REGULATION OF NEUTROPHIL FUNCTIONS BY TUMOR NECROSIS FACTOR-ALPHA

by

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A thesis submitted to the University of Adelaide in fulfilment of the requirements for the degree of Doctor of Philosophy.

Department of Medicine,
University of Adelaide,
October, 1989.

For my parents.

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DECLARATION

I hereby declare that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text. No part of this thesis has been submitted to any other University for any degree or diploma. I consent to this thesis being made available for photocopying and loan, if accepted for the award of the degree.

Y.H. ATKINSON

PREFACE

Aspects of the work herein have been published as follows:

JOURNAL ARTICLES:

ATKINSON, Y.H., Marasco, W.A., Lopez, A.F., and Vadas, M.A. (1988) Recombinant human tumor necrosis factor-alpha regulation of f Met-Leu-Phe receptor affinity and function on human neutrophils. *J. Clin. Invest.* **81**:759-765

ATKINSON, Y.H., Lopez, A.F., Marasco, W.A., Lucas, C.M., Wong, G.G., Burns, G.F., and Vadas, M.A. (1988) Recombinant human granulocyte-macrophage colony-stimulating factor (rH GM-CSF) regulates f Met-Leu-Phe receptors on human neutrophils. *Immunol.* **64**:519-525

ATKINSON, Y.H., Marasco, W.A., Lopez, A.F., and Vadas, M.A. (1989) The regulation of human neutrophil responses to f Met-Leu-Phe by GM-CSF. *Progress In Leukocyte Biology* (in press)

ATKINSON, Y.H., Krilis, S., Murray, A.W., Vadas, M.A., and Lopez, A.F. Human tumor necrosis factor-alpha directly stimulates arachidonic acid release in human neutrophils. (submitted)

REVIEW:

Vadas, M.A., Lopez, A.F., Shannon, M.F. and ATKINSON, Y.H. (1987) Human myeloid growth factors. *Aust. J. Immunol. & Cell Biol.* **65**:141-145.

ABSTRACTS FOR CONFERENCES:

ATKINSON, Y.H., Marasco, W.A., Lopez, A.F., and Vadas, M.A. Recombinant human granulocyte-macrophage colony-stimulating factor and tumor necrosis factor-alpha regulate the expression of f Met-Leu-Phe receptors on human neutrophils. Australian Society of Immunology, Newcastle, December, 1986.

ATKINSON, Y.H., Vadas, M.A., and Lopez, A.F. Recombinant human tumor necrosis factor-alpha. Regulation of f Met-Leu-Phe receptor expression and function on human neutrophils. Eleventh International RES Congress, Hawaii, October, 1987.

ATKINSON, Y.H., Lopez, A.F., and Vadas, M.A. Tumor necrosis factor-alpha and cytochalasin B. Similarities in their regulation of neutrophil function and binding of f Met-Leu-Phe. Australian Society of Immunology, Canberra, February, 1988.

