Schroder et al 1990), MGSA-gro and possibly other peptides (review Matsushima and Oppenheim 1989). Although the data would suggest that NAP-1/IL-8 may be involved in the direct stimulation of PMN respiratory burst activity, the evidence is far from conclusive. Certainly the highest concentration of bioactive recombinant human NAP-1/IL-8 could still not reproduce the effects of either the MNCM or fractions eluted from Sephadex G-75 and G-50 columns.

This work outlines the problems associated with using recombinant human cytokines, as the responses of three different preparations of one cytokine varied. Exclusion of bioactivity of other cytokines, due to a failure to demonstrate responses should be guarded against. To try and confirm observations alternative investigations should be carried out, for example use of specific antibodies to abrogate effects.

Summary

Stimulated MNC cells are capable of producing cytokines that can have differential effects upon PMN function. TNF caused a significant enhancement in PMN respiratory burst when cells were exposed to a second agonist. The mechanism by which TNF could bring about this effect was unclear, although preliminary evidence suggested that this cytokine was not acting via increasing the affinity or number of cell surface receptors. TNF alone did not consistently induce PMN superoxide anion production. Another low molecular weight factor in MNCM was capable of directly stimulating PMN respiratory burst and this factor was thought to be NAP-1/IL-8.

CHAPTER FOUR: RESULTS

4.1. Effect of TNF on PMN Adhesion Molecule Expression

4.1.1. CD11b Expression

TNF has been shown to increase the ability of PMN to adhere to endothelium and penetrate monolayers *in vitro* (Moser *et al* 1989). This cytokine has also been shown to increase the adhesiveness of PMN to other surfaces (Seow *et al* 1987). A number of other factors , including C5a, LTB_4, LPS and PMA and the cytokine IL-1 appear to increase the adhesive capacity of these cells by increasing the expression of adhesion molecules (Kishimoto *et al* 1989). The chemotaxins C5a des arg and FMLP have also been shown to increase the adhesion of neutrophils to endothelium (Tonnesen *et al* 1984). The adhesion of neutrophils and migration from the circulation has been demonstrated in a rabbit model to occur by CD-18-dependent and -independent mechanisms, with the CD18-dependent adhesion occurring in the systemic circulation and both mechanisms being involved in the pulmonary circulation (Doerschuk *et al* 1990). *In vitro* both alpha and beta subunits of the CD11/CD18 family have been demonstrated to be of physiological importance in the adhesion of PMN (Anderson *et al* 1986).

TNF has the potential to increase the degree of margination and migration observed by increasing the expression of these

adhesion molecules. The use of flow cytometry together with monoclonal antibodies to a variety of adhesion molecules allowed for the effect of TNF on adhesion molecule expression to be determined. After a 15 minutes incubation at 37°C the cells were washed and antibody binding carried out. The results were calculated as the percentage shift in mean fluorescence with CD11b expression in the presence of TNF over those cells incubated in control buffer, as seen in figure 39. The ability of TNF to increase the expression of adhesion molecules was investigated; as shown in figure 40, TNF exerted a dose dependent increase in the expression of CD11b over controls. At concentrations as low as 10U ml^{-1}, this cytokine was capable of significantly ($p<0.025$) increasing the expression of these adhesion molecules by 65.48% $\pm$ 10.70%.

The time course for increased expression of this adhesion molecule was investigated using TNF at a concentration of 10U ml^{-1}. Figure 41 shows that increased expression was seen to occur as early as 5 minutes with no further significant increases being observed after 25-40 minutes.

CD11b forms the alpha sub-unit of the MAC-1 complex (C3bi receptor). This particular adhesion molecule has been associated with the membrane of neutrophil secondary granules (Bainton et al 1987), it was therefore possible that increases in CD11b expression were associated with PMN secondary

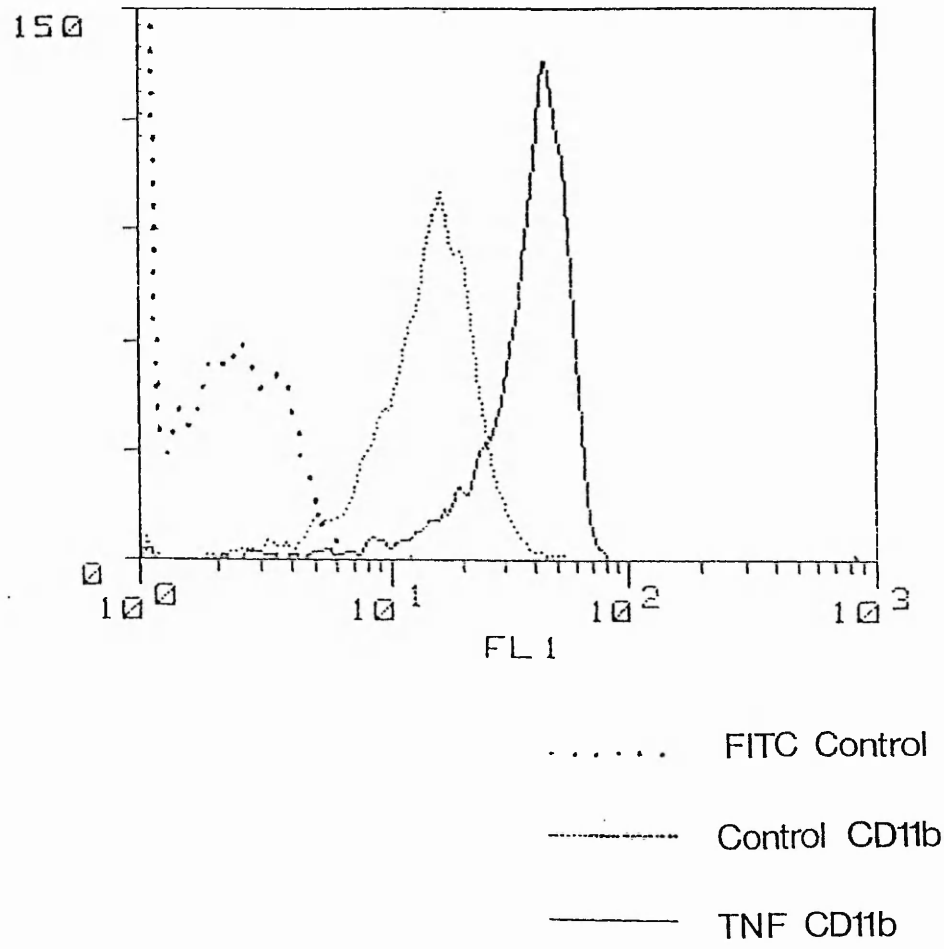

Figure 39: The effect of 10U ml^{-1} of TNF on the expression of CD11b. The expression is calculated as the percentage shift in the peak of mean fluorescence in the presence of TNF over PMN incubated in control medium.

The results are from one representative experiment.

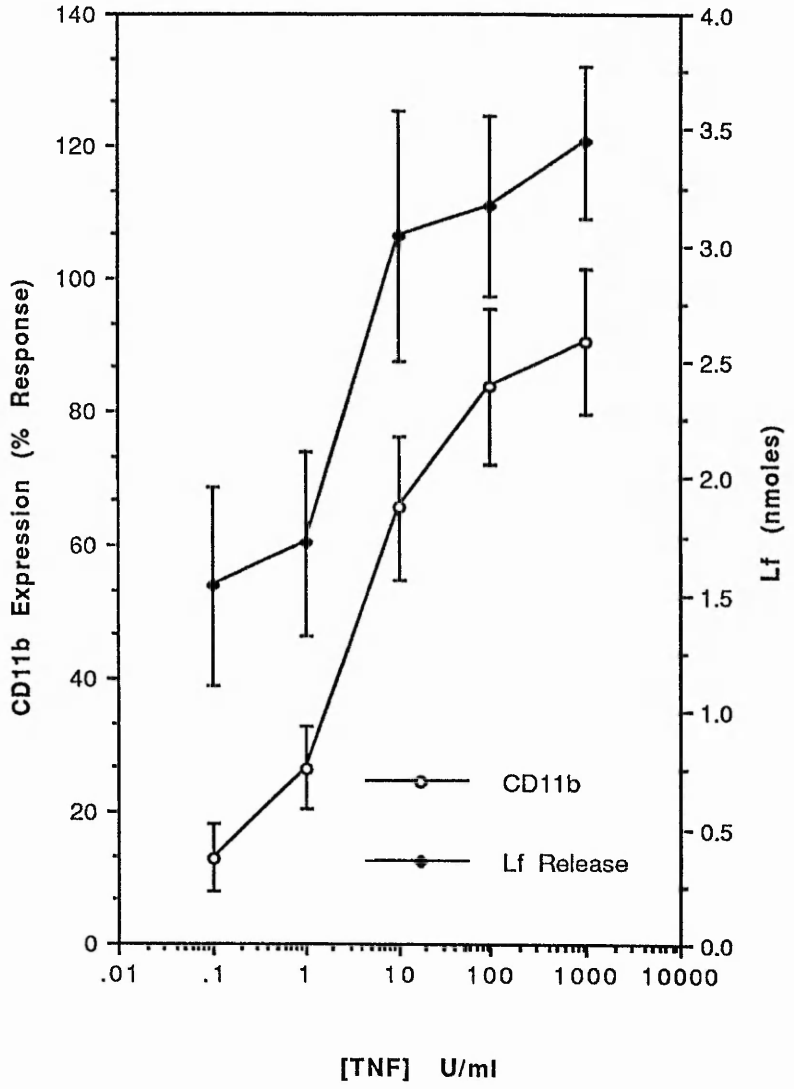

Figure 40: The effect of increasing concentrations of recombinant human TNF on CD11b expression and Lf release. The results represent the means of 7 separate experiments ± SEM using different donor PMN.

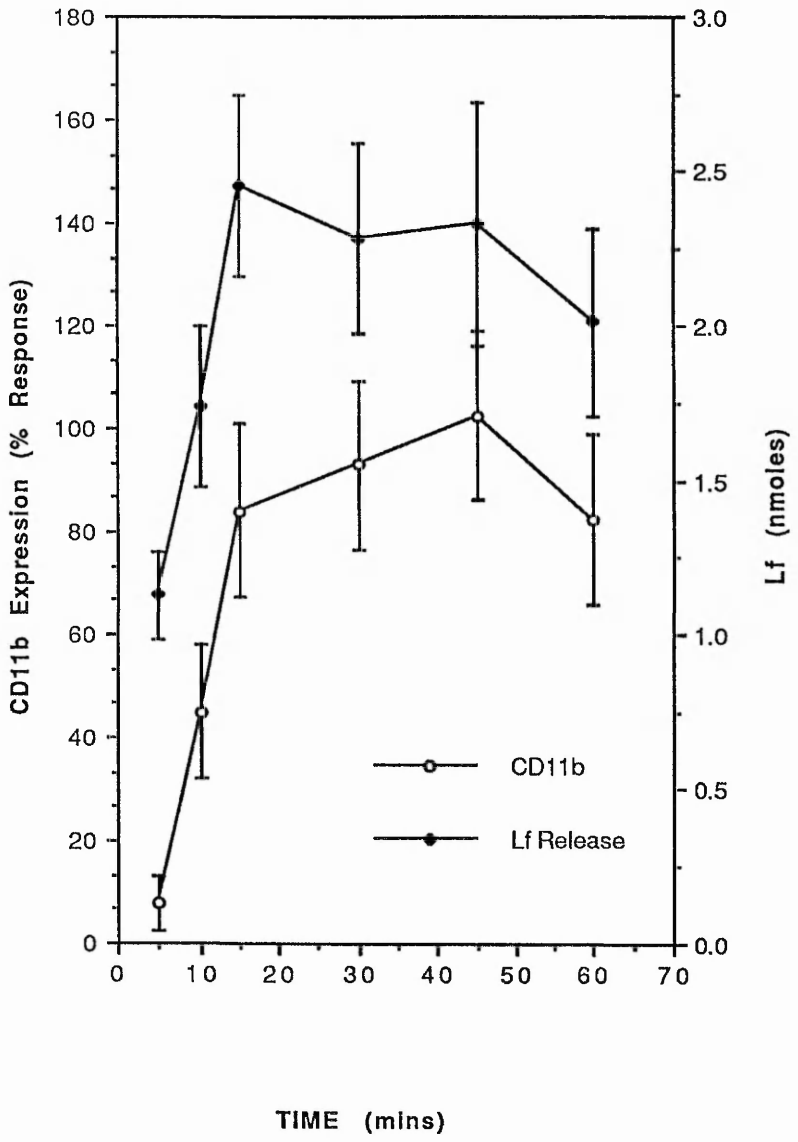

Figure 41: The effect of incubation times on both CD11b expression and Lf release. PMN were incubated with 10U ml^{-1} TNF for the times shown.

The results represent the means of 8 separate experiments ± SEM, carried out on different donor PMN.

degranulation.

4.1.2. Effect of TNF on Lactoferrin Release

In addition to monitoring the expression of CD11b with increasing concentrations of TNF, the amount of Lf released from these cells was also investigated. An example of a standard curve for the ELISA is shown in figure 42. After the 15 minutes incubation at 37°C, the cells were centrifuged down at 400g for 10 minutes and the supernatant collected. Figure 40 shows the amount of Lf released from these cells in response to increasing concentrations of TNF, these data showed a dose dependent increase in the amount of secreted Lf. Lysis of PMN by either sonication or Triton X100 treatment allowed for measurement of total Lf, this resulted in the measurement of 13.26nmoles $\pm$ 1.42 per 10^6 PMN ml^{-1} (n=8, mean $\pm$ SEM). There was a strong correlation ($p<0.001$) between increased CD11b expression and Lf release in response to TNF (figure 43). As shown in the previous chapter, low concentrations of recombinant human TNF (10U ml^{-1} and less) primed PMN for increased respiratory burst activity. In these experiments 10U ml^{-1} TNF also caused significant increases in both CD11b expression and Lf release. As with CD11b expression this concentration was used for investigating the effect of different incubation times (at 37°C) on these two parameters. Figure 41 shows that increased Lf release showed similar

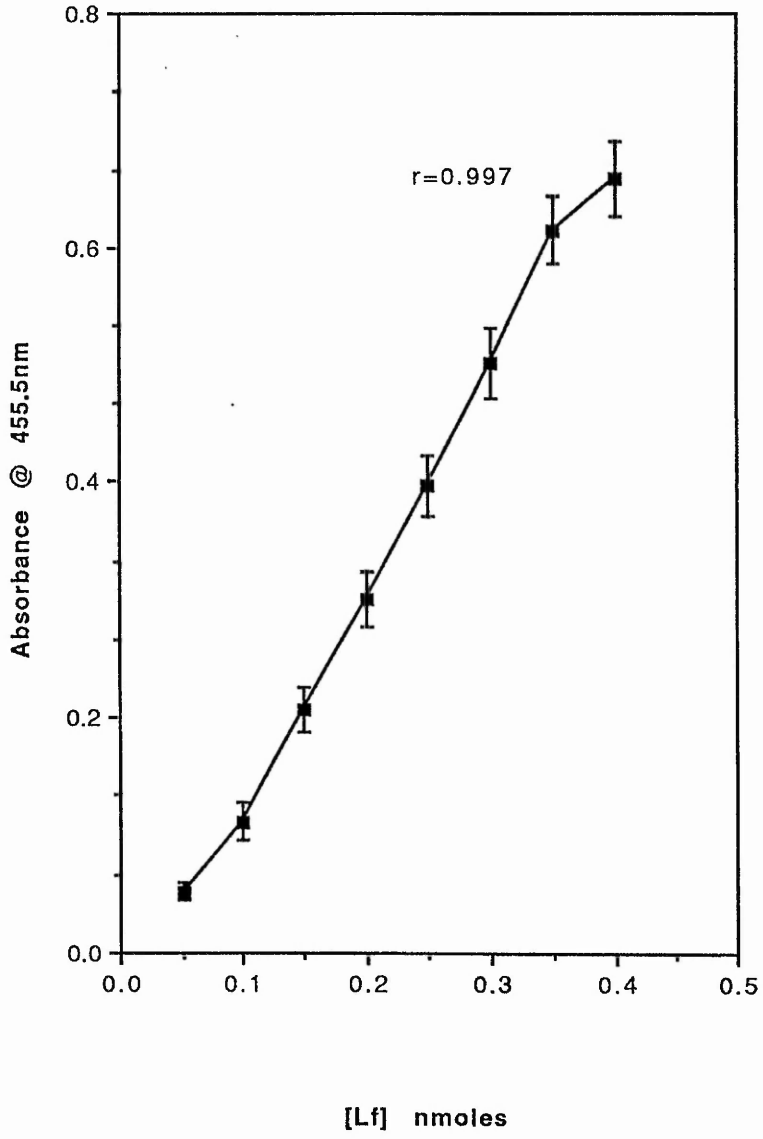

Figure 42: Lactoferrin ELISA standard curve; increased concentrations of Lf correlates with increased absorbance. The results represent the means of 15 separate standard curves ± SEM.

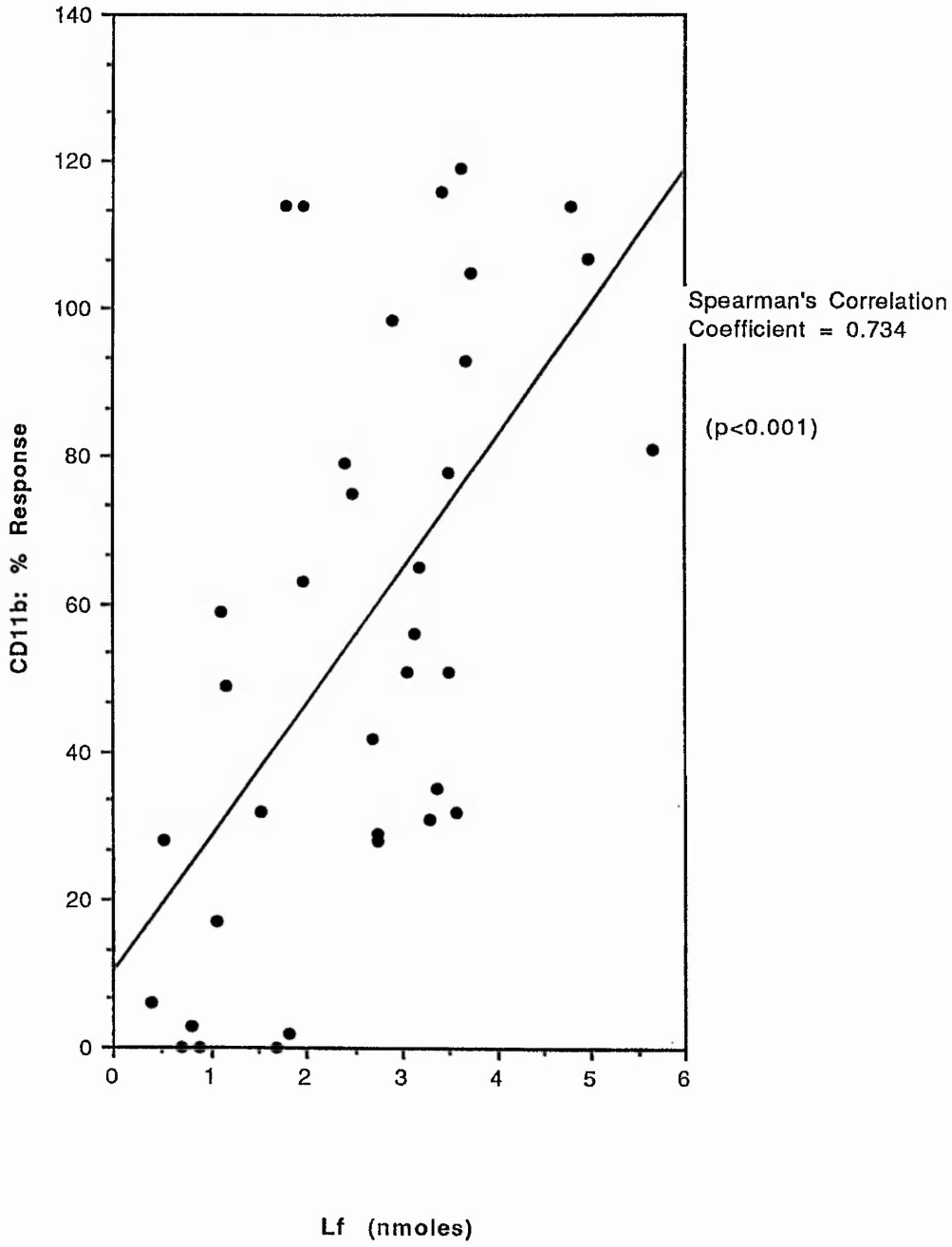

Figure 43: Correlation between CD11b and Lf release with increasing concentrations of TNF.
The results are from 7 different donor PMN.