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ABBREVIATIONS

$^1O_2$ - singlet oxygen

$[Ca^{2+}]_i$ - intracellular free calcium

ADP - adenosine 5'-diphosphate

ATP - adenosine 5'-triphosphate

BHT - butylated hydroxytoluene

BSA - bovine serum albumin

cAMP - cyclic adenosine monophosphate

CGD - Chronic granulomatous disease

CHS - Chediak-Higashi syndrome

CSF - colony-stimulating factor

CTC - chlortetracycline

DAG - diacylglycerol

EGTA - ethylene glycol- bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid

ETYA - 5,8,11,14-eicosatetraynoic acid

f MET-LEU-PHE - *N*-formylmethionylleucylphenylalanine

FAD - flavin adenine dinucleotide

FCS - foetal calf serum

G-CSF - granulocyte colony-stimulating factor

GM-CSF - granulocyte-macrophage colony-stimulating factor

GTP - guanosine 5'-triphosphate

H - human

H_2O_2 - hydrogen peroxide

HBSS - Hanks' balanced salt solution

HEPES - *N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

HETE - hydroxyeicosatetranoic acid

HPETE - hydroperoxyeicosatetranoic acid

HPLC - high performance liquid chromatography

IL - interleukin

IP_3 – inositol trisphosphate

Kd - apparent dissociation constant

LPL - lipoprotein lipase

LPS - lipopolysaccharide

LT - leukotriene

M-CSF - macrophage colony-stimulating factor

MPO - mycloperoxidase

NAD - nicotinamide adenine dinucleotide

NADH - nicotinamide adenine dinucleotide, reduced form

NADPH - nicotine-adenine dinucleotide phosphate

NBT - nitroblue tetrazolium

NDGA - nordihydroguaiaretic acid

O_2^- - superoxide anion

OAG - 1-*O*-alkyl-2-acyl-*sn*-glycerol

OD - optical density

$OH^{\cdot}$ - hydroxyl radical

PAF - platelet-activating factor

PBS - phosphate buffered saline

PG - prostaglandin

PI - phosphatidylinositol

PIP_2 - phosphatidylinositol-4,5-bisphosphate

PMA - phorbol 12-myristate 13-acetate

r - recombinant

SOD - superoxide dismutase

TCA - trichloroacetic acid

TNF-α - tumor necrosis factor-alpha

TPA - 12-*O*-tetradecanoylphorbol-13-acetate

ZAS - zymosan activated serum

SUMMARY

Neutrophils are essential for the successful development of an inflammatory reaction, and a defect in any of their inflammatory functions reduces their microbicidal capacity. Since TNF-α is produced by macrophages at the site of infection, its influence on neutrophil functions was studied.

In the first series of experiments, two functions were studied - the generation of superoxide anion, and neutrophil locomotion. rH TNF-α was unable to directly induce the release of superoxide anion from neutrophils, but could enhance the response to the chemotactic peptide *N*-formylmethionylleucylphenylalanine (f Met-Leu-Phe). Three characteristics of priming emerge from this study. Firstly, the response to f Met-Leu-Phe varies between donors, and this affects the subsequent degree of enhancement induced by rH TNF-α. Secondly, the cells must be preexposed to rH TNF-α for an enhanced response to f Met-Leu-Phe to occur, and thirdly, washing the rH TNF-α from the cells prior to stimulating them with f Met-Leu-Phe did not abolish priming. Control experiments were performed to determine the specificity of this response. When cells were stimulated in the presence of superoxide dismutase, priming by rH TNF-α was abolished, indicating that neutrophils specifically increase their production of superoxide anion in response to priming by rH TNF-α. The possibility of contamination by LPS was discounted by incubating the cells in the presence of boiled and untreated rH TNF-α. It was found that the primed response, but not the response to f Met-Leu-Phe was abolished by boiling rH TNF-α. To define whether priming was confined to the f Met-Leu-Phe response, the effect of rH TNF-α was tested on two other activators of neutrophil function. Preincubation with rH TNF-α enhanced the production of superoxide anion in response to zymosan activated serum (crude C5a), but not to phorbol myristate acetate.

Another aspect of neutrophil function required for the development of an inflammatory response is the chemotactic migration from the blood to the site of infection. Although rH TNF-α was not itself chemotactic for neutrophils, preincubation with this cytokine inhibited the chemotaxis of neutrophils towards a source of f Met-Leu-Phe. The inhibition of chemotaxis was dependent on both the concentration of rH TNF-α used, and the time of preincubation. In addition, the inhibition of chemotaxis was not confined to f Met-Leu-Phe, because rH TNF-α was also shown to inhibit the migration towards a gradient of zymosan activated serum.

The second series of experiments were designed to further define the influence of rH TNFα on neutrophil superoxide generation and chemotaxis, and attempt to define the mechanism by which rH TNF-α enhanced superoxide production but inhibited chemotaxis. rH TNF-α was found to enhance superoxide production in a concentration-dependent manner. In parallel experiments, a titration of f Met-Leu-Phe in the presence or absence of rH TNF-α revealed that neutrophils generate more superoxide anion at concentrations of f Met-Leu-Phe from 10^{-6} to 10^{-8}M. As with the inhibition of chemotaxis, enhancement of superoxide generation by rH TNF-α occurred in a time -dependent manner, and exhibited similar kinetics when compared to the chemotactic response. Unstimulated neutrophils possess f Met-Leu-Phe receptors with high and low affinities, which are thought to be responsible for the chemotactic and superoxide reponses respectively. The regulation of f Met-Leu-Phe receptor expression on human neutrophils was therefore studied. Upon incubation with rH TNF-α, the high affinity f Met-Leu-Phe receptor was lost, and a single lower affinity receptor population was expressed. Although the affinity of the receptors was altered, the total number of f Met-Leu-Phe receptors remained unchanged. The changes in receptor expression were consistent with the increase in superoxide production and the decrease in chemotactic responsiveness.