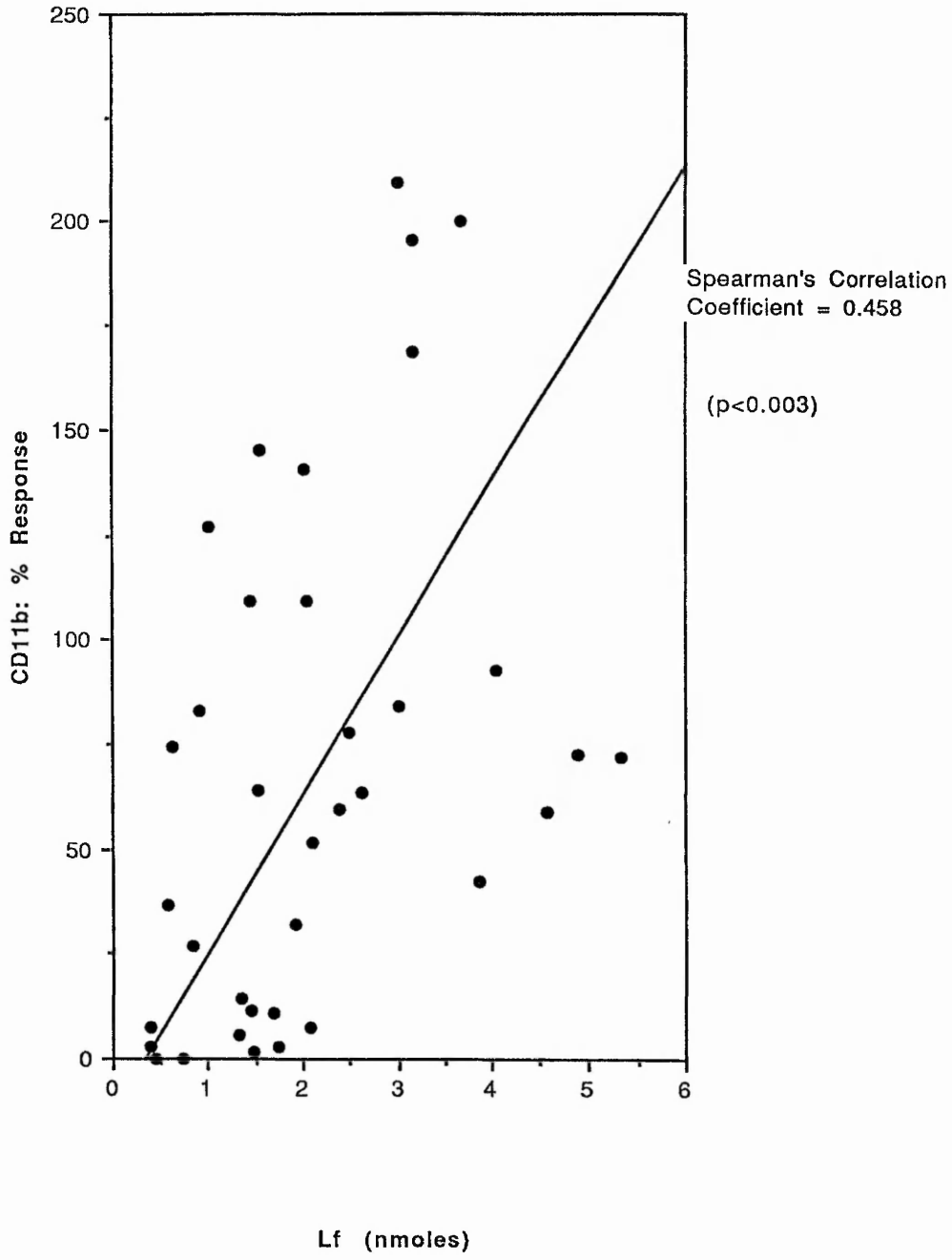

Figure 44: Correlation between CD11b expression and Lf release for PMN exposed to 10U ml^{-1} TNF at 37°C with constant agitation increasing incubation times.

The results are from experiments carried out on 8 individual donor PMN.

kinetics to increased CD11b expression and as with the dose response to TNF, there was a strong correlation ($p<0.003$) between increased expression of the adhesion molecule and secondary degranulation (figure 44).

4.1.3. Effect of Washing on CD11b Expression and Lactoferrin Release

As seen with TNF induced priming of respiratory burst activity, the increase in both adhesion protein expression and Lf release could not be reversed by washing the TNF from the reaction mixture. Figure 45 shows that almost identical results for both CD11b expression and Lf release were obtained when the cells were pre-incubated with TNF for 15 minutes at $4^{o}C$ before washing and warming to $37^{o}C$, or when they were incubated just at $37^{o}C$ prior to determination of receptor expression or Lf release.

4.1.4. Superoxide Anion Production

The ability of these same donor PMN to generate superoxide anions in response to TNF was also investigated. Figure 46 confirms the results from the previous chapter, where high concentrations of TNF (100 to 1000U ml^{-1}) elicited a variable response. Some, but not all, donor PMN were induced to generate superoxide anions. At 10U ml^{-1} TNF, no respiratory burst activity from any of the donor PMN was detected. However,

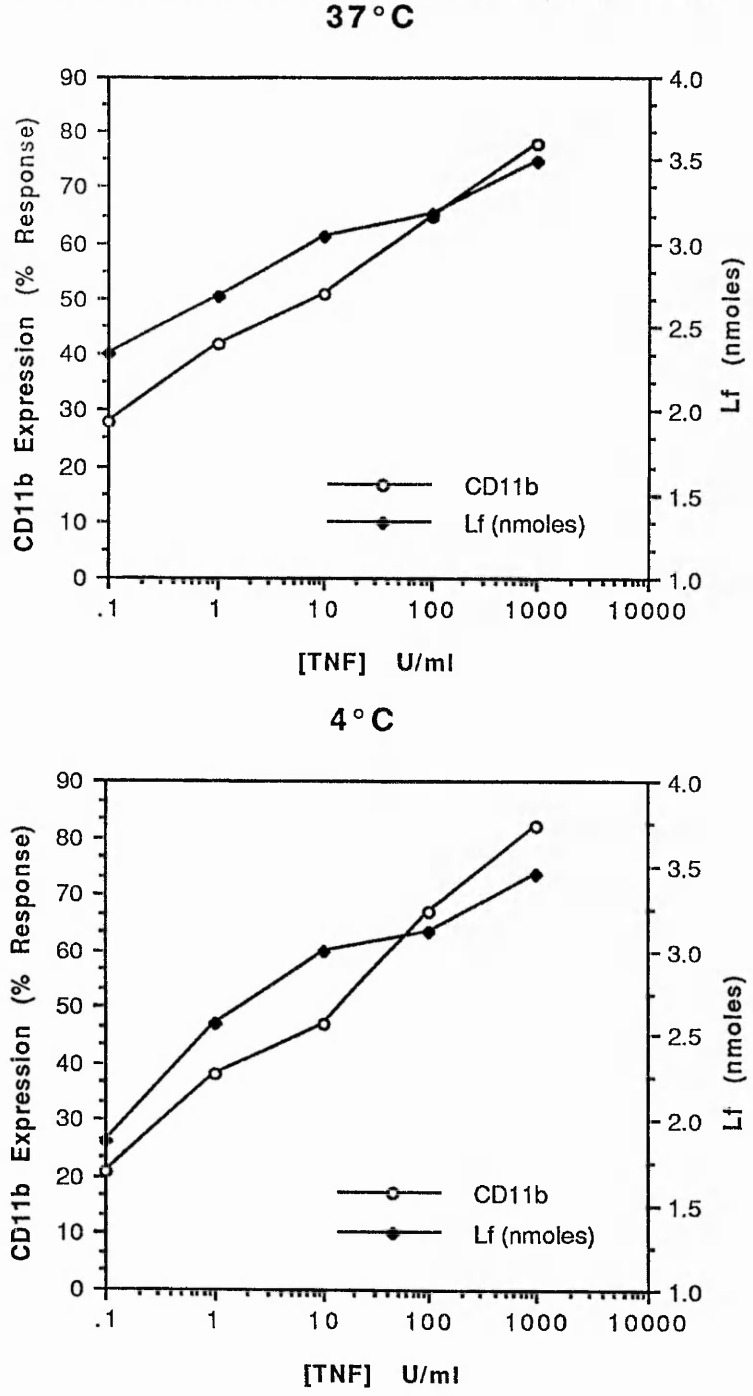

Figure 45: The effect of different preincubation conditions on the expression of CD11b and Lf release in response to increasing concentrations of TNF. The top panel shows the results of a 37°C pre-incubation for 15 minutes with TNF, while the lower panel shows the effect of cells preincubated with TNF for 15 minutes at 4°C followed by washing and warming to 37°C. The results are from one PMN donor representative of four similar experiments.

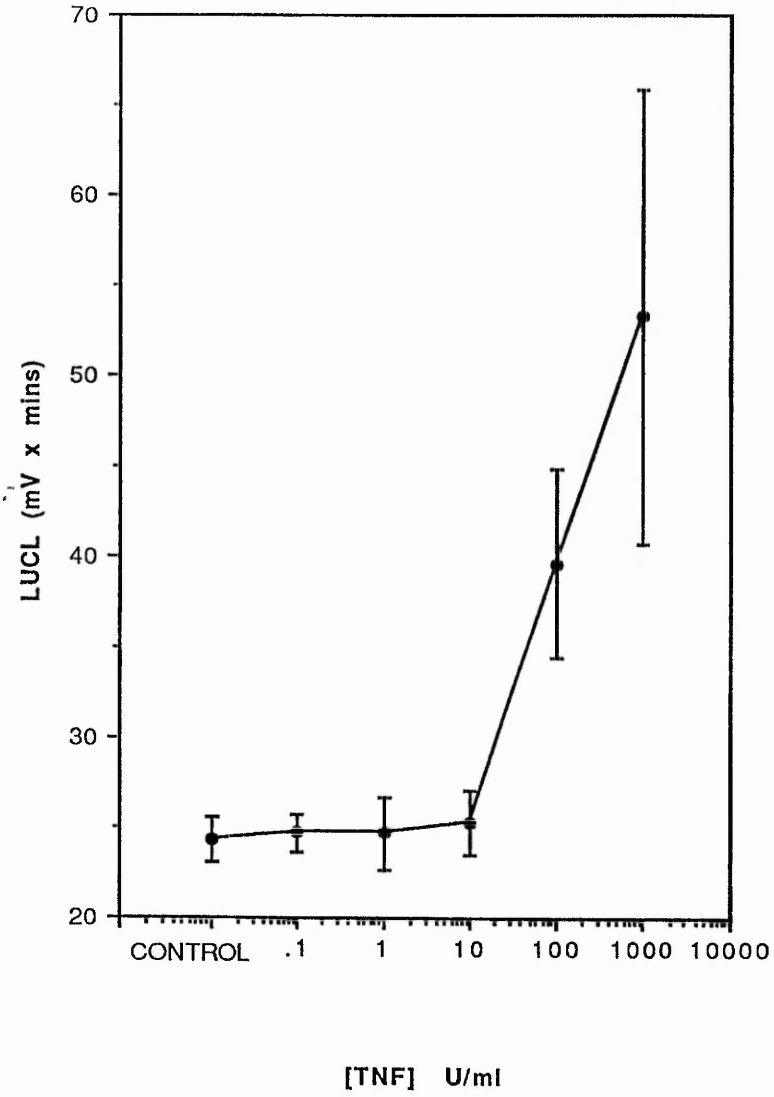

Figure 46: The effect of increasing concentrations of TNF on PMN LUCL.

The results represent the means of 7 separate experiments ± SEM

this concentration of TNF was capable of inducing a significant increase in both CD11b expression and secondary degranulation in these same cells.

4.1.5. CD11a, CD11c and CD18 Expression

As CD11b represents only one of the alpha sub-units of this integrin family we also investigated the effects of 10U ml^{-1} TNF on expression of the beta sub-unit (CD18) and the CD11a and CD11c alpha subunits. The PMN were incubated for 15 minutes at 37°C with 10U ml^{-1} of TNF, figure 47 shows that TNF induced a significant increase in CD18 expression, 161.46% $\pm$ 34.09% ($p<0.001$). CD11a and CD11c showed slight, but not significant, increases in expression compared with controls. The expression of CD11b and CD18 in response to 10U ml^{-1} TNF was of the same magnitude.

4.2. Effect of FMLP on CD11b Expression and Lf Release

The formyl peptide FMLP has been shown to exert chemotactic activity at low concentrations, while stimulating the neutrophil respiratory burst at higher concentrations (Gallin 1980). We have already shown (chapter 3) that a concentration of FMLP at 10^{-5} to 10^{-6}M optimally stimulates superoxide anion production under the assay conditions used. Chemotaxis involves polarizing the cells with receptors capping to one end of the neutrophil. Therefore, to determine whether the effect of

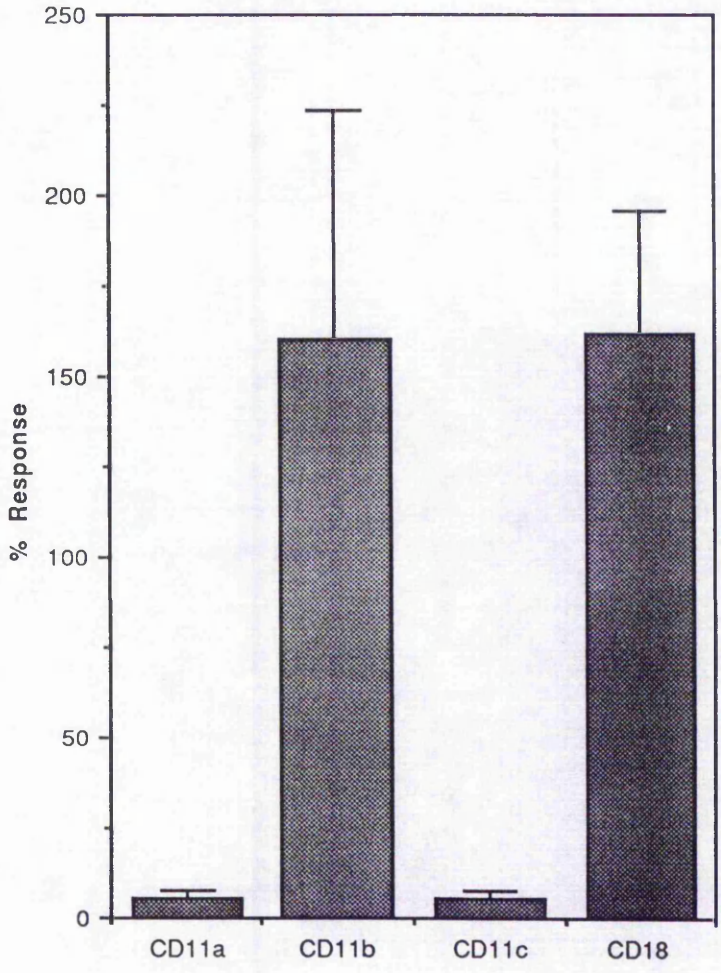

Figure 47: The effect of 10U ml^{-1} TNF on the expression of CD11a,b,c and CD18. The % response for each was calculated as the shift in mean fluorescence of TNF treated cells over control PMN incubated in control buffer.

The results represent the means of 5 separate experiments ± SEM, carried out on different individual donor PMN.

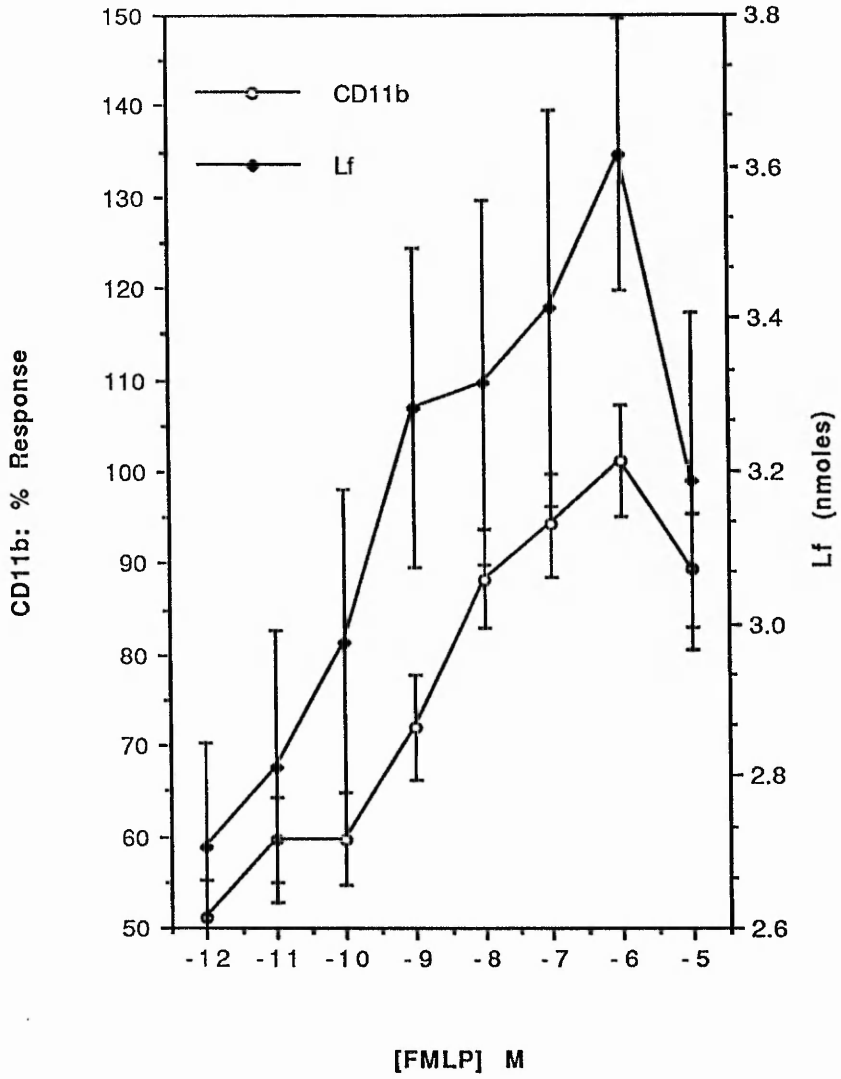

Figure 48: The effect of increasing concentrations of FMLP on PMN CD11b expression and Lf release after a 30 minutes incubation at 37°C.

The results represent the means of 4 separate experiments ± SEM, carried out on different individual donor PMN.

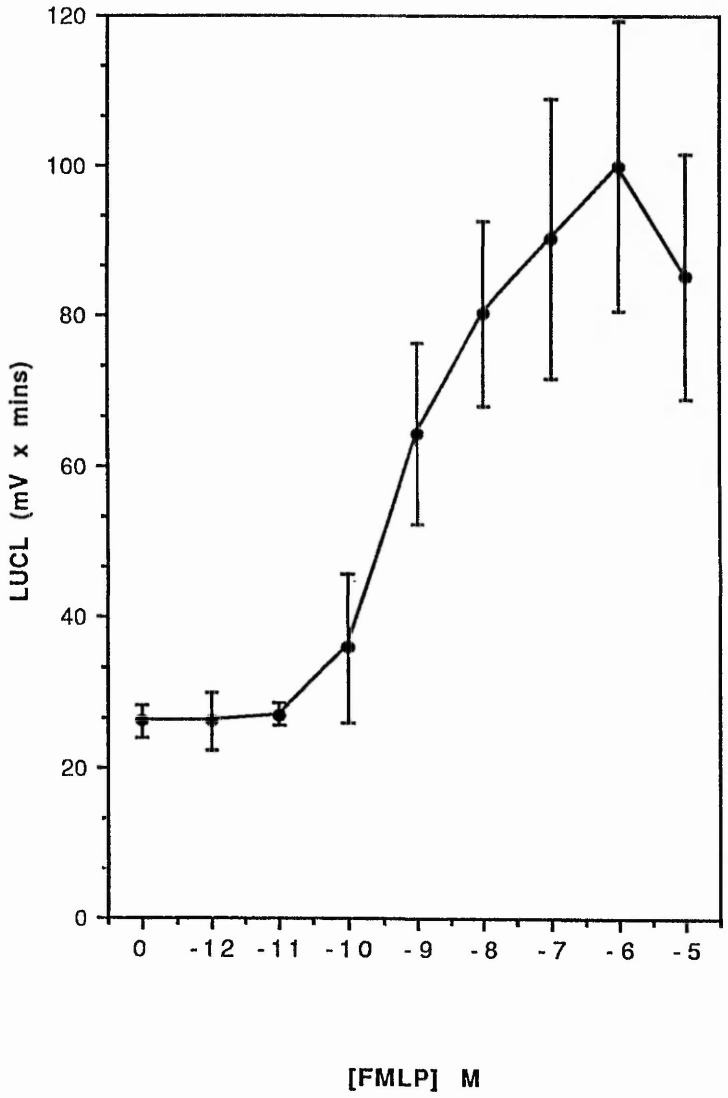

Figure 49: The effect of increasing concentrations of FMLP on PMN LUCL.

The results represent the means of 4 separate experiments ± SEM.

chemotactic concentrations of FMLP is to increase receptor expression from secondary granules, CD11b expression and Lf release were investigated.