To investigate whether the regulation of superoxide generation, chemotaxis, and f Met-Leu-Phe receptor expression was unique to rH TNF-α, the effect of recombinant human

granulocyte-macrophage colony-stimulating factor (rH GM-CSF) on neutrophil responses and receptor expression was studied. As with rH TNF-α, rH GM-CSF enhanced the respiratory burst in a concentration- and time-dependent manner. Preincubation with rH GM-CSF also inhibited chemotactic migration in a time-dependent manner, which paralleled that shown for superoxide anion generation. F Met-Leu-Phe receptor affinity but not number, was also altered in a manner similar to that shown for rH TNF-α. Interestingly, the time course of change in receptor expression was similar to the time courses for superoxide generation and chemotaxis. These observations imply that the regulation of f Met-Leu-Phe receptor affinity may contribute to the regulation of neutrophil functions by rH TNF-α.

The other microbicidal mechanism of neutrophils is the release of lysosomal enzymes into phagosomes and the extracellular environment. To further define the regulation of neutrophil function by rH TNF-α, its influence on neutrophil degranulation was studied. In contrast to superoxide anion production, rH TNF-α directly stimulated neutrophil degranulation. Preincubation with rH TNF-α also enhanced the response to f Met-Leu-Phe, and although the effect was additive, it varied amongst donors as for superoxide generation. Preincubation with rH TNF-α influenced neutrophil degranulation in response to f Met-Leu-Phe in a concentration-dependent manner, and exhibited an additive effect at all f Met-Leu-Phe concentrations tested. The cell surface expression of the granule associated receptor for C3bi (CR3) was also studied. As with degranulation, rH TNF-α directly stimulated the expression of this molecule, but the effect was never as potent as that observed for f Met-Leu-Phe.

Neutrophil degranulation is dependent on extensive actin rearrangements within the neutrophil cytoskeleton, therefore the effect of rH TNF-α on actin polymerisation was studied. Incubation of neutrophils with rH TNF-α stimulated the rapid polymerisation of actin which peaked within 10 seconds and depolymerised within two minutes. Although rH TNF-α-induced polymerisation was kinetically similar to f Met-Leu-Phe-induced actin

polymerisation, both the magnitude of the response and the depolymerisation time were always less. The pattern of actin polymerisation was similar between rH TNF-α and f Met-Leu-Phe, and a titration of rH TNF-α revealed that this effect could only be detected at 1000 and 100 u/ml, which is higher than that required for other functions. In addition to a direct effect on actin polymerisation, preincubation with rh TNF-α also enhanced f Met-Leu-Phe-induced actin polymerisation in a concentration-dependent manner.

The neutrophil functions that rH TNF-α directly affects, i.e. cell surface receptor expression, degranulation and actin polymerisation, all require some alteration in the lipid composition of the cells. In addition to this, certain neutrophil lipids act as second messengers in the signal transduction pathways. Therefore, to determine whether rH TNFα could influence lipid metabolism, the release of arachidonic acid from neutrophils was studied. Incubation with rH TNF-α induced the release of arachidonic acid from neutrophils. Interestingly, this effect was equipotent with that observed for f Met-Leu-Phe, and appears to be confined to the cytokines that enhance superoxide production in response to f Met-Leu-Phe. As with the other effects observed, the stimulation of arachidonic acid release from neutrophils was both time- and concentration-dependent. Of particular note, the time course of rH TNF-α-induced arachidonic acid release appears to be temporally related to that observed for the enhancement of superoxide anion generation and inhibition of chemotaxis. In addition, although rH TNF-α induces the release of arachidonic acid from neutrophils, it does not appear to stimulate its metabolism. These observations suggest a possible mechanism of priming of peripheral blood neutrophil functions by rH TNF-α.