Figure 48 shows that concentrations of FMLP as low as 10^{-11}M were capable of significantly increasing ($p<0.05$) CD11b expression. As with TNF there was also a concomitant release of Lf. Superoxide anion production from these cells did not occur at these low concentrations (figure 49).

4.3. Effect of TNF on Intracellular Calcium Ions

TNF has been reported to induce increases in intracellular Ca^{2+} only when PMN are stimulated under adherent conditions (Richter et al 1988). As both CD11b expression and Lf release could be measured from cells in suspension intracellular Ca^{2+} was determined in the presence of this cytokine using fura-2-am fluorimetry. Figure 50 shows that no changes in intracellular Ca^{2+} were seen using TNF at 1000U ml^{-1}. The responsiveness of cells were confirmed using 10^{-6}M FMLP as a control.

4.4. Discussion

In addition to the priming of PMN respiratory burst activity TNF has been shown to exert a number of other effects on neutrophil function, including increasing the phagocytic activity of cells (Shalaby et al 1986). Increases in

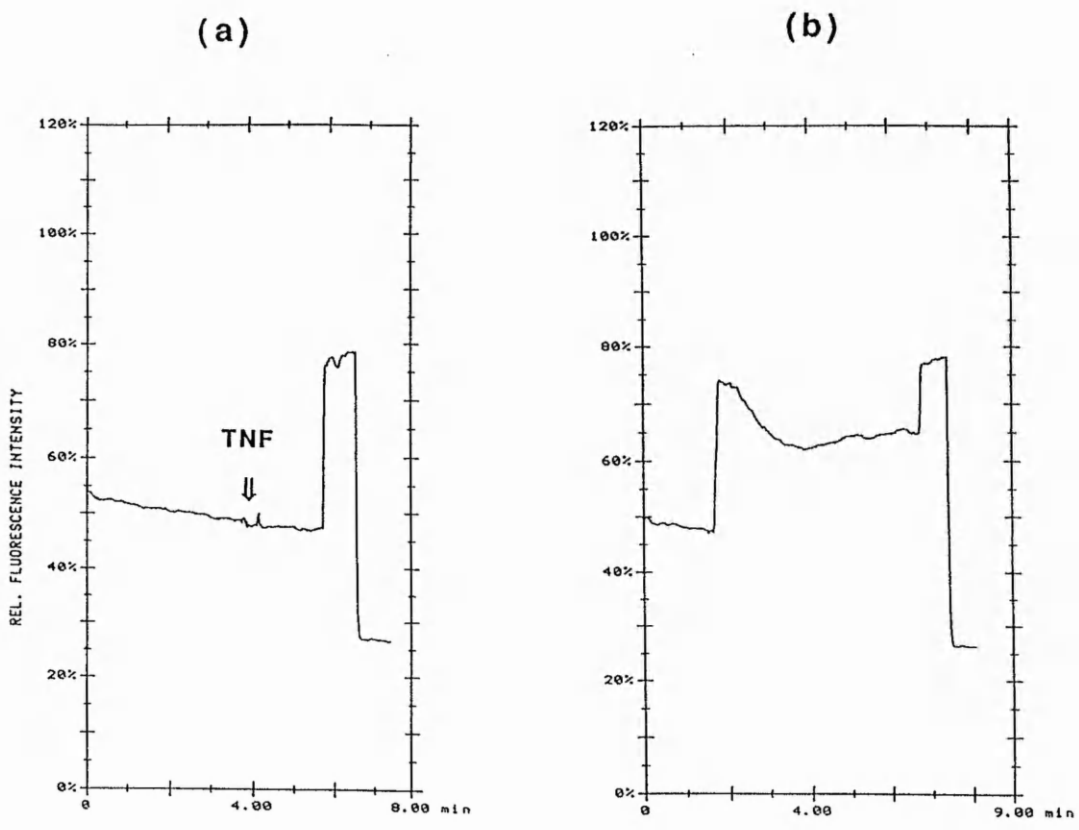

Figure 50: A comparison between the effect of 1000U ml^{-1} TNF and 10^{-6}M FMLP on intracellular calcium ions. Panel (a) shows the effect of TNF and panel (b) FMLP

The results are from one experiment representative of four similar ones carried out on different donor PMN.

phagocytosis may be due to increased expression of both FcR receptors and C3bi receptors. The latter, in addition to binding opsonised bacteria and particles is also involved in adhesion of PMN to endothelial cells. The ability of TNF (10U ml^{-1}) to irreversibly induce increased adhesion molecule expression, at concentrations insufficient for the induction of respiratory burst activity, suggests that TNF can modulate different PMN responses, depending on the concentration of the agonist.

The formyl peptide FMLP showed a similar pattern of activity, with so called chemotactic concentrations of FMLP ($<10^{-8}M$) inducing increased adhesion protein expression and Lf release, while higher concentrations ($>10^{-8}M$) stimulated superoxide anion production. Other investigators have reported the use of cytochalasin b to induce maximal/measurable secondary degranulation in response to FMLP stimulation (Petrequin et al 1987). However the data presented here shows that FMLP was capable of stimulating Lf release (at detectable levels) from cells in suspension in the absence of cytochalasin b. The difference between these results and those reported by others are difficult to explain, but may simply be a result of the techniques used to measure secondary degranulation. The use of a sensitive ELISA allowed for the determination of Lf down to a concentration of 5pmoles.

Adherent PMN have been reported to release significantly more Lf than those held in suspension (Schleiffenbaum and Fehr 1990). Under adherent conditions PMN were found to release 65% more of their transcobalamine (a secondary granule protein) content, in response to TNF while cells in suspension showed limited degranulation. Our findings would suggest that TNF is capable of inducing the increased expression of adhesion molecules and inducing secondary degranulation. The amount of Lf released in response to TNF was approximately 25% of the total cellular content per 10^6 PMN ml^{-1}. Due to the differences in the methods employed to measure secondary degranulation it is difficult to compare these figures with those of other investigators. Although attempts were made to maintain PMN in suspension through constant agitation on a rotating wheel at 37°C, the possibility that some cells were adhering cannot be excluded.

Ginis and Tauber (1990) demonstrated that adhesion as a priming event did not necessarily initiate cell effector function. Since neutrophils adhered to plastic, generated increased superoxide anion production by a PKC dependent mechanism. While cells adhered to fibronectin did not show any increased activity. Cells adherent to both surfaces did show increased responsiveness when exposed to FMLP when compared to neutrophils held in suspension. These differences may reflect activation and/or mobilization of distinct receptors depending on the experimental conditions.

PMN held in suspension did not show an increase in intracellular calcium concentration in response to 1000U ml^{-1} TNF. Other studies have shown that TNF is capable of inducing calcium transients in adherent PMN together with increased secondary degranulation (Richter et al 1989). Although we were unable to demonstrate any increase in intracellular Ca^{2+} in these PMN, Lf was released in a dose dependent fashion in response to TNF in cells being constantly agitated. This would suggest that the effect of TNF on secondary degranulation acts independently of Ca^{2+}. Again these differences may result from the techniques used, and the possibility of some cells adhering during the incubation cannot be excluded. Wright et al (1991) working with PMN in suspension showed that, in addition to upregulating CD14 expression, TNF was capable of increasing the cell surface expression of CR3 (CD11b/CD18) with cells maintained in suspension, thus confirming our data.

These two agonists (TNF and FMLP) were capable of inducing similar responses in the cells with respect to CD11b expression and Lf release. Low concentrations of FMLP may prime cells through their ability to induce secondary degranulation. If FMLP receptors are stored in the secondary granules (Fletcher and Gallin 1983) then low concentrations of FMLP may cause the required increased expression of receptors and bring about enhancement of respiratory burst activity when exposed to higher concentrations of the agonist or other stimuli. However, while FMLP has been shown to exert its effects through the

induction of secondary messengers in the cell including increased calcium, IP_3 and DAG, TNF is not reported to act via any of these secondary messenger systems. The intracellular messenger signals that mediate the PMN response after binding of TNF to its receptor on the cell surface are unknown. However the bioactivity of TNF shows that it exerts profound effects upon PMN function. If TNF only induces calcium transients in adherent PMN this would allow for PMN to respond maximally at the site of infection or injury, and would prevent circulating PMN from producing toxic oxygen radicals if exposed to TNF in the circulation. However, if adherent PMN do release toxic oxygen radicals when attached to the endothelium, this could result in tissue damage. Therefore while it would be desirable for PMN in the tissues to show enhanced and prolonged respiratory burst activity (Nathan 1987) and also increased phagocytosis and killing (Herrmann et al 1990), this effect at the endothelial surface could be damaging.

The effect of inflammatory cytokines, such as TNF and IL-1, on the induction of increased expression of adhesion molecules on endothelial cells has been well documented (Pober et al 1986, Bevilacqua et al 1985). However, in order for leucocytes such as neutrophils to adhere and migrate into the tissues, these cells must also be induced to express receptors for the ligands on endothelial cells. The adherence of stimulated neutrophils is dependent upon both CD11a/CD18 (Pohlman et al 1986) and CD11b/CD18 (Smith et al 1988, Zimmerman et al 1988).

Unstimulated neutrophils have also been shown to adhere to endothelial cells, and the mechanism for this is reported to differ from that of stimulated neutrophils. Unstimulated neutrophil adherence is thought to depend upon ICAM-1 interacting with CD11a/CD18. As we could detect no increase in the expression of CD11a it is possible that this adhesion molecule is maximally expressed at the cell surface. This expression of CD11a would therefore allow the cells to take part in neutrophil-endothelial binding in the absence of neutrophil stimulation. However, there is evidence to suggest that the action of warming cells from 4°C to 37°C is sufficient for the induction of adhesion molecule expression (Lacal et al 1988). They demonstrated that warming neutrophils results in both secondary and tertiary degranulation which would thus increase the expression of adhesion molecules and other receptors, so that cells manipulated in this way cannot be thought of as resting. Our data also showed that warming the cells induced increased expression of CD11b and CD18, although no increase in either CD11a or CD11c was observed, it is possible that these latter molecules are indeed maximally expressed on circulating peripheral blood PMN.

The adherence of PMN to endothelium appears to be a complicated process, in addition to the role of members of the integrin family already mentioned, another cell surface receptor is regulated by cytokines and chemotactic peptides, namely the LAM-1 molecule. This adhesion protein contains a lectin-like

domain which binds to a specific glycoconjugate on target cells (Tedder et al 1989). The regulation of this molecule on neutrophils in response to TNF differs from CD11b/CD18 expression. Spertini et al (1991) showed that TNF caused increased expression of CD11/CD18 while at the same time induced shedding of LAM-1 molecules. LAM-1 has been suggested to be principally involved in the initial interaction between leucocytes and endothelium, whereas other adhesion molecules appear to have a role in subsequent adhesion and diapedesis into tissues following LAM-1 shedding (Griffin et al 1990). As TNF affects endothelial expression of adhesion proteins and those on the surface of leucocytes, it suggests that this inflammatory cytokine is important in modulating the migration of cells into tissues.

These data would suggest that low concentrations of TNF increase the ability of PMN to adhere to endothelial cells. The fact that the concentrations of TNF required to induce increased adhesiveness do not elicit a respiratory burst would prevent any damage to the endothelium from toxic oxygen radicals. The CD11b/CD18 complex has been shown to mediate activated PMN binding to endothelium (Doerschuck et al 1990) and the adherence dependent stimulation of PMN H_2O_2 production (Nathan 1989). This complex has also been associated with the binding of LPS (Wright et al 1989), a putative LPS binding site has been suggested. In addition, CD11b/CD18 forms the C3bi receptor which binds opsonised particles. TNF may therefore

exert its many effects on neutrophil function through regulating the expression of this and other receptors stored within the intracellular pool.

We have shown that the increased expression of CD11b and CD18 in response to TNF is associated with secondary degranulation; there was a strong correlation between increased expression and the amount of Lf released from PMN held in suspension. This contradicts the data of Richter et al (1989) suggesting that adherence was required before secondary degranulation occurred in response to TNF stimulation. Although the CD11b/CD18 complex (MAC-1) has been associated with the membranes of the gelatinase containing tertiary granules (Lacal et al 1988), immunocytochemical studies have demonstrated co-localisation of CD11b/CD18 with Lf (Bainton et al 1987).

The reason for Lf release concomitant with the upregulation of adhesion molecules and other receptors is difficult to explain. It may be that Lf is involved in increasing the ability of PMN to adhere to endothelium. Lf is a highly charged molecule and its release may act to negate the electrostatic repulsion between PMN and endothelium and allow receptor-ligand interactions to take place. However, Lf has also been shown to inhibit the production of cytokines and may therefore be acting as part of a negative feedback loop.

CHAPTER FIVE: RESULTS

5.1. Effect of Lactoferrin on TNF secretion

The iron-binding protein Lf, secreted from neutrophils, has been shown to inhibit the production of colony stimulating activity (Broxmeyer et al 1978), and acts as a negative feedback mechanism of myelopoeisis (Broxmeyer et al 1980). This inhibitory activity is a result of inhibiting GM-CSF production (Broxmeyer and Platzer 1984) and appears to be dependent upon the degree of iron saturation (Broxmeyer et al 1978). Slater and Fletcher (1986) showed that 50% Fe-saturated Lf inhibited 3-way MLC proliferation to the same degree as Lf released from lysed PMN. In accordance with these findings increasing concentrations of 50% Fe-saturated Lf were incubated with 1ug ml^{-1} LPS stimulated MNC. After 5 hours of culture the MNCM were assayed for immunoreactive TNF by ELISA. Figure 51 shows the effect of Lf on the amount of TNF released and demonstrates that concentrations of Lf 10^{-9}M and 10^{-10}M were the most effective at inhibiting the secretion of this cytokine into the culture medium, with 10^{-9}M inhibiting by 42.78% $\pm$ 4.71 and 10^{-10}M inhibiting by 41.49% $\pm$ 5.28% (n=8 $\pm$ SEM).

It has been suggested that TNF is at least partially responsible for stimulating the secretion of IL-1 from MNC (Dinarello et al 1986), therefore IL-1 was also measured in these cultures. As figure 51 shows, Lf present in the culture

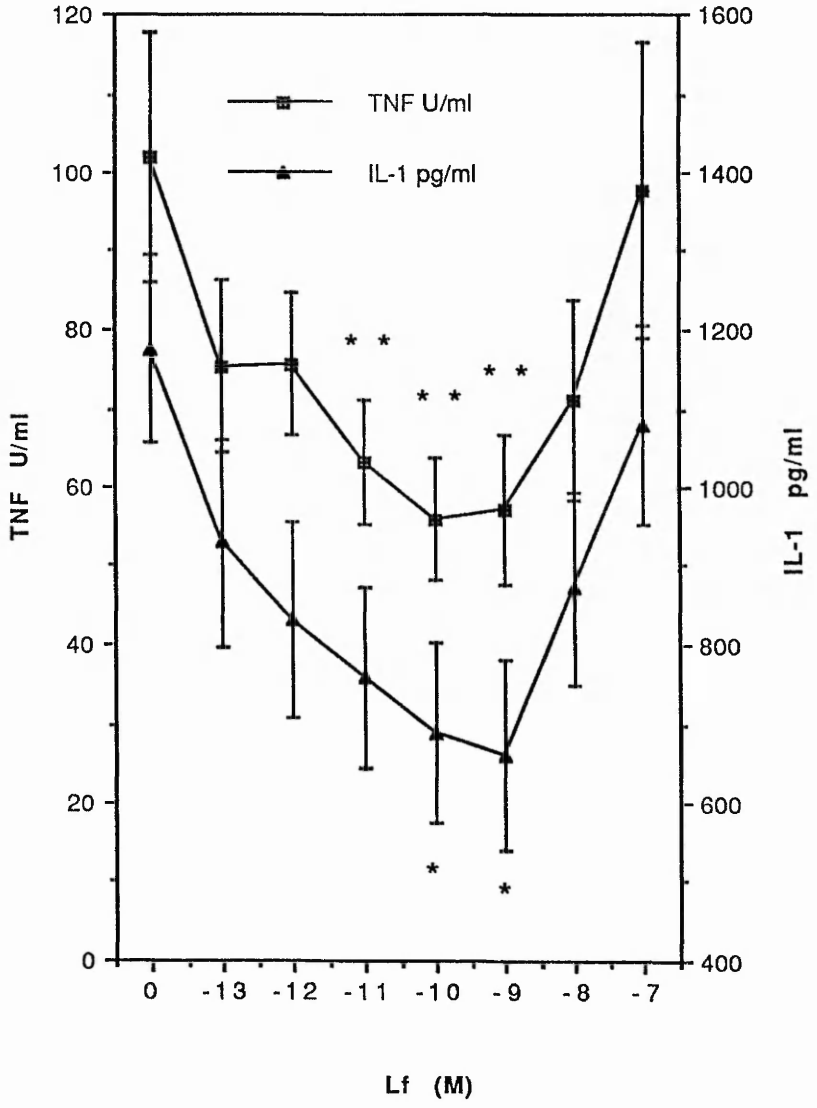

Figure 51: The effect of increasing concentrations of 50% Fe-saturated Lf on the production of TNF and IL-1 from MNC stimulated for 5 hours in the presence of 1ug ml^{-1} LPS.
The results represent the means of 8 separate experiments $\pm$ SEM, and are significant at * $p<0.05$ and ** $p<0.025$, when compared with control cells incubated in the absence of Lf.

medium also exerted a dose dependent inhibition of IL-1 production, as measured by ELISA. The pattern of IL-1 inhibition was similar to that seen with TNF, 10^{-9}M Lf inhibited IL-1 secretion by 45.29% ± 7.49% and 10^{-10}M Lf inhibiting by 40.59% ± 3.85% (n=8 ± SEM).

5.1.1. Effect of Anti-Lf

Further experiments were carried out using 50%-Fe-saturated Lf at a concentration of 10^{-10}M to determine whether the inhibitory effect of Lf was a direct effect on TNF and IL-1 production. Firstly, MNC stimulated with LPS were incubated in the presence and absence of 10^{-10}M Lf. In addition a polyclonal anti-Lf antibody was added as required, while pre-immune rabbit serum was used as a control. Again supernatants were collected after 5 hours in culture and the amount of immunoreactive TNF assayed. As figure 52 shows the presence of Lf resulted in a 39.15% ± 7.98% (n=10 ± SEM, p=0.032) reduction in the amount of TNF assayed in the medium compared with controls. IL-1 production was reduced from 1322pg ml^{-1} ± 147 to 755pg ml^{-1} ± 98 (down 42.89%: n=7 ± SEM, p=0.032) in the presence of 10^{-10}M Lf. Incubation with the anti-Lf antibody returned the amount of secreted TNF and IL-1 to that of controls. Control wells incubated in the presence of pre-immune rabbit serum did not affect the inhibitory activity of Lf. None of the antibody treatments had any direct stimulatory effect on the production of either cytokine suggesting that the amount of endotoxin

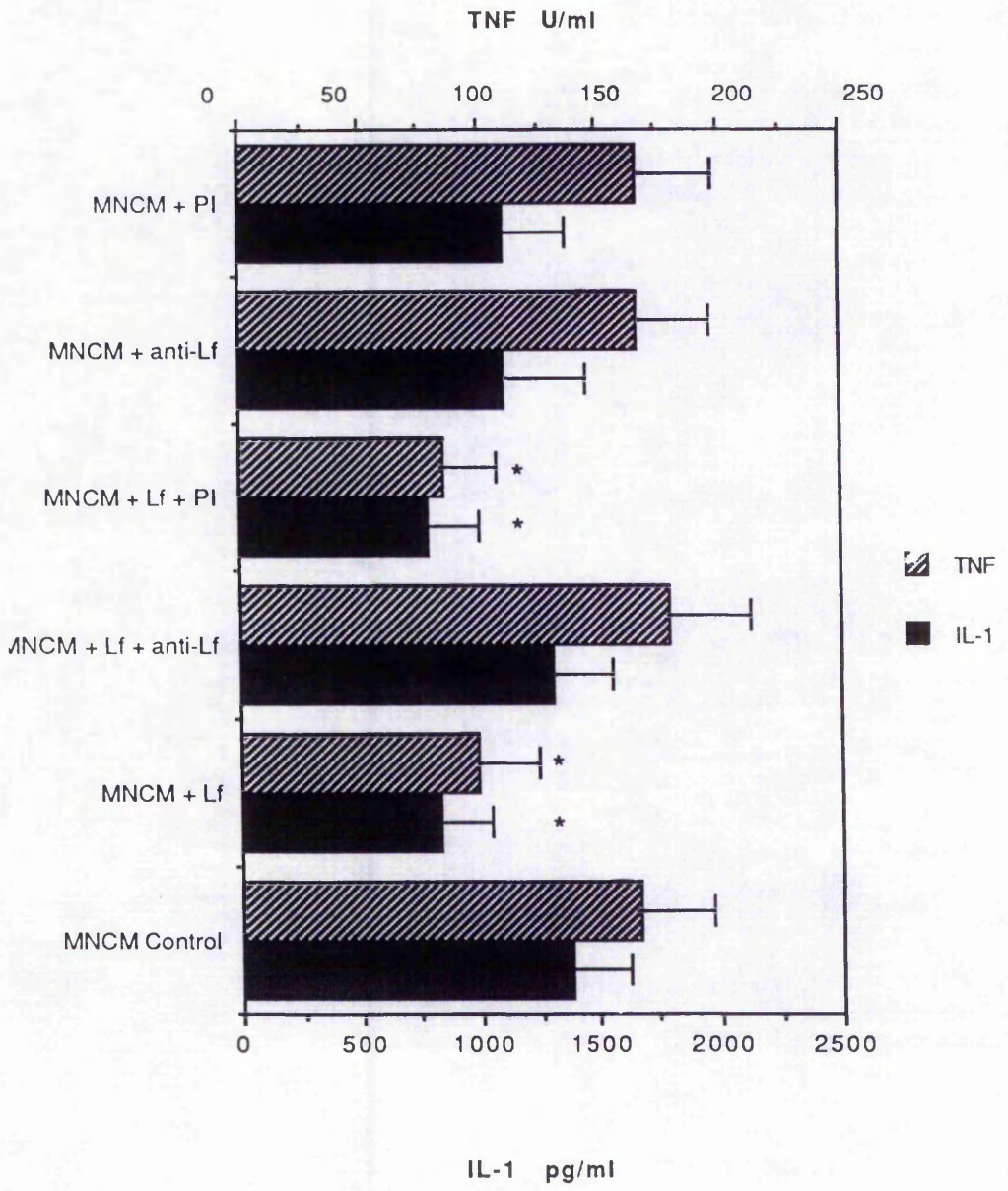

Figure 52: The effect of a rabbit polyclonal antibody to human Lf on the inhibitory action of 10^{-10}M Lf with respect to TNF (▨) and IL-1 (●). PI represents preimmune serum.
The results represent the means ± SEM for 10 and 7 separate experiments respectively; * $p<0.05$.

in these preparations was very low. This was confirmed using the LAL assay which showed that all preparations contained less than 20pg ml^{-1} endotoxin.