CHAPTER 1

INTRODUCTION

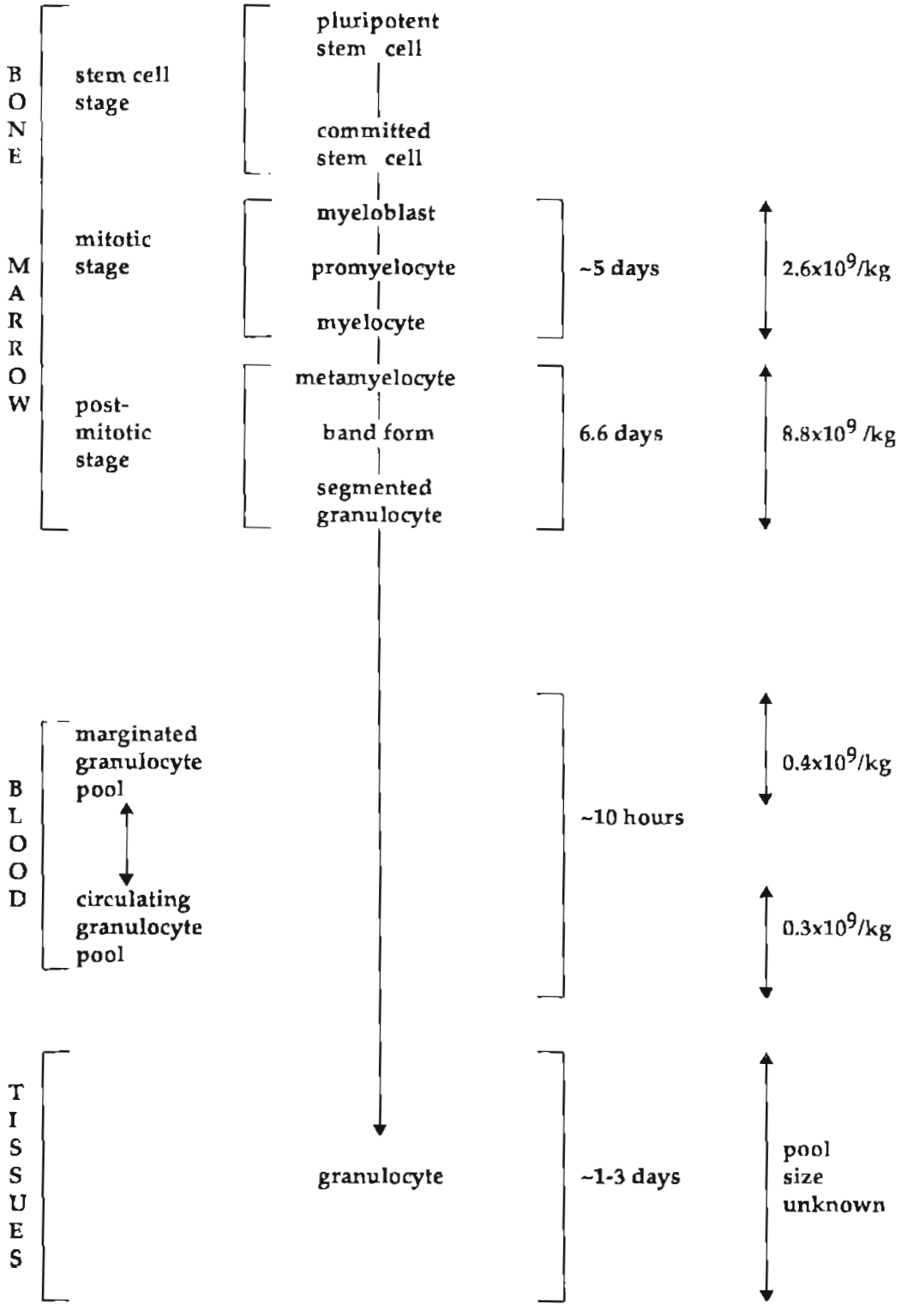
SECTION 1.1 GENERATION OF PERIPHERAL BLOOD NEUTROPHILS

Human polymorphonuclear neutrophils are cells whose life is spent in three environments: bone marrow, blood, and tissues. Their primary function is to infiltrate tissues from the blood in order to phagocytose and kill invading micro-organisms. About 55 to 60% of the bone marrow is dedicated to the production of the neutrophil. There have been three major stages of differentiation defined in the bone marrow (Figure 1.1). The pluripotent stem cells constitute the most primitive stage of this process. These cells are capable of either differentiating or self-renewal by proliferation. The second stage of differentiation in the bone marrow consists of progenitor cells which are committed to the neutrophil lineage. Although these cells still have proliferative potential, they can only differentiate into end cells when triggered by specific stimuli. The third stage of granulopoiesis consists of distinct cell populations. These cells are either fully differentiated or are undergoing the last few divisions to reach maturity.

Neutrophils develop in the bone marrow from a myeloblast, which undergoes a process of proliferation and differentiation (Figure 1.1). Proliferation consists of approximately five divisions, and only occurs during the first three stages of neutrophil maturation (blast, promyelocyte, and myelocyte). The cells become "end cells" after the myelocyte stage, i.e. no longer capable of mitosis. They then enter a storage pool within the bone marrow from which they are released into the blood about five days later, where they circulate for about ten hours. Approximately 100 billion neutrophils enter and leave the circulation daily in normal adults (Dancey, et al., 1976: Walker and Willemze, 1980). It is unknown how long they survive in the tissues following emigration, but it is thought that they only last for one to two days. The normal sites of destruction are unknown, but a study by Jamuar and Cronkite (1980) reported that granulocytes tended to concentrate in the spleen.

FIGURE 1.1

Granulocyte maturation occurs in three stages.


BONE MARROW
stem cell stage
mitotic stage
post-mitotic stage
pluripotent stem cell
committed stem cell
myeloblast
promyelocyte
myelocyte