5.2. Effect of Lf in Mixed Lymphocyte Cultures

As demonstrated previously incubating MNC in the presence of LPS stimulates these cells to secrete inflammatory cytokines. In addition to using LPS which is a stimulus representative of an infectious agent it is possible to stimulate cells using an immunological stimulus. This can be achieved using mixed lymphocyte cultures (MLC); incubating cells from three separate donors initiates an immune response through the recognition of "foreign" antigens on the surface of the different donor leucocytes. It has previously been shown that Lf inhibits the production of a growth factor in MLC (Slater and Fletcher 1986). Since, cell proliferation in MLC is considered to be a T cell phenomenon driven by IL-2 (Ilonen and Kartunen 1984), it was thought that this may represent the factor whose release was inhibited by Lf. Slater (1988) confirmed that this factor was indeed IL-2 and that the release of this lymphokine is inhibited in the MLC in the presence of 50% Fe-saturated Lf. The production of IL-2 from T lymphocytes is dependent upon IL-1 secretion from monocytes (Durum et al 1987, Dinarello et al 1986). It is therefore possible that the inhibition of IL-2 secretion may be an indirect effect of Lf acting via the modulation of IL-1 and perhaps TNF release from monocytes.

Therefore MLC were generated and cell proliferation was measured using tritiated thymidine uptake. Duplicate cultures were set up for the measurement of secreted IL-1 and TNF at each time point. Cultures were also set up in the presence of 10^{-10}M Lf, with the addition of anti-Lf or pre-immune controls

Figure 53 shows that the proliferation of the MLC was inhibited in the presence of 10^{-10}M Lf, this inhibition could be reversed by incubating MLC with an anti-Lf polyclonal antibody. Control experiments with preimmune control serum showed that Lf was again capable of inhibiting the proliferation of the cultures. Increasing incubation times resulted in a more pronounced inhibitory activity of Lf, inhibition was significant at 24, 48 and 72 hours, with maximal inhibition occurring at 72 hours.

Using an ELISA technique for the measurement of IL-1 it was possible to show a reduction of this cytokine in MLC supernatants in the presence of Lf (figure 54, panel a). The effect of Lf on IL-1 production was detected as early as 5 hours (although not statistically significant), with maximal inhibitory activity observed at 72 hours. The addition of the anti-Lf antibody neutralized the inhibitory activity of the Lf with respect to IL-1 production.

As TNF is at least partially responsible for stimulating the production of IL-1 from monocytes (Dinarello et al 1986), the effect of Lf on TNF production was also investigated. Again the

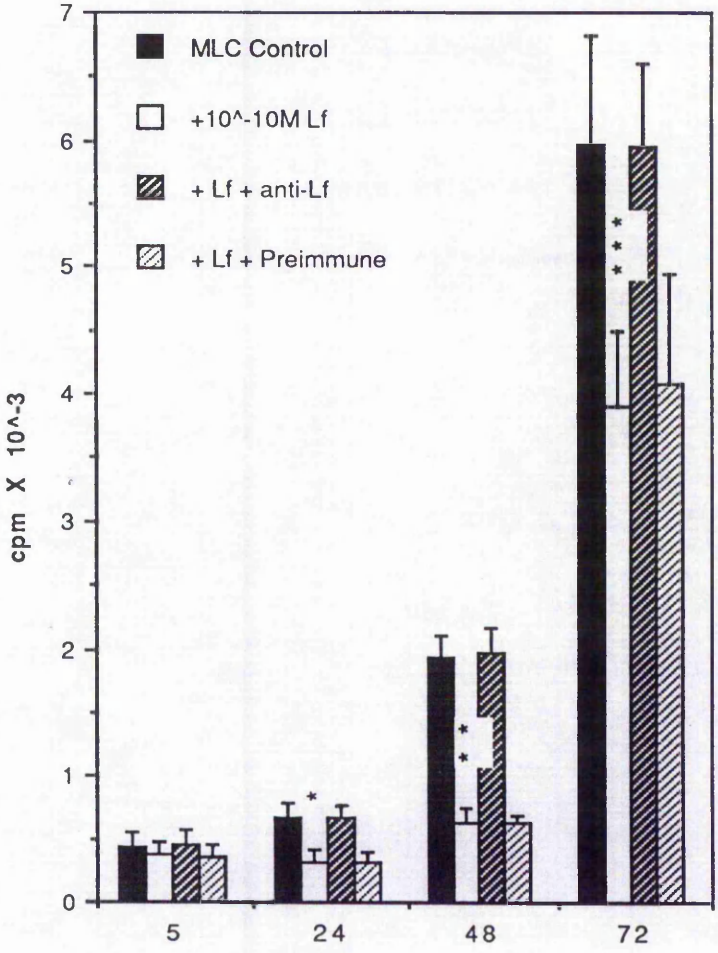

Figure 53: The effect of 10^{-10}M Lf on proliferation of the MLC, as determined by tritiated thymidine uptake. MLC were incubated for the times shown in complete medium (●), complete medium plus Lf (○), plus Lf plus anti-Lf (▨) and plus Lf plus pre-immune serum (▨).

The results represent the means of 5 separate experiments ± SEM, and were significant at * $p<0.05$, ** $p<0.025$ and *** $p<0.001$.

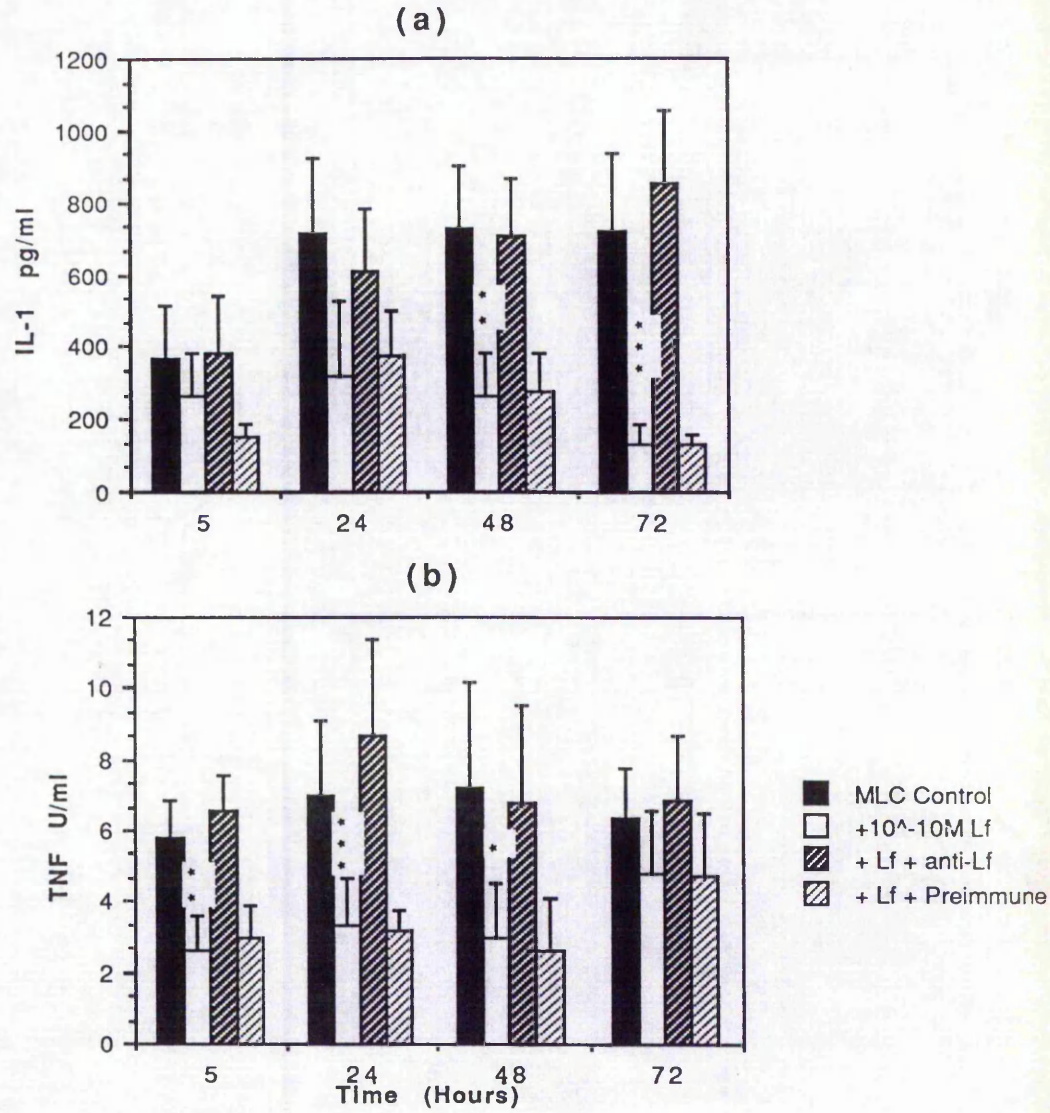

Figure 54: The effect of 10^{-10}M Lf on IL-1 (panel a) and TNF (panel b) secretion from MLC incubated for increasing times. The results shown are for complete medium (●), complete medium plus Lf (○), Lf plus anti-Lf (▨) and Lf plus pre-immune serum (▨).

The results represent the means of 5 separate experiments ± SEM, and results were significant at * $p<0.05$, ** $p<0.025$ and *** $p<0.001$.

presence of 10^{-10}M Lf inhibited TNF production (figure 54, panel b). While abrogating the effect of Lf the anti-Lf did not have any direct effect on proliferation of the MLC or affected TNF secretion, nor did the pre-immune serum. Lf exerted a dose dependent inhibition of immunoreactive TNF assayed in the conditioned media from MLC as early as 5 hours (figure 55). However whilst significant inhibition of TNF production occurred at all the time points, significant inhibition of MLC proliferation was only observed after 24, 48 and 72 hours in culture.

As ELISA techniques were used to determine the concentration of Lf assayed in the conditioned media from both LPS stimulated MNC and MLC, it was important to confirm that Lf in the culture medium was not interfering with antigen-antibody binding in the assay technique. Therefore control culture medium containing 10^{-10}M Lf was added to the various concentrations of TNF and IL-1 standards. As figure 56 shows Lf did not affect either of the ELISA methods with respect to the assay of the two cytokines.

5.3. Discussion

These data would suggest that Lf acts to inhibit the secretion of a number of inflammatory mediators from activated MNC. Broxmeyer and Platzer (1984) showed that the time point at which Lf is added to cultures is important for inducing the

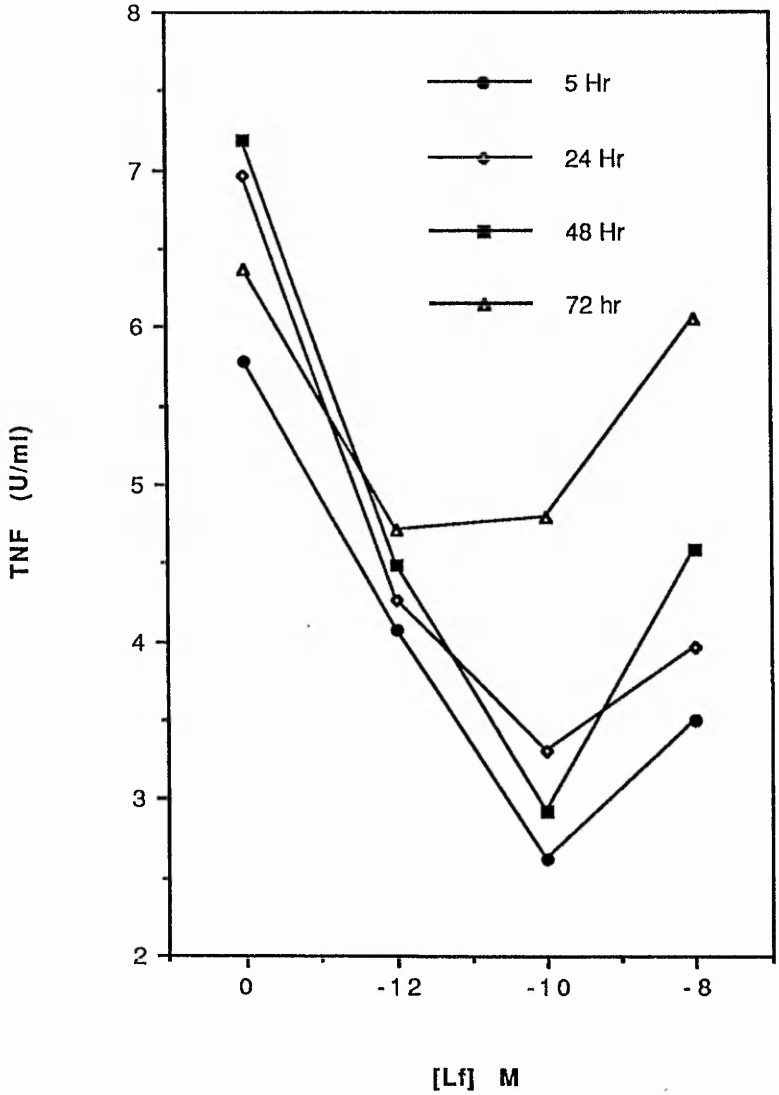

Figure 55: The effect of increasing concentrations of 50% Fe-saturated Lf on TNF secreted in MLC. At each of the time points shown the plates were spun down and the supernatant harvested, immunoreactive TNF was assayed by ELISA.
The results represent the means of 6 separate experiments.

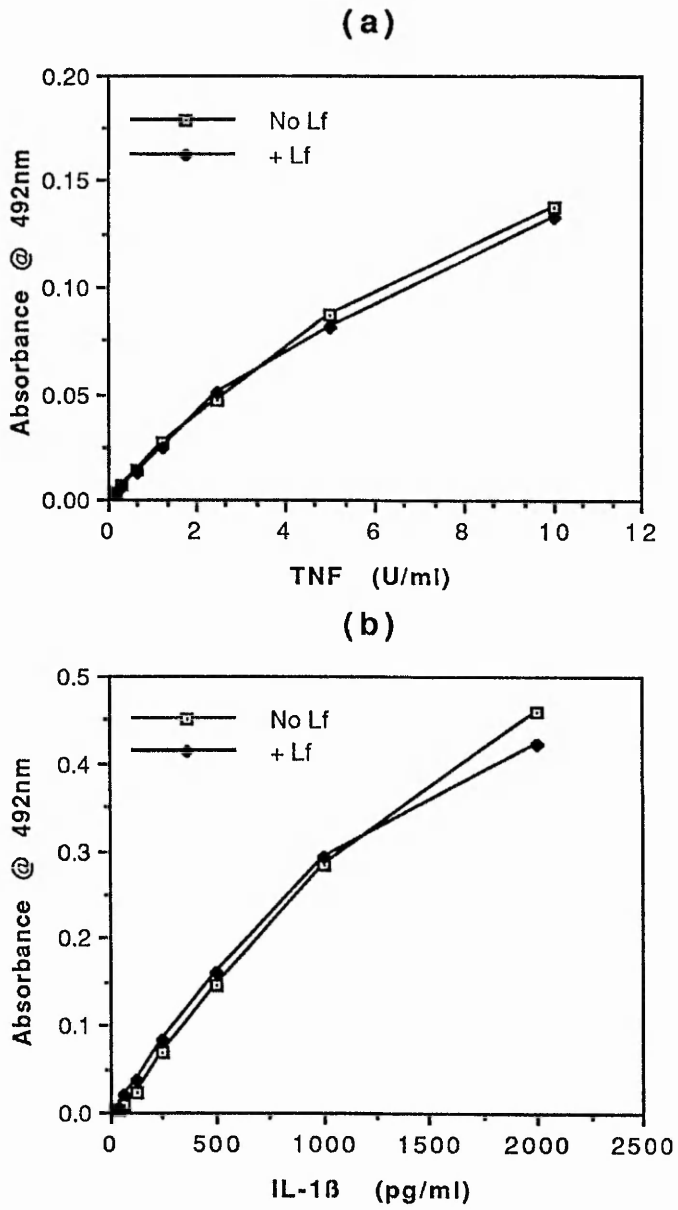

Figure 56: The effect of 10^{-10}M Lf on the ELISA standard curves for both TNF and IL-1.

The results are from one experiment representative of three .

inhibition of colony stimulating activity. Inhibition could only be observed when Lf was added at the beginning of the culture period. This supports the view that Lf in MLC is acting indirectly on IL-2 production from T cells by affecting monocyte function. It has been shown that Lf inhibits GM-CSF via the inhibition of IL-1 production from monocytes (Zucali et al 1989). The requirement for IL-1 in the production of IL-2 from activated T cells is well established (Durum et al 1984, Dinarello et al 1986). It has also been suggested that TNF can stimulate the production of IL-1, and that IL-1 production may be partially dependent upon the initial secretion of TNF (Dinarello et al 1986).

It would therefore appear that Lf may act to inhibit the production of IL-2 and CSFs through the inhibition of other cytokines secreted earlier in response to an inflammatory stimulus. In order to confirm this hypothesis we firstly studied the effect of this protein under conditions known to induce the secretion of IL-1 and TNF, namely LPS stimulated MNC. Under these conditions Lf was found to inhibit TNF and IL-1 release in a dose dependent fashion. However, human monocytes have also been shown to express an IL-1 inhibitor that functions as a receptor antagonist by binding to IL-1 receptors on T lymphocytes, synovial cells and chondrocytes (Arend et al 1990). It was therefore possible that Lf may have been acting to induce the synthesis of this or some other inhibitor. Previous work carried out by Slater (1988) showed that the

activity of IL-1 in the NOB-1/CTLL-2 assay was not affected by the presence of Lf. This would suggest that Lf was not acting via this mechanism but was somehow directly inhibiting cytokine production. PGE_2 was not responsible for the inhibition being observed in these MLC and MNC cultures as indomethacin was present in the culture medium. Indomethacin is a non-steroidal anti-inflammatory agent which acts to inhibit the production of PGE_2 from cells. To try and confirm the direct action of Lf on cytokine production all experiments with Lf were also carried out in the presence of a neutralising antibody. In all cases the inhibitory effects of Lf were completely abrogated by the addition of this antibody to the cultures. A rabbit pre-immune serum was used as a control and under these conditions the inhibitory effect of Lf was again observed.

The effect of increasing concentrations of Lf above 10^{-8}M resulted in stimulation of both TNF and IL-1 release. Bagby and Bennett (1982) showed that the inhibitory action of Lf on colony stimulating activity could be abrogated at high concentrations of the Fe-binding protein. This loss of inhibitory activity was associated with polymerization of Lf which in turn lead to stimulation.

LPS has been shown to induce the release of Lf from PMN *in vitro* in a dose dependent fashion, this effect of LPS could be augmented by the addition of MNC to the incubation mixture (Koivuranta-Vaara *et al* 1987). This effect only occurred as a

result of the addition of monocytes and the stimulatory factor was thought to be TNF. This confirms the ability of MNC products to cause the release of Lf from PMN, and the secretion of Lf at this time seems important in regulating subsequent cytokine production. Our data shows that Lf inhibits both TNF and IL-1 production, and the inhibition of IL-1 release may be an indirect effect of Lf acting via reducing TNF secretion.

The inhibitory effect of Lf was also observed in MLC suggesting that Lf exerted its effect independent of the type of stimulus for cytokine production. It was also possible that Lf may have been inhibiting the MLC by acting initially to reduce TNF secretion. TNF was detected in the supernatants as early as 5 hours after the initiation of the culture. This confirms the published work by Shalaby et al (1988) where TNF was detected in one-way MLC supernatants within one hour of initiating the cultures, with peak levels being achieved after 4 hours. With our MLC the reduced proliferation of cultures with Lf was blocked in the presence of an antibody to this Fe-binding protein, suggesting that it was indeed Lf that was exerting this inhibitory effect and not some other factor which may be present.

There was a significant reduction in immunoreactive levels of TNF in MLC with 10^{-10}M Lf at all the time points tested. Although IL-1 production was reduced at 5 hours the release of this cytokine was not significantly inhibited until 24 hours.

Slater (1988) showed that IL-2 production was significantly inhibited at 48 and 72 hours, which correlated with the significant inhibition by Lf of MLC proliferation. This confirms the importance of IL-2 as the key factor in the induction of cell proliferation in these cultures. However, as both TNF and IL-1 induce IL-2 secretion this implies that both of these MNC products are important in proliferation of the MLC. Overall since Lf exerts its inhibitory effects only when added at the beginning of the culture period, and since IL-2 secretion in these cultures occurs later than TNF and IL-1 it is possible that Lf acts on MNC to inhibit the production of these two cytokines which, in turn, leads to inhibition of IL-2 and reduced proliferation of the MLC.

TNF and IL-1 have been shown to enhance leucocyte adhesion to HUVEC (Pohlman et al 1986, Moser et al 1989), IL-1 has been shown to stimulate GM-CSF production from endothelial cells (Broudy et al 1987). Both TNF and IL-1 stimulate the production of a number of pro-inflammatory proteins from human fibroblasts (Beresini et al 1988) and also augment IL-6 secretion from endothelial cells (Jirik et al 1989). TNF has also been shown to enhance certain T cell responses (Scheurich et al 1987). In addition, TNF alone can induce chemotaxis of neutrophils and monocytes (Ming et al 1987) and enhance neutrophil killing capacity (Shalaby et al 1985). Therefore TNF and IL-1 appear to have profound effects on promoting the inflammatory response through the induction of a number of proteins that act as pro-

inflammatory agents. Interestingly, some of the molecules whose production is stimulated by TNF and IL-1 can also act as negative regulators of inflammation. IL-6 has been shown *in vitro* to inhibit LPS induced TNF production from monocytes (Aderka *et al* 1989). TNF is also capable of inducing PGE_2 from a number of cells including synovial cells and dermal fibroblasts (Dayer *et al* 1985), and PGE_2 inhibits cytokine production. Both PGE_2 and Lf have been shown to regulate macrophage and granulocyte proliferation (Pelus *et al* 1979). Therefore it is not unreasonable for pro-inflammatory mediators to induce the production of negative regulators at the same time. Lf receptors at 1.6×10^6 per cell have been demonstrated on monocytes (Birgens *et al* 1983), and Lf appears to be able to bind to these cells in a saturable and reversible manner. The inhibition of the release of TNF and IL-1 by Lf secreted from neutrophils at an inflammatory site, could potentially lead to a reduction in a number of related pro-inflammatory phenomena. This inhibitory feedback mechanism would prevent excessive neutrophil recruitment and activation, which if allowed to continue unchecked could be potentially harmful.

The demonstration that Lf inhibits the release of TNF and IL-1 could link many of its various reported functions. The consequent inhibition of IL-2 production may explain the inhibitory effects of Lf on natural killer cell activation (Nishiya and Horwitz 1982). The inhibition of antibody formation could also be explained by the effects of Lf on IL-1,

since B cell activation can be stimulated either directly by IL-1, or indirectly via T cells. Considered together with the effects of IL-1 and TNF on GM-CSF production by fibroblasts and endothelial cells, Lf has the potential to function as a potent regulator of inflammation and immunity.

Lf acts to control MNC responses during inflammation by reducing cytokine production, this would also have effects upon PMN whose responses are upregulated by inflammatory mediators. In some inflammatory conditions PMN activation can cause tissue damage and therefore drugs which inhibit the actions of these cells can be useful in preventing exacerbation of the inflammatory response. One such drug is pentoxifylline (PTOX), a methylxanthine used in the treatment of claudication. The effects of PTOX on PMN function are discussed in the following chapter.

CHAPTER SIX: RESULTS

Effect of Pentoxifylline on PMN Function

6.1. Effect of Slow Release Pentoxifylline

6.1.1. Lucigenin-Enhanced Chemiluminescence

To investigate the effects of PTOX ex vivo, 8 normal healthy volunteers received 1 x 400mg slow release PTOX in tablet form. Blood was taken before ingestion and at 1.5, 5, 24 and 48 hours after ingestion into 3mM EDTA. The purification of PMN was carried out as detailed in Methods 2.1.

To investigate the effect of ingested PTOX on respiratory burst activity, PMN isolated at each time point were stimulated with ZAS and FMLP and superoxide anion production was monitored using LUCL. Figure 57 shows that ingestion of 400mg of slow release PTOX resulted in significant depression ($p<0.009$) of respiratory burst activity in response to both stimuli at 1.5 hours post-ingestion. The response to ZAS was still significantly depressed ($p<0.02$) after 5 hours while the FMLP response, although still reduced, was not significantly different from control PMN prior to ingestion.

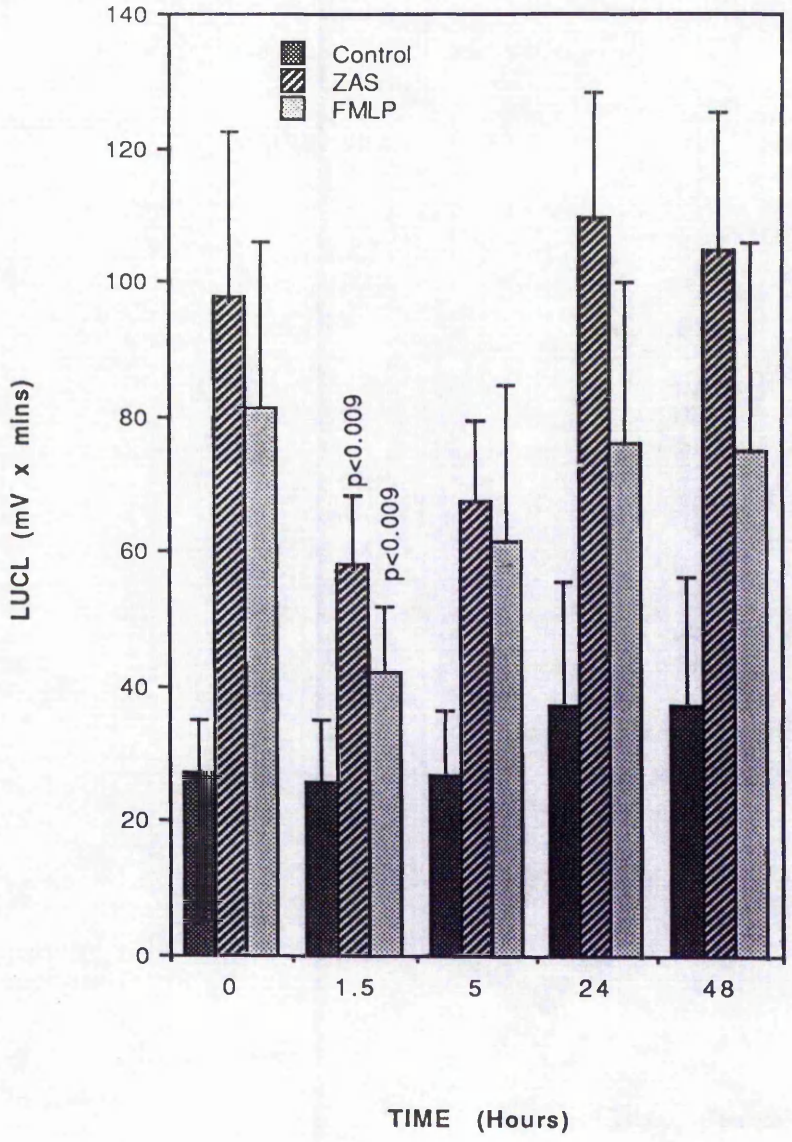

Figure 57: The effect of ingestion of 1 x 400mg slow release PTOX on PMN LUCL ex vivo. PMN, once isolated, were stimulated with either ZAS or FMLP or left unstimulated (control). The results represent the means of 8 separate experiments ± SEM.

6.1.2. Plasma Levels of PTOX and Metabolites.

PTOX is rapidly and extensively metabolised *in vivo*, studies on the kinetics of both oral PTOX and intravenous administration of the drug have shown that cumulation of PTOX in plasma was minimal. The levels of the active 5'-hydroxylated metabolite was found to be higher than those of the parent drug after both routes of administration (Beermann *et al* 1985). To find out whether the depression of respiratory burst activity correlated with levels of PTOX and/or its metabolites, the levels of these methylxanthines were measured in the plasma. Figure 58 shows that the highest levels of all four methylxanthines measured were detected at 1.5 and 5 hours post ingestion. At 24 hours only BL-194 and MET V were detectable in the plasma. Comparisons between these data and the results of LUCL showed that there was a significant correlation between depressed superoxide anion production and the levels of all three metabolites but not the parent drug. As shown in figure 59 there was a correlation between the levels of MET V measured and depressed chemiluminescence ($p<0.04$). To confirm the relative activities of these methylxanthines *in vitro* investigations were carried out using physiologically relevant concentrations.

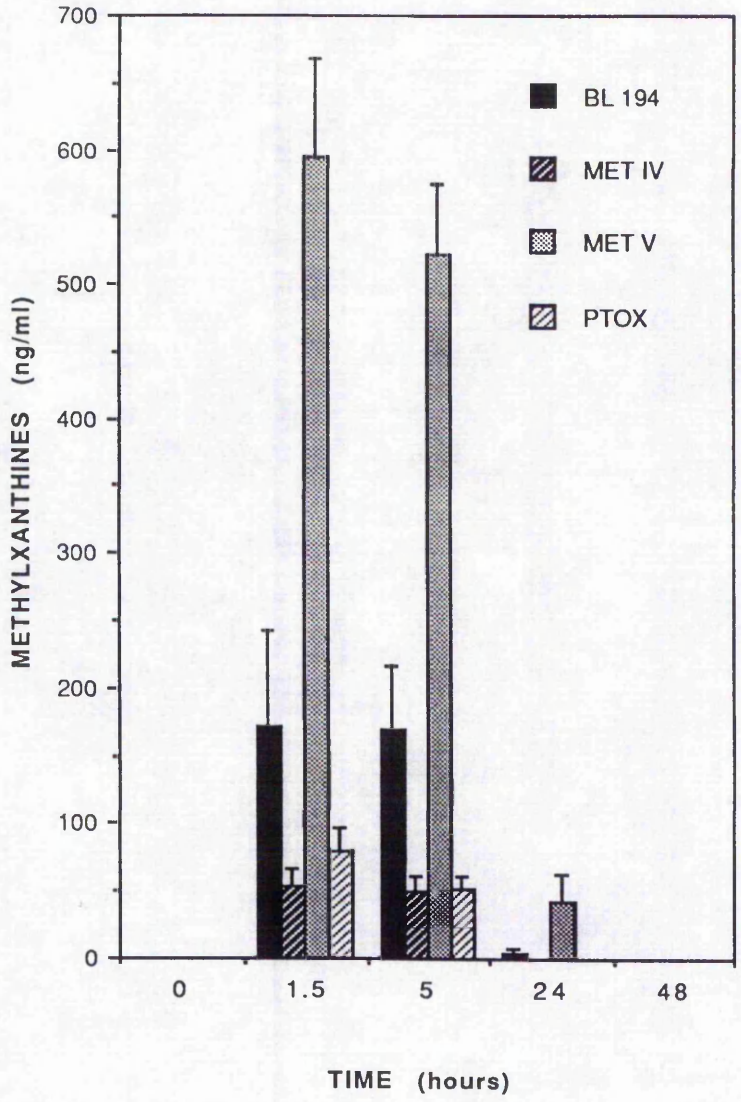

Figure 58: The concentrations of plasma levels of methylxanthines (PTOX and metabolites) measured in the plasma of subjects receiving 1 x 400mg slow release PTOX.
The results represent the means of 8 separate experiments ± SEM.

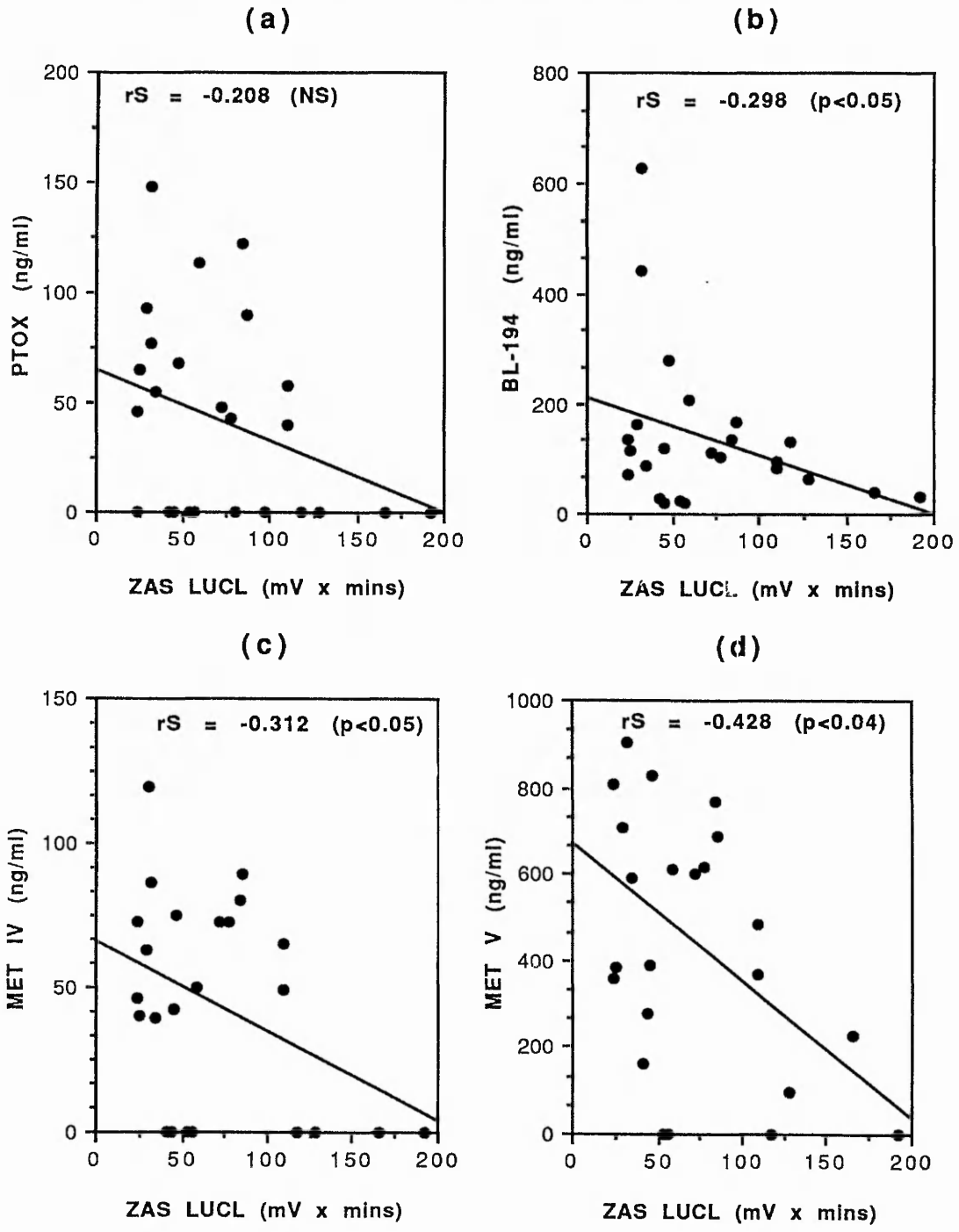

Figure 59: Correlations between circulating levels of methylxanthines and depressed chemiluminescence for (a) PTOX, (b) BL-194, (c) MET IV and (d) MET V.
The results are from 8 separate experiments.

6.1.3. *In Vitro* Effects of PTOX and Metabolites

6.1.3.1. Lucigenin-Enhanced Chemiluminescence

Figure 60 shows that all three metabolites at a concentration of 100ng ml^{-1} were capable of significantly reducing PMN respiratory burst activity in response to FMLP stimulation. Unlike the *ex vivo* data there did not appear to be a concentration dependent effect of methylxanthines *in vitro*. At 100ng ml^{-1} the parent drug did not show a marked reduction in superoxide anion production in response to this stimulus.

6.1.3.2. Lactoferrin Release

Previous studies have shown that *in vitro* PTOX is capable of reducing Lf release from PMN (Slater *et al* 1988). PMN require minimal stimulation in order to release their secondary granule contents compared to that required for the induction of respiratory burst activity. The effect of PTOX and these metabolites was investigated on PMN warmed from 4°C to 37°C, in the absence of any further stimulation, and also in response to stimulation with ZAS. Lf released into the supernatant was assayed by ELISA (as previously described). Figure 61 shows that only those PMN incubated with either MET IV or MET V showed a significant reduction in Lf released as a result of

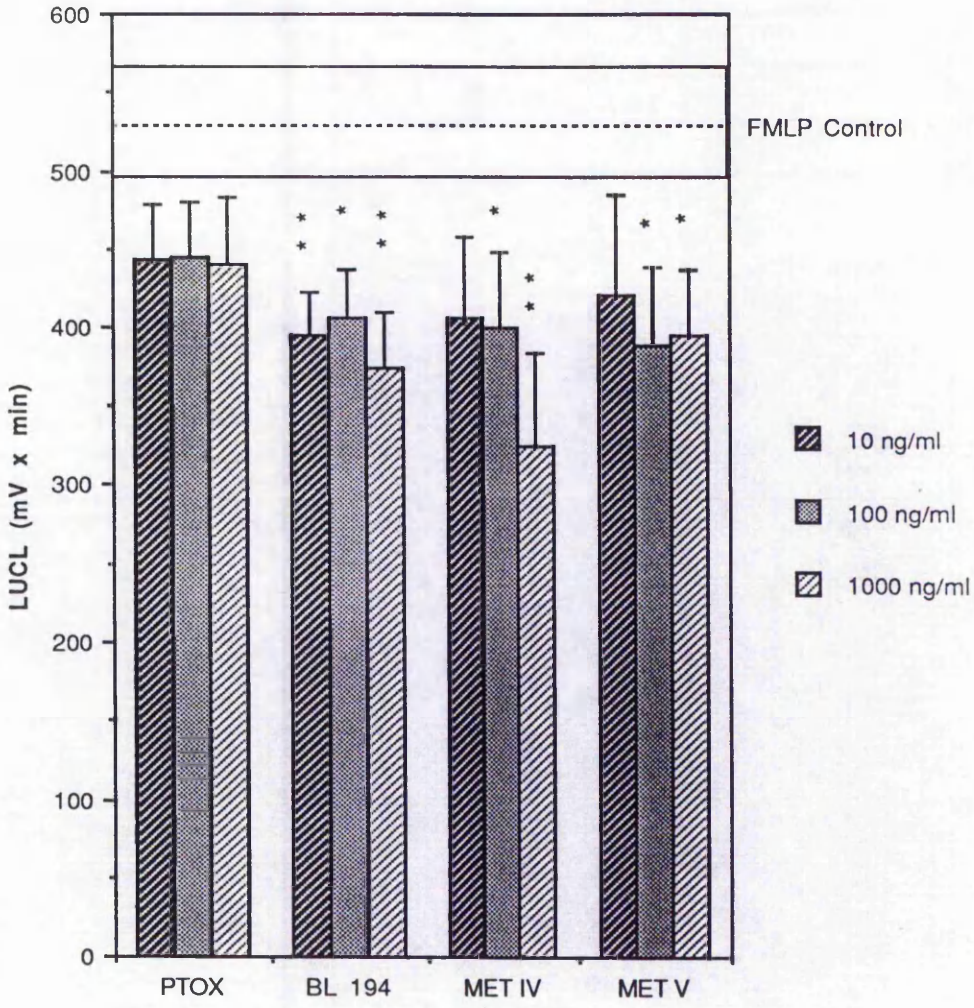

Figure 60: The in vitro effect of increasing concentrations of methylxanthines on LUCL in response to 10^{-6}M FMLP stimulation. The PMN were incubated with the methylxanthines for 30 minutes at 37°C prior to addition of stimulus.
The results represent the means of 7 separate experiments ± SEM, and were significant at, * $p<0.05$, ** $p<0.025$.

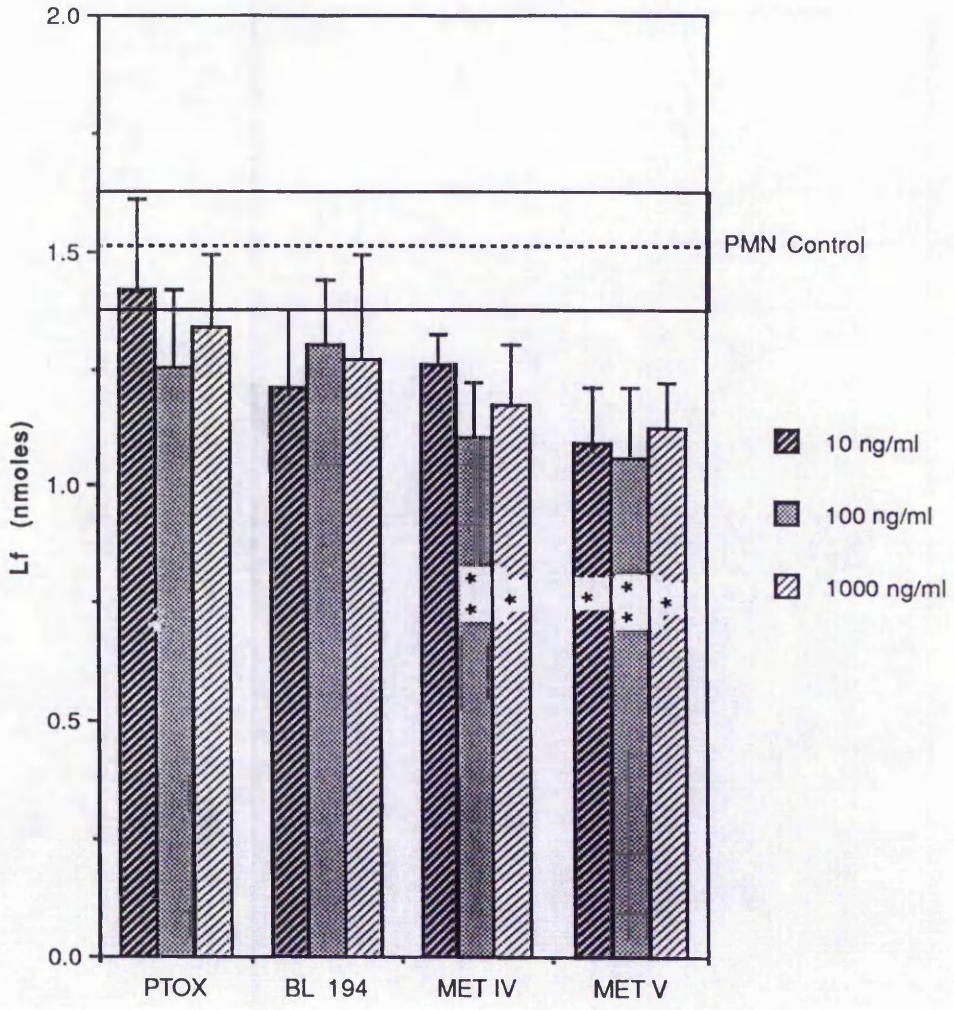

Figure 61: The effect of increasing concentrations of methylxanthines on Lf release. PMN were warmed from 4°C to 37°C, at this higher temperature cells were incubated with constant agitation for 30 minutes, then centrifuged down and Lf assayed in the supernatants.

The results represent the means of 7 separate experiments ± SEM, results were significant at * $p<0.04$, ** $p<0.03$.

simply warming the cells and incubating them for 30 minutes at 37°C.

When PMN were incubated under the same conditions as above but with the addition of ZAS only MET IV and MET V showed significant ($p<0.025$) reductions in the amount of Lf released compared with controls (figure 62).

6.1.3.3. CD11b and CD18 Expression

The adhesion molecules CD11b and CD18 are associated with the membranes of secondary granules (Bainton *et al* 1987), therefore, as MET IV and MET V were capable of significantly reducing Lf release adhesion molecule expression on these PMN was also investigated. Figure 63 shows that with cells subjected only to warming in the presence of each of the methylxanthines tested, only MET V was capable of causing a significant reduction in the expression of both adhesion molecules. At a concentration of 100ng ml^{-1} CD11b expression was reduced by 29.58% $\pm$ 5.61% and CD18 expression was reduced by 22.91% $\pm$ 3.31%.

When PMN were incubated in the presence of ZAS for 30 minutes there was a 2-3 fold increase in the expression of both CD11b and CD18 (figure 64). When each of the methylxanthines were tested under these conditions for their effects upon expression of these adhesion molecules all methylxanthines, including PTOX

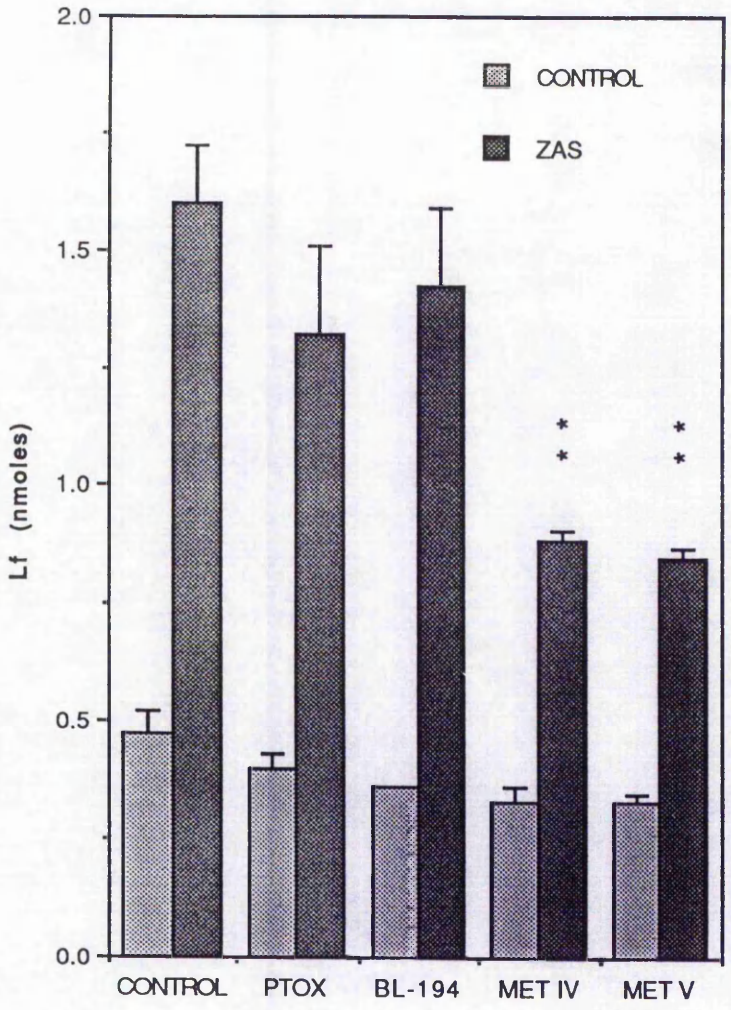

Figure 62: The effect of methylxanthines, all at a concentration of 100ng ml^{-1} on Lf release in response to ZAS stimulation compared with controls.

The results represent the means of 4 separate experiments $\pm$ SEM, * $p<0.025$.

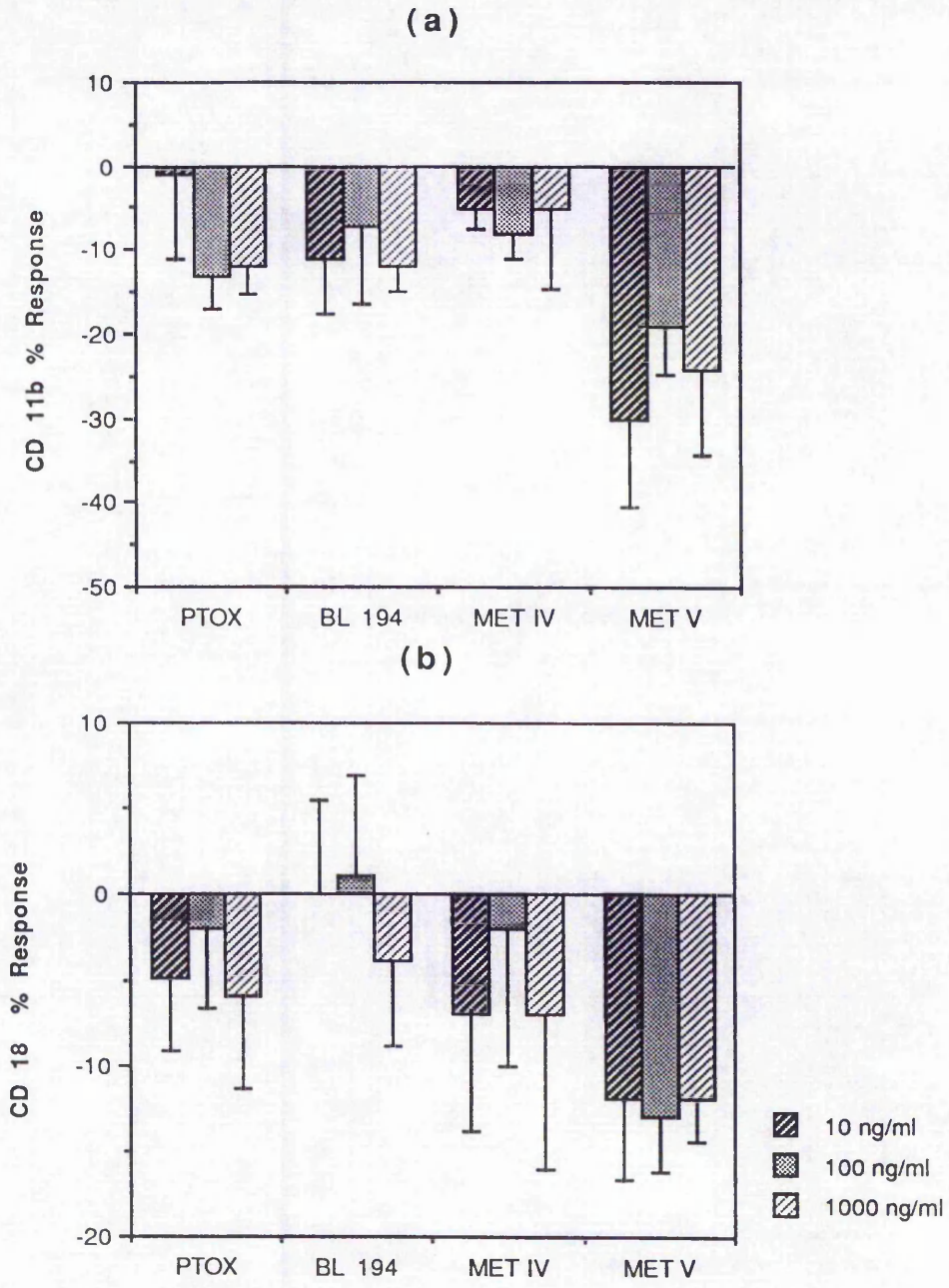

Figure 63: The effect of increasing concentrations of methylxanthines on CD11b and CD18 expression. The PMN were warmed from 4°C to 37°C, and incubated at this higher temperature for 30 minutes before determination of adhesion molecule expression.

The results represent the means of 6 separate experiments ± SEM, and are calculated as the percentage shift in mean fluorescence over controls.

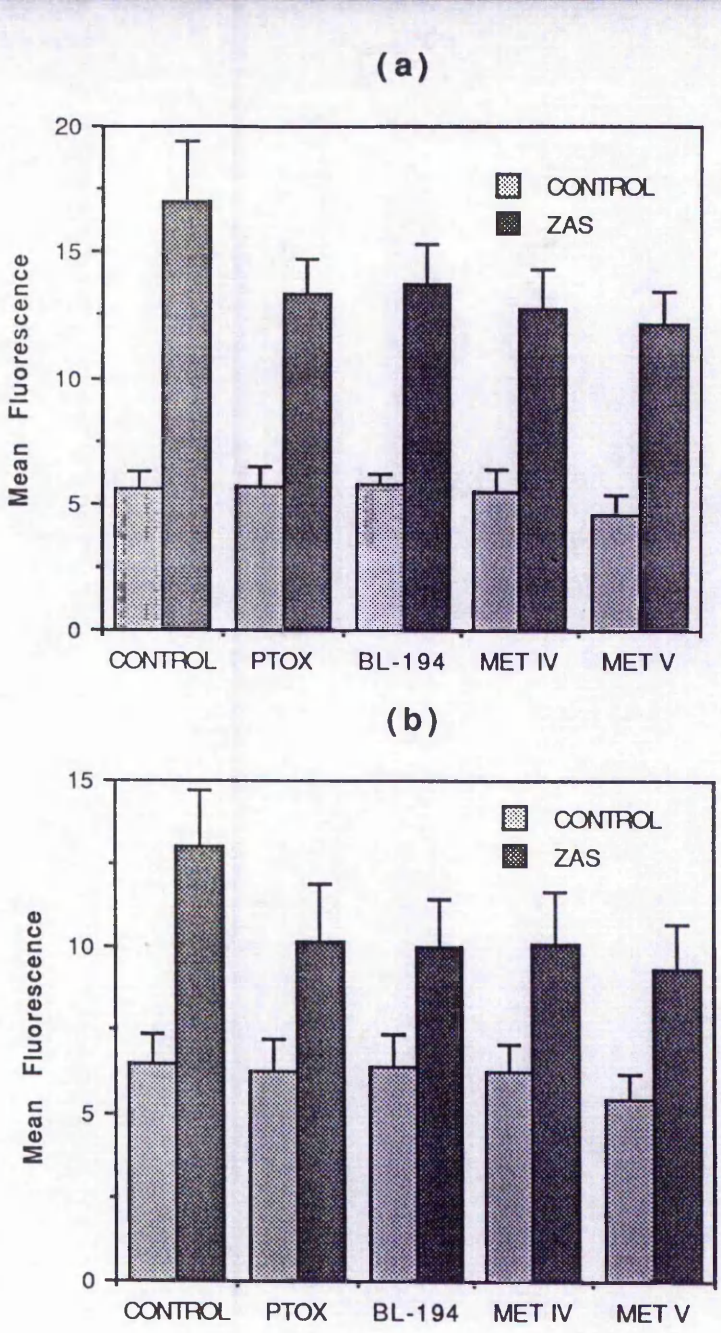

Figure 64: The effect of methylxanthines (at 100ng ml^{-1}) on CD11b (panel a) and CD18 (panel b) expression on ZAS stimulated cells compared with controls.

The results represent the means of 4 separate experiments $\pm$ SEM, with results expressed as mean fluorescence units.

inhibitory to the same degree. As shown in figure 62, there was a significant reduction in the amount of Lf released in the presence of MET IV and MET V together with reduced expression of adhesion molecules in the presence of ZAS (figure 64).

6.2. Discussion

Previous *in vitro* investigations have shown that PTOX is capable of inhibiting a number of aspects of neutrophil function, including Lf release (Slater *et al* 1988), myeloperoxidase release and superoxide anion production (Currie *et al* 1990). PTOX has also been shown to affect monocyte function by depressing secretion of TNF (Sullivan *et al* 1988, Strieter *et al* 1988). However the concentrations of PTOX used in these investigations were far higher (10ug to 1mg ml^{-1}) than those achievable *in vivo*.

In this study plasma concentrations of PTOX did not exceed 100ng ml^{-1}, and therefore according to the *in vitro* data available would be ineffective at depressing normal leucocyte function. However in subjects receiving a 400mg slow release tablet of PTOX, depression of PMN respiratory burst activity *ex vivo* in response to ZAS and FMLP stimulation was observed. As PTOX is extensively metabolised it seemed likely that the methylxanthine metabolites of PTOX may be exerting an effect. Of the metabolites assayed in the plasma MET V was detected at the highest concentrations. The strongest correlation between

depressed superoxide anion production and levels of circulating methylxanthines was found with MET V, although both of the other metabolites assayed (BL-194 and MET IV) showed significant correlations. However, there was no significant correlation between depressed respiratory burst activity and the plasma levels of the parent drug. Yukawa et al (1989), working with eosinophils, showed that micromolar concentrations of methylxanthines potentiated superoxide anion production, while higher concentrations inhibited the generation of these toxic oxygen radicals. If the same were true for neutrophils then it would be predicted that the plasma concentrations of methylxanthines would either potentiate PMN responses or have no effect. The implication of these ex vivo data would suggest that part of the effect of PTOX on circulating PMN is due to its more active metabolites. Although these data suggested that MET V was the most effective methylxanthine in vivo, further investigations carried out in vitro were required to confirm these findings.

When methylxanthines were added to PMN in luminometer cuvettes immediately prior to or after ZAS stimulation, no effect on respiratory burst activity was observed (data not shown). However when cells were preincubated with methylxanthines at 37^{o}C for 30 minutes prior to stimulation with ZAS, all three metabolites induced significant reductions in superoxide anion production at the physiologically relevant concentration of 100ng ml^{-1}. None of the concentrations of PTOX used exhibited

this inhibitory activity. These data appear to confirm the ex vivo results which indicated that it is the metabolites and not the parent drug that are effective at reducing PMN respiratory burst activity.

PTOX has been used in the treatment of venous leg ulcers where it has been shown to improve ulcers healing when used in conjunction with compression bandaging (Colgan et al 1990). It is possible that PTOX reduces tissue damage through the action of its metabolites by inhibiting the production of toxic oxygen radicals. The methylxanthine metabolites may also decrease the numbers of PMN in the marginated pool. Adhesion of PMN to endothelium is regulated through the expression of CD11/CD18 on the surface of PMN and also expression of the corresponding ligand on endothelial cells. As secondary degranulation allows for increased expression of these adhesion molecules at the cell surface, methylxanthine metabolites may prevent excessive recruitment of cells into an inflammatory site by inhibiting secondary degranulation and thereby reducing adhesion molecule expression. MET V was effective at reducing both Lf release and CD11b and CD18 expression, this would suggest that this particular metabolite is effective at reducing the adhesiveness of the PMN.

MET V was found at the highest concentrations in the plasma of the subjects and was also detectable up to 24 hours post-ingestion. These investigations would suggest that MET V was

the most effective at reducing PMN activity both in vivo and in vitro.

The mechanism by which methylxanthines exert their inhibitory activity is thought to be through inhibition of phosphodiesterase (PDE) activity. Inhibiting the activity of this enzyme results in increased levels of cAMP. Therefore, if the responsiveness of PMN is mediated via cAMP dependent pathways methylxanthines are more likely to exert an inhibitory effect. None of the methylxanthines tested were capable of completely inhibiting PMN responses at physiological concentrations. Studies carried out on a number of animal tissues have shown that different methylxanthines appear to act on different PDEs (Stefanovich 1974). The data showed that PTOX exhibited different inhibitory activities on PDE isolated from different organs. As certain tissues and cells have also been shown to possess different forms of the enzyme it is possible that there is more than one isoform of PDE in PMN. If this were the case then the most effective of the metabolites, MET V, may be acting on just one PDE. This would result in continued hydrolysis of cAMP to 5'-AMP by PDE unaffected by the methylxanthine used. There are other secondary messenger systems involved in neutrophil activation which would be involved in cell responsiveness. This may explain why only approximately 30% inhibition was achieved in vitro with the metabolites or ex vivo with the drug. Another explanation would be that at these concentrations of methylxanthines

reduced PMN responses is not a result of PDE inhibition. PTOX has a 50% inhibitory activity for PDE of 10^{-3}M to 10^{-4}M (Stefanovich 1974). Therefore, the concentrations found in vivo are not high enough to be acting as PDE inhibitors, unless the IC_{50} for the metabolites is lower than that for the parent drug.

PTOX has been shown to be effective in a number of inflammatory conditions, both with respect to its effects upon PMN function and particularly through its action on inhibiting TNF production. TNF has been implicated in septic shock and ARDS (Tracey et al 1988), while studies carried out on the in vivo effects of LPS induced TNF production (Zabel et al 1989) showed that infusion of human subjects with PTOX reduced circulating levels of TNF induced by the LPS treatment. As TNF has been shown to enhance PMN function, it is possible that the methylxanthine metabolites of PTOX may act at two levels. Firstly, methylxanthines act directly on the PMN by reducing a number of biological functions and also to reduce TNF production from monocytes, and thus reduce the amount of priming agent available to these cells. Under these conditions methylxanthines may provide a greater degree of inhibition.

It is probable that PTOX will depress PMN responses to inflammatory stimuli in vivo and may reduce unwanted tissue damage. It is quite possible that this is its mechanism of action in potentiating the healing of varicose ulcers. Nash et

al (1988), showed that PTOX was effective at improving the healing of this type of ulcer when used in conjunction with compression bandaging. Repeated dosing of the drug improves walking distance in patients with claudication, and these effects are associated with modulation of leucocyte function. These are long term effects despite the fact that neither PTOX nor its metabolites remain in the circulation or in the tissues for prolonged periods of time. It is possible that methylxanthines, after a brief exposure to cells, cause irreversible inhibition of leucocyte function preventing the recruitment of cells from the marrow into the circulating pool, margination and migration into the tissues and activation of cells at the site of inflammation.

CHAPTER SEVEN: CONCLUSIONS

This work has shown that TNF is produced early on (5-6 hours) in response to an inflammatory stimulus when MNC are cultured *in vitro* in the presence of LPS. This TNF in MNCM is capable of markedly enhancing PMN respiratory burst activity when cells are exposed to a second agonist. This priming requires only a short preincubation with the TNF, which appears to bind irreversibly to receptors on the cell surface and cannot be removed by washing. The priming effects of both MNCM and recombinant human TNF did not appear to affect the affinity of either ZAS or FMLP receptors as neither dose response curve showed any shift after cells had been incubated with the priming agents. The secondary granules are reported as acting as an intracellular pool of FMLP receptors (Fletcher and Gallin 1983). Therefore it would be expected that agents that cause secondary degranulation would increase the expression of these receptors and also the CD11/CD18 molecules involved in adhesion. The results in chapter 4 show that recombinant human TNF was capable of increasing secondary degranulation together with increased CD11b/CD18 expression. The results shown in chapter 3 indicate that there was a vast increase in the amount of superoxide anions suggesting that the number of cells responding were increased or alternatively there was an increase in NADPH-oxidase activity. As the secondary granule membrane contains cytochrome b_{-245}, an essential part of the oxidase (Ambruso *et al* 1990), it may be that the increased

secondary degranulation seen with low concentrations of TNF results in a greater activation of the oxidase allowing for increased reduction of molecular oxygen to form superoxide anions. TNF has also been shown to increase the accumulation of phosphatidic acid in cells subsequently stimulated with FMLP (Bauldry et al 1991). TNF appeared to increase phospholipase D activity with minimal production of diacylglyceride. The priming activity of TNF may therefore result from increased production and activation of secondary messenger systems and/or increased numbers of responsive cells resulting in a dramatic increase in PMN respiratory burst activity.

While the priming effect of MNCM could be attributed to TNF, another low molecular weight factor(s) appeared to be active in the direct stimulation of PMN respiratory burst activity. The neutrophil chemotactic peptide NAP-1/IL-8, first isolated from LPS stimulated MNC (Yoshimura et al 1987) seemed the most likely candidate, although it is possible that other low molecular weight peptides (Matsushima and Oppenheim 1989) may also be involved. The results in chapter 3 show that antiserum to NAP-1/IL-8 reduced the direct effect of MNCM but did not completely abrogate the direct stimulatory activity. The involvement of other low molecular weight peptides was partially confirmed when fractions eluted from a Sephadex G-75 column were investigated for their ability to stimulate PMN superoxide anion production. Of two peaks eluting with a UV absorbance and with a molecular weight of less than 12.3kD,

only the activity of one could be reduced in the presence of the NAP-1/IL-8 antiserum.

This work has also outlined the problems of working with recombinant human cytokines *in vitro*, in an attempt to "mimic" the regulatory processes at work *in vivo*. Of three different recombinant NAP-1/IL-8 preparations tested for bioactivity in vitro, only one was capable of inducing a dose dependent increase in PMN respiratory burst activity (as reported by Thelen *et al* 1988). Neither of the other two preparations had this effect upon PMN function. This could explain the disparity in the literature whereby some investigators (Thelen *et al* 1988, Baggiolini *et al* 1989) show NAP-1/IL-8 to be a potent direct stimulus of respiratory burst and others do not (Djeu *et al* 1990). Although bioactivity with respect to the induction of calcium transients was demonstrated in all three preparations, the relative potency of each was vastly different. These differences may result in the purification and lyophilisation procedures used. The bioactivity of MNCM, with respect to PMN superoxide anion production was much greater than that seen NAP-1/IL-8, and it may be that the secreted form of the peptide in MNCM is more biologically active than the commercial preparations currently available.

The same may be true for other cytokines and could also explain the differing reported functions of TNF, IL-1, G-CSF and GM-CSF, for example with respect PMN chemotaxis and respiratory

burst activity. TNF has been reported to only induce secondary degranulation (in the absence of cytochalasin b) in adherent PMN (Richter et al 1988). However, the results shown in chapter 4 show that low concentrations induced both Lf release and increased adhesion protein expression in cells held in suspension.

There are CD11/CD18-dependent and -independent mechanisms of PMN adherence (Doerschuk et al 1990, Anderson et al 1991), the ability of TNF to increase the expression of CD11b and CD18 molecules suggests that this cytokine is capable of increasing the CD11/CD18-dependent adhesive capacity of PMN. In vivo this may result in an increase in the marginated pool and thereby the number of cells entering the tissues. However, it must be stressed that the expression of adhesion molecules is not the only requirement for increased adherence. Lo et al (1991) showed that TNF acted upon endothelial monolayers to increase PMN binding, and that the endothelium was acting as the stimulus for increased CD11b/CD18 expression on PMN. They also suggested that the expression of ELAM-1 is necessary for EC to activate the CD11b/CD18 receptors on PMN. It is therefore possible that TNF can cause a rapid increase in CD11b/CD18 expression on PMN, with a more prolonged exposure to this cytokine activates the endothelial cells to express ELAM-1 which then activates the CD11b/CD18 on the PMN and allows the cells to adhere.

TNF released early in response to infection/injury may cause an increase in the marginated pool. As the cell moves towards the site of infection, and presumably along a concentration gradient of this and other pro-inflammatory agents, the cells become increasingly primed such that when the neutrophils reach the injurious agent they can quickly and efficiently eradicate it.

The role of Lf released at the same time as the cells are being upregulated may be simply due to negating the electrostatic repulsion between neutrophil and endothelium (Gallin 1980). The results in chapter 5 would suggest it to be a potent negative regulator of cytokine production. Other investigations have shown it to be an inhibitor of IL-2 production (Slater 1988) and GM-CSF via the inhibition of IL-1 (Zucali _et al_ 1989). We would suggest that the concentrations of this Fe-binding protein likely to be found _in vivo_ are capable of regulating inflammation through the initial inhibition of TNF production from monocytes. This in turn would lead to a reduction in IL-1, IL-2 and possibly GM-CSF. Lf would therefore appear to act in much the same way as PGE_2, both agents are released in response to TNF and both are capable of acting as negative regulators of inhibition of cytokine production.

Finally, we have looked at the effects of methylxanthines on PMN function. There are certain conditions where tissue injury is associated with an increased influx and activation of

neutrophils (Schmid-Schonbein and Engler 1986). Therefore in these circumstances it is desirable to minimize the amount of damage by reducing PMN recruitment and activation. The data in chapter 6 shows that PTOX acts *in vivo*, via metabolites to reduce PMN respiratory burst activity. In vitro investigations showed that metabolites IV and V were most effective at reducing respiratory burst activity, secondary degranulation and adhesion molecule expression in the presence of physiologically relevant stimuli. The concentrations of each of the methylxanthines used are likely to be found in the circulation after administration of PTOX. Although the inhibition varied according to the donor PMN, cellular responses were never completely inhibited. This is possibly as a result of activation of other secondary messenger pathways not involving cAMP and PDE. It may also be that these concentrations of methylxanthines are not sufficient to inhibit PDE, but may act through another mechanism which is more sensitive to lower concentrations of methylxanthines. However, these investigations confirm the clinical findings that PTOX is a valuable tool in the treatment of peripheral vascular disease as a result of its actions upon leucocyte function.

It is possible to upregulate and inhibit PMN responses using leucocyte products and drugs. In addition Lf secreted from PMN is capable of reducing cytokine production from MNC. This study has investigated some of the pathways involved in the promotion and negative control of the inflammatory response with respect

to early events associated with acute inflammation and highlights the importance of MNC products in PMN activation.

APPENDIX 1

1. Buffers for TNF and GM-CSF ELISAs

a) 50mM Sodium carbonate/bicarbonate Coating Buffer pH 9.5

0.1M Na_2CO_3 (A) 5.3g Na_2CO_3 in 500mls d.H_2O.

0.1M $NaHCO_3$ (B) 4.2g $NaHCO_3$ in 500mls d.H_2O

For 250mls pH 9.5 buffer add 75mls of solution A to 175 mls of solution B. Correct to pH 9.5.

b) Phosphate Buffered Saline pH 7.4

0.5M NaH_2PO_4 (A) 39.0g in 500mls d.H_2O (store at 4°C)

0.5M Na_2HPO_4 (B) 35.5g in 500mls d.H_2O (store at RT)

For use add 15mls of solution A to 75mls of solution B, add 39.5g of NaCl (0.15M) and make up to 5 litres with d.H_2O and pH to 7.4.

c) 0.1M Citrate/phosphate Substrate Buffer pH 5.0

0.1M citric acid (A) 10.5g citric acid (monohydrate) in 500mls d.H_2O (store at 4°C)

0.2M Na_2HPO_4 (B) 14.2g in 500mls d.H_2O (store at RT)

For use add 48.5mls of solution A to 51.5mlsof solution B and adiust to pH 5.0.

2. Buffers for Lactoferrin ELISA

a) 50mM Sodium carbonate/bicarbonate Coating Buffer pH 9.5

See TNF ELISA above.

b) Phosphate Buffered Saline pH 7.4

See TNF ELISA above.

c) Borate Saline Buffer pH 8.6

Boric acid	5.5g
Sodium tetraborate	9.5g
NaCl	29.2g

Dissolve above reagents and make up to 1 litre with d.H_2O adjust to pH 8.6

d) 0.02M Phosphate Substrate Buffer pH 6.8

0.5M NaH_2PO_4 (A) 39.0g in 500mls d.H_2O (store at 4°C)

0.5M Na_2HPO_4 (B) 35.5g in 500mls d.H_2O (store at RT)

For use add 8.75mls of solution A to 11.25 mls of solution B and make up to 500mls with d.H_2O adjust to pH 6.8

e) Colour Reagent

Add 1mg ml^{-1} 5'-amino salycyclic acid to 0.02M phosphate buffer pH 6.8, add 1mg activated charcoal per 20mls buffer. Dissolve colour reagent by constant agitation on a rotating wheel for 1 hour at RT. Filter (Whatman No.1) prior to addition of 200ul of 1% (v/v) H_2O_2 per 20mls of subtrate.

f) Stopping Solution

3M NaOH	60.0g NaOH in 500mls d.H_2O.

APPENDIX 2: List of Suppliers

All the tissue culture plastics used were by Falcon, supplied by Fahrenheit Lab. Supplies.

Centrifuge tubes were supplied by Sterilin Ltd.

Sterile polypropylene tubes were supplied by Gibco Nunc Ltd

Foetal calf serum was obtained from Sera-Lab lot number 601103-A.

Amersham International plc, Buckinghamshire, UK. (020404) 4444.
BDH Ltd, Hampshire, UK. (0703) 619171.
Beckman-RIIC Ltd, Buckinghamshire, UK. (0494) 41181.
British Biotechnology Ltd, Oxfordshire, UK. (0865) 748747.
Costar distributed by Northumbria Biologicals Ltd.
Dakopatts Ltd, Buckinghamshire, UK. (0494) 452016.
Dynatech Laboratories Ltd, Sussex, UK. (0403) 814565.
Fahrenheit Lab Supplies (Midlands) Ltd, Nottinghamshire, UK. (0602) 391227.
Flow Laboratories Ltd, Hertfordshire, UK. (0923) 774666.
Genzyme Antibodies distributed by Koch Light, Hertfordshire, UK. (07072) 75733.
Gibco Nunc Ltd, Paisley, UK. (0491) 889 6100.
National Institute for Biological Standards and Control, Hertfordshire, UK. (0707) 54753.

Northumbria Biologicals Ltd, Northumberland UK. (0670) 732992.

Pharmacia Ltd, Buckinghamshire, UK. (0908) 661101.

Sartorius Ltd, Surrey. UK. (081) 642 8691.

Sigma Chemical Company Ltd, Dorset, UK. (0202) 733114.

Sterilin Ltd, Middlesex, UK. (081) 572 2468

Whatman Labsales Ltd, Kent, UK. (0622) 674821.

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British Journal of Haematology, 1991, 77, 158–164 ADONIS 000710489100026B

The priming effects of the products of stimulated mononuclear cells on the response of neutrophils to C5a des arg

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Received 31 March 1990; accepted for publication 25 September 1990

Summary. Certain recombinant human cytokines have been shown to enhance polymorphonuclear leucocyte (PMN) responses to subsequent stimulation. Mononuclear cells (MNC) from normal healthy individuals were stimulated for 5 h with 1 μg/ml bacterial lipopolysaccharide (LPS) in order to induce production and secretion of inflammatory cytokines into the surrounding medium. These mononuclear cell conditioned media (MNCM) were then used to prime PMN isolated from healthy volunteers. Preincubating the PMN with MNCM for 15 min at 4°C followed by washing and warming to 37°C caused a 344% increase ($n=26$) in the rate of superoxide anion production in response to zymosan-activated serum (ZAS), a source of C5a des arg. This effect could not be reproduced with recombinant human forms of interleukin 1 beta (Il-1 beta) or granulocyte-macrophage-colony stimulating factor (GM-CSF), although, with the latter, there was some effect when the preincubation stage was carried out for 60 min at 37°C. Only recombinant human tumour necrosis factor-alpha (rh-TNF-alpha) gave a similar PMN priming effect to that seen with MNCM. This effect could not be reversed by washing away either the MNCM or rh-TNF-alpha. The priming effect could be markedly reduced (74·8%, $n=6$) by employing the use of polyclonal antibody to TNF-alpha in the preincubation step; assaying for TNF-alpha in these MNCMs showed that the degree of priming corresponded to the amount of TNF-alpha present. Rh-TNF-alpha alone appeared to have very little direct stimulatory effect on respiratory burst activity.

The results show that TNF-alpha produced by LPS stimulated MNC after 5 h binds to a PMN surface receptor in the cold and warming of the cells to 37°C allows for an immediate and dramatic response to ZAS stimulation. This suggests that TNF-alpha is the important cytokine upregulating PMN responses to other physiological mediators, including C5a des arg during the early phases of an inflammatory reaction.

Recombinant human cytokines, particularly TNF-alpha, gamma interferon (Shalaby *et al*, 1985; Perussia *et al*, 1987) and colony stimulating factors (Weisbart *et al*, 1986) enhance polymorphonuclear granulocyte (PMN) responses to subsequent stimulation. Some chemoattractants such as formylmethionylleucylphenylalanine (FMLP), leukotriene B4 and platelet activating factor have similar effects at low concentrations: at higher concentrations they also act as potent direct stimuli (Ingraham *et al*, 1987; Wymann *et al*, 1987). *In vivo* it is unlikely that any of these factors are independent of the others and therefore it is necessary to examine their effects in combinations and at concentrations likely to occur as part of the inflammatory response. For this reason we have stimulated mononuclear cells (MNC) with bacterial lipopolysaccharide (LPS) and examined both the direct effect of the products on PMN respiratory burst and how they alter the PMN response to the activated complement component C5a des arg. It is hoped that this may provide an *in vitro* model to shed light upon PMN responses during inflammation.

The results suggest that quantitatively the most important factor released by mononuclear cells within the first hours of stimulation is TNF-alpha. This is a 17000 dalton protein originally noted for its cytotoxicity but now known as a potent chemotaxin (Ming *et al*, 1987). There is conflicting evidence as to whether it can directly stimulate the PMN respiratory burst (Berkow *et al*, 1987; Tsujimoto *et al*, 1986) but it certainly enhances responses by priming to subsequent stimulation (Atkinson *et al*, 1988).

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METHODS

Isolation of MNC and preparation of mononuclear cell-conditioned media (MNCM). EDTA anti-coagulated blood from normal healthy donors was separated using a rapid single-step technique. The blood was layered onto a Ficoll–Hypaque solution of 1·114 g/ml density (Monopoly Resolving

Medium; Flow Laboratories). After centrifugation at 400 *g* for 30 min the leucocytes resolved into two bands; MNCs forming a band at the interface with neutrophils collected in a second band below the first. During the centrifugation erythrocytes sedimented to the bottom of the tube. The MNC fraction was collected and washed twice in RPMI 1640 medium (Flow Laboratories). The viability of the MNCs was greater than 98%, as judged by trypan blue exclusion. The cells were then suspended at 3×10^6 ml in complete medium comprising RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (all Flow Laboratories).

After incubation in 12-well plastic tissue culture plates in the presence/absence of LPS (*E. coli* 055:B5, Sigma Chemical Co.) at 37°C plus 5% CO_2, the medium was removed from each well, the non-adherent cells spun down and the resulting cell free MNCMs were filtered through a 0·2 μm filter and stored at −70°C until required.

Isolation and priming of PMN. The PMN were isolated from peripheral blood as mentioned above. The PMN band was collected and washed in cold sterile phosphate-buffered saline (PBS) pH 7·4 at 4°C. PMN collected in this way were always of a purity greater than 97% with a viability greater than 99% as determined by trypan blue exclusion.

Priming was carried out by suspending cells at 1×10^6 ml in MNCM or recombinant human cytokines, the latter being prepared in the complete medium used for preparing MNCM. The PMN were incubated for 15 min at 4°C before washing in cold sterile PBS and resuspension in cold RPMI 1640 containing 0·1% bovine serum albumin (BSA fraction V; Sigma), prior to determination of superoxide anion production. The recombinant human cytokines used in the priming experiments included rh-TNF-alpha, rh-Il-1 beta (kindly supplied by Dr A. Meager, NIBSC) and rh-GM-CSF (Glaxo).

In some experiments rabbit anti-human polyclonal antibodies to TNF alpha, interleukin 1 beta (IL-1 beta), and granulocyte-macrophage colony stimulating factor (GM-CSF) (Genzyme) were used to block the actions of these cytokines, if present in MNCM. Preimmune rabbit serum was used as a control; all antibodies were present only in the preincubation step carried out at 4°C. The final dilutions of antibodies and all antibody preparations were free of sodium azide.

PMN stimulation and measurement of superoxide anion production. Superoxide anion production was determined by lucigenin enhanced chemiluminescence (LUCL) using a Bio-orbit 1251 luminometer. Briefly, 400 μl of PMN suspension were added to a cuvette containing 50 μl of $2{\cdot}5 \times 10^{-5}$ M lucigenin (Sigma Chemical Company) diluted in sterile PBS pH 7·4. The cuvettes containing the PMN were warmed to 37°C in the luminometer and chemiluminescence measured continually with or without addition of zymosan activated serum (ZAS) at a final concentration of 10% by volume. To ensure that increases in chemiluminescence were due to superoxide anion production, these experiments were carried out in the presence and absence of 50 μg/ml superoxide dismutase (Sigma Chemical Co.). Results were expressed as the rate of increase in chemiluminescence 21 min after addition of any stimulus as at this time the rate of increase was maximal. All measurements were carried out in duplicate and the results represented the mean.

A standard supply of ZAS was produced according to the method of Fernandez *et al* (1978). Briefly, blood was taken from 12 healthy volunteers and allowed to clot in serum tubes at room temperature. These samples were then spun down at 1000 *g* for 15 min. The serum supernatant was harvested and then pooled together. The pooled serum was then incubated with zymosan-A (Sigma Chemical Co.) at a concentration of 1 mg/ml on a rotating wheel at 37°C for 1 h. The zymosan particles were removed by centrifugation at 1000 *g* for 15 min, followed by filtration of the supernatant twice through 0·2 μm millipore filters. The filtration step was carried out twice to ensure removal of any residual zymosan particles. The ZAS was then aliquoted and stored at −70°C until required.

Assay of TNF-alpha. TNF-alpha bioactivity was measured in MNCM using the 1 d L-929 mouse fibroblast cell line assay (Matthews & Neale, 1987), in the presence of actinomycin D (Sigma Chemical Company). The same polyclonal antibody was used to neutralize TNF-alpha in this assay as in priming experiments.

It should be noted that all buffers and reconstituted reagents were tested for endotoxin (ET) contamination using the limulus amoebocyte lysate (LAL) assay. All solutions used were determined to contain less than 10 pg/ml of contaminating ET with the exception of the Ficoll–Hypaque separation medium which contained less than 20 pg/ml of contaminating endotoxin. However, this is still less than the reported 100 pg/ml or more of LPS required to prime PMN for enhanced respiratory burst activity (Guthrie *et al*, 1984).

RESULTS

Preliminary experiments show that priming with MNCM at 37°C caused loss of cells due to aggregation, cell numbers could not be recovered after washing and there was loss of measurable chemiluminescence. Aggregation did not occur following priming and washing at 4°C. The result was considerably greater enhancement of the chemiluminescence response to ZAS after priming at 4°C rather than at 37°C and subsequently all priming was carried out at 4°C.

Kinetics of priming PMN at 4°C

Fig 1 shows that priming with MNCM is rapid and almost complete within 5 min at 4°C rising to a plateau by 15 min. The MNCM used in these experiments was produced by 5 h stimulation of MNC with 1 μg/ml LPS. The control experiments using the medium containing 1 μg/ml LPS shows that this is capable of priming even at 4°C but at a much lower level and priming continues to increase over 40 min.

Kinetics of priming factor production

The time course for production of priming factors from LPS stimulated MNC is shown in Fig 2. Maximal release of priming factors occurred after 5 h and this time was chosen for subsequent experiments. TNF-alpha was released from MNC into the supernatant with exactly the same time course as priming factors (Fig 2).

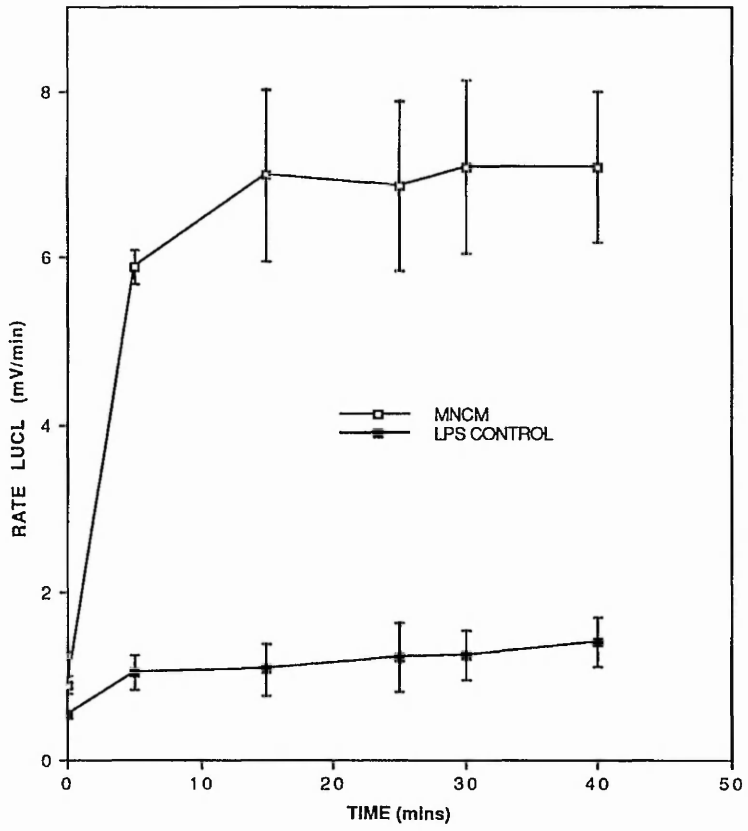

Fig 1. The kinetics of the priming effect observed after incubation with MNCM at 4°C followed by washing and stimulation with ZAS at 37°C. A constant pool of MNCM from several different donors was tested against six different donor PMN. Results represent means ±SEM from these six experiments.

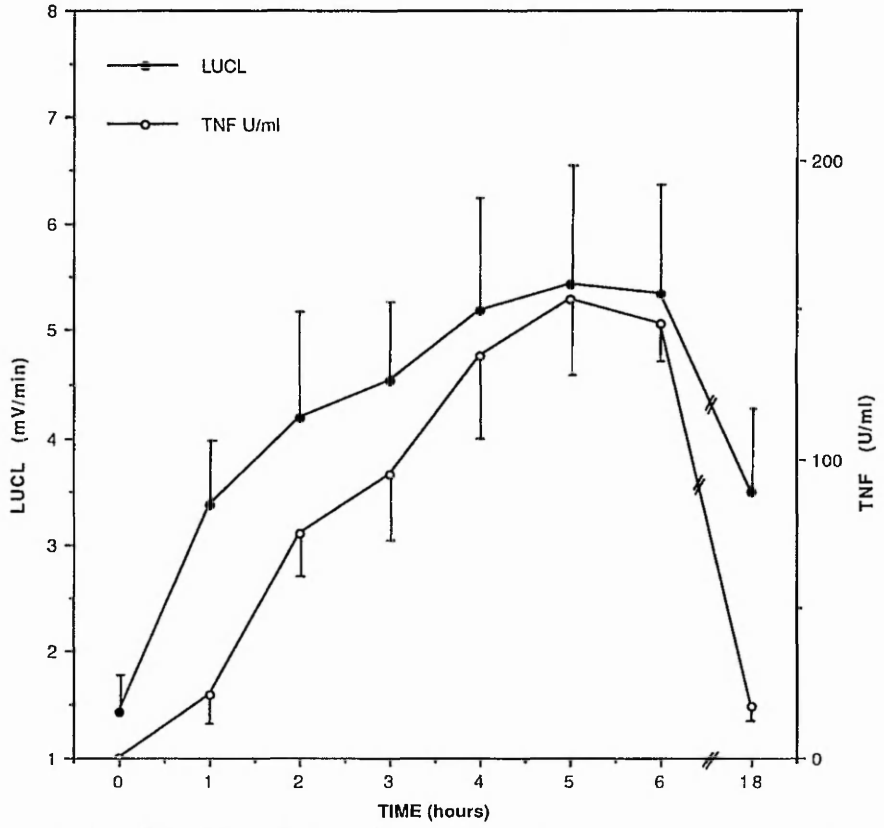

Fig 2. Time course for the production of TNF-alpha and priming factors from LPS stimulated MNC. MNCM at each time point were preincu with PMN at 4°C and after washing, the rate of increase LUCL in response to ZAS was determined. The levels of TNF-alpha were assayed same MNCM used for these priming experiments. Results represent the means of six separate experiments ±SEM.

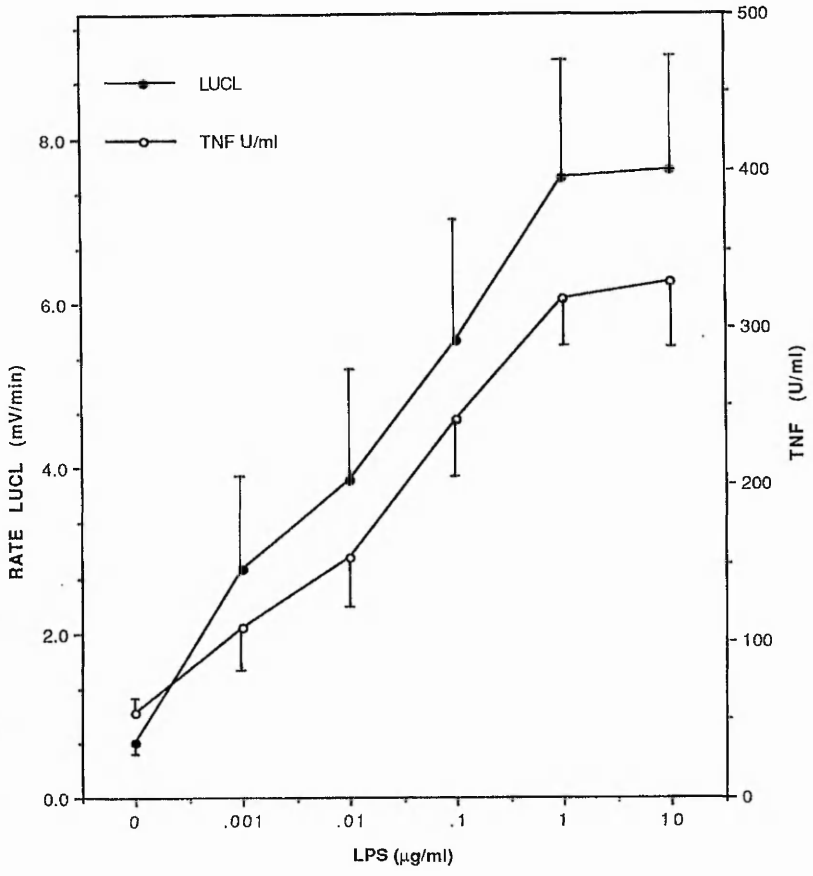

Fig 3. The dose dependent production of both priming factors and TNF-alpha from MNC stimulated with increasing concentrations of LPS. The levels of TNF were assayed in the same MNCM used for priming. Results represent the means of six separate experiments ±SEM.

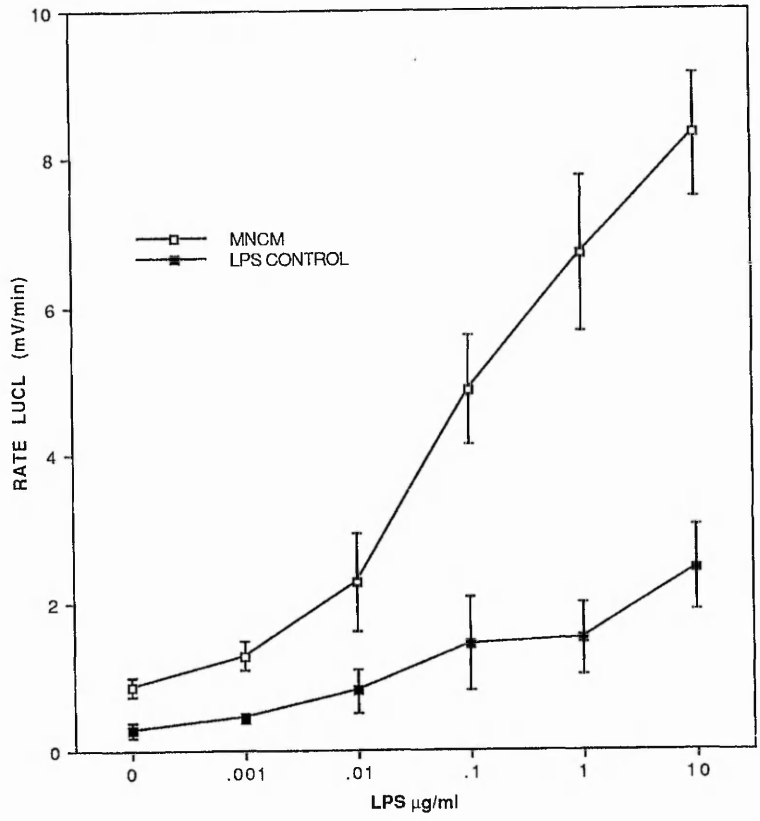

Fig 4. The dose dependent increase in production of priming factors in MNCM generated with increasing concentrations of LPS compared with the priming effect of control medium containing LPS alone. Results represent the means ±SEM from 26 separate experiments.

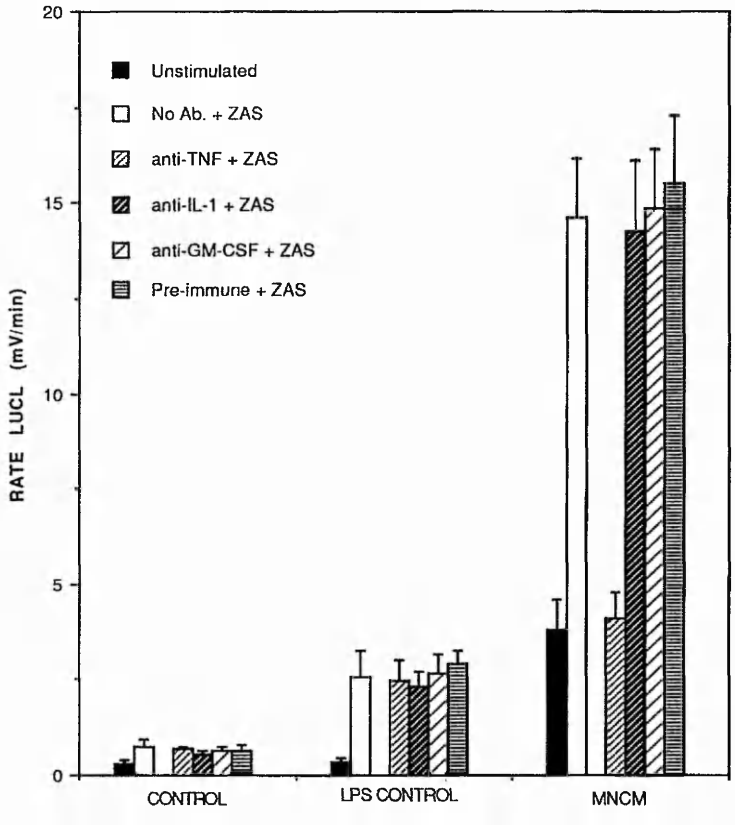

Fig 5. The effect of cytokine antibodies on the priming of PMN responses by MNCM and the direct effect of MNCM on superoxide anion production. All cells were preincubated for 15 min at 4°C with either complete medium (CONTROL), complete medium plus 1 μg/ml LPS (LPS CONTROL) or MNCM. For each of these groups incubations were also carried out in the presence of anti-TNF alpha, anti-IL-1 beta, anti-GM-CSF and preimmune rabbit serum. PMN were washed and warmed to 37°C and then stimulated with ZAS. The rate of increase in LUCL of the ZAS stimulated PMN was compared with those not subjected to further stimulation. The results represent the means of six separate experiments ± SEM.

Dose–response relationship for LPS

Fig 3 shows dose dependent increase in release of priming factors from MNC stimulated with increasing concentrations of LPS. The release of TNF-alpha shows exactly the same relationship. Medium containing LPS also primes neutrophils for their subsequent response to stimulation with ZAS even though the PMN were exposed to this medium for only 15 min at 4°C. However, Fig 4 shows that MNCM generated by stimulation of MNC with increasing concentrations of LPS produce far more priming than medium containing LPS alone. The optimum concentration for discrimination between MNCM and LPS was 1 μg/ml.

TNF-alpha

The data presented in Figs 2 and 3 shows a close correlation between production of priming factors and release of TNF-alpha into the MNCM. Consequently the recombinant cytokines TNF-alpha IL-1 beta, and GM-CSF were tested for their ability to prime PMN. Of these only TNF-alpha primed at 4°C for 15 min and 1000 U/ml TNF-alpha reproduced the priming effect of a pool of MNCM (data not shown). While recombinant GM-CSF had no effect at 4°C when the experiment was repeated at 37°C for 60 min there was slight dose dependent priming with 76% enhancement in superoxide production in response to ZAS (data not shown); this may be compared with the priming effect of TNF-alpha at 4°C for 15 min which produces more than 400% enhancement of superoxide production in response to ZAS.

Further evidence of the quantitative importance of TNF-alpha was produced by addition of a polyclonal antibody to TNF-alpha which reduced the priming effect by 74·8% (r of six separate experiments). The concentration of anti used was sufficient to neutralize completely primin 1000 U ml rh-TNF-alpha. When antibodies were against IL-1 beta and GM-CSF, with a pre-immune seru control, no inhibition of the priming effect was observec 5).

MNCM directly stimulates the PMN respiratory burst

When PMN which have been primed with MNCM for 15 at 4°C were washed, resuspended in RPMI and si warmed to 37°C without any subsequent stimulation release of superoxide begins more quickly and proceeds faster rate than is seen with unprimed PMN stimulated ZAS. Fig 5 shows that this direct effect of MNCM is not d TNF-alpha as it is not blocked by antibody and preincub with recombinant TNF-alpha followed by washing warming causes only very slight activity of the respira burst. LPS does not explain the direct effect as PMN prim a medium containing 1 μg/ml LPS, washed and warme not show any respiratory burst activity. There was als direct effect when recombinant human TNF-alpha ar beta were added to the LPS (data not shown) suggesting the activity observed was not due to an additive or syner effect of these cytokines either with each other or with

DISCUSSION

LPS stimulated MNC produce a large number of cytol which may be important at different times in an inflan

tory response according to the kinetics of their release. We have looked for biological effects of MNC products on PMN, particularly priming of PMN to enhance their response to the second stimulus C5a des arg in ZAS. Priming activity in the supernatant of LPS stimulated MNC appears rapidly and reaches a maximum after 5 h before gradually declining. The priming effect appears to be due to TNF-alpha as this cytokine is released with the same kinetics and dose response as priming activity. Investigations carried out on purified populations of monocytes show the mRNA for both TNF-alpha and IL-1 beta are produced early in LPS stimulated monocytes reaching a maximum at 6 h and then declining, with levels of secreted cytokines being maintained up to 18 h (Burchett *et al*, 1988). Our MNC cultures did not involve purification of the monocyte population, the decreased levels of bioactive TNF after 5–6 h suggests increased production of prostaglandin E_2 (PGE_2) which has been shown to reduce LPS induced functionally active TNF (Spengler *et al*, 1989). In our experiments recombinant TNF-alpha but not IL1-beta reproduced the priming effect of MNCM and priming by MNCM was almost completely abrogated by a polyclonal antibody to TNF-alpha. GM-CSF is produced later than TNF-alpha and IL1-beta by the action of the cytokines on T lymphocytes and possibly monocytes within the MNC. Although GM-CSF is claimed to be a potent priming factor (Fleischmann *et al*, 1986; Fletcher & Gasson, 1988) it had no effect on subsequent response to C5a when incubated with MNC at 4°C followed by washing and warming; it had very little effect at 37°C even after 60 min incubation. The data suggests that TNF-alpha is the most important priming factor produced in the first few hours after stimulation of MNC.

The fact that PMN are primed following exposure to MNCM for only 5 min at 4°C and that the effect is maximal after only 15 min argues that TNF-alpha binds to its receptor in the cold and is not displaced by washing. Studies using ^{125}I-labelled TNF-alpha support this as binding at 4°C is rapid and reaches a steady state after 20 min (Pichyangkul *et al*, 1987). When the PMN are warmed to 37°C there is an immediate enhancement of response to the second stimulus ZAS. C5a des arg in ZAS was chosen as the second PMN stimulus as complement activation is likely to be an early and transient event when bacteria invade tissue or the blood stream. How TNF-alpha primes the response to C5a des arg is not known. The C5a receptor is expressed on PMN and there is no evidence for translocation of an intracellular pool to the cell's surface as occurs with the CR3 receptor. TNF-alpha has been shown to prime for stimulation with FMLP by changing the affinity but not the number of receptors (Atkinson *et al*, 1988) and the same may apply to C5a receptors. It is also possible that the TNF affects the transduction pathway as the fact that cells can be primed for increased responses to phorbol esters (Berkow *et al*, 1987; Berkow & Dodson, 1988) suggests that protein kinase C, the phorbol ester receptor, is translocated into the cell membrane.

The LPS used in these experiments to stimulate release of cytokines from MNC was also capable of priming PMN at 4°C for subsequent enhanced response to C5a after washing and warming. The kinetics of its action differed from TNF-alpha as the effect continued to increase with continuing incubation at 4°C after 15 min. The fact that PMN were primed under these conditions indicates that LPS, like TNF-alpha, combines with a receptor on the surface of PMN and there is evidence to suggest that it actually binds to part of the CR3 receptor (Wright *et al*, 1989).

Incubation at 4°C for 15 min with either LPS or recombinant TNF-alpha had only a very slight effect on superoxide anion production after washing and warming unless the second stimulus, ZAS, was applied. By contrast, incubation in the cold with MNCM followed by washing caused the production of superoxide to begin more quickly and to proceed at a faster rate when the cells were warmed even without addition of a second stimulus. This direct effect of MMCM was actually greater than the response of PMN which had been incubated with LPS in the cold and then stimulated with ZAS. It could not be explained either by LPS or TNF-alpha as preincubation with either of these had only a very slight effect on superoxide anion production when incubation with them in the cold was simply followed by washing and warming. The data suggests that there is another factor produced by MNC after stimulation with LPS for 5 h; this factor also binds to PMN in the cold, is not displaced by washing and has a direct stimulatory effect when the cells are warmed. Furthermore, it seems unlikely to prime as the priming activity in MNCM was almost completely abrogated by polyclonal antibody to TNF-alpha. The likely identity of this direct stimulant of PMN respiratory burst is neutrophil activating factor (NAF) sometimes called IL-8. Thelen *et al* (1988) and Baggiolini *et al* (1989), reported stimulation but, more recently, data from Djeu *et al* (1990) could not show IL-8 to be a stimulant of PMN superoxide anion production. However, NAF/IL8 is produced by MNCM in response to IL-1 beta and TNF-alpha and although it is released into the supernatant later than these cytokines it should be present after incubation with MNC with LPS for 5 h (Yoshimura *et al*, 1987a, b; Matsushima *et al*, 1988). NAF/IL8 receptors have been reported and binding should withstand washing to allow stimulation of the respiratory burst when PMN are warmed to 37°C and these were the conditions used in these experiments.

Finally, the figures show wide standard errors and this was due to variation in PMN responses from individual donors while the pattern seen with each individual was consistent (data not shown). These differences may be related to the number or affinity of PMN surface receptors for TNF-alpha and/or C5a des arg. There are varying reports as to the number of TNF-alpha receptors present on human PMN although the findings on affinity binding seem to be consistent (Perussia *et al*, 1987; Pichyangkul *et al*, 1987). These varying reports may reflect real differences between individuals which would explain the differences in our observations but their physiological significance is not known.

ACKNOWLEDGMENT

This work was supported by a grant from the Leukaemia Research Fund and Hoechst (U.K.) Ltd.

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