metamyelocyte
band form
segmented granulocyte
~5 days
6.6 days
2.6x10^9/kg
8.8x10^9 /kg
BLOOD
marginated granulocyte pool
circulating granulocyte pool
~10 hours
0.4x10^9/kg
0.3x10^9/kg
TISSUES
granulocyte
~1-3 days
pool size unknown

SECTION 1.2 THE ANATOMY OF THE NEUTROPHIL

The resting neutrophil exhibits a condensed multi-lobed nucleus (most commonly three, but varying from one to five or more) surrounding the centrosome, which is located at the approximate geometric centre of the cell. The centrosome consists of two centrioles from which microtubules radiate outward, and the Golgi complex. Scattered in the periphery of the neutrophil cytoplasm are the azurophilic (primary) granules, and the specific (secondary) granules. Table 1 (page 18) lists the components of human neutrophil granules reported from both cytochemical and isolation techniques. The primary granules are commonly called peroxidase-positive granules and contain a variety of neutral proteases and lysosyme (Spitznagel et al., 1974; Baggiolini, 1972; Strominger and Ghuysen, 1967), β-glucuronidase (Bainton, Nichols and Farquhar, 1976), phospholipase A_2 (Franson, Patriarca and Elsbach, 1974) and cationic proteins (Zeya and Spitznagel, 1966; Odeberg and Olsson, 1975; Drazin and Lehrer, 1977). It was originally reported that primary granules contain the antimicrobial substance lactoferrin (Spitznagel et al., 1974), an observation which has been recently confirmed by Cramer et al. (1985). The secondary granules contain a vitamin B_{12} binding protein (Kane and Peters, 1975), phospholipase A_2 (Weiss et al., 1978), and approximately two-thirds of the cells' lysosyme (Spitznagel et al., 1974). In addition, the specific granules have also been reported to contain the receptors for the chemoattractant *N*-formylmethionyl-leucylphenylalanine (f Met-Leu-Phe, Fletcher and Gallin, 1983; Jesaitis et al., 1982), the receptor for the complement fragment C3bi (CR3 receptor, O'Shea et al., 1985) and laminin (Yoon et al., 1987). There has also been a tertiary granule population tentatively identified, which has been reported to contain gelatinase (Dewald, Bretz and Baggiolini, 1982).

The granules are compartmentalized away from the plasma membrane by a cortical actin network (Southwick and Stossel, 1983). This prevents indiscriminate fusing of the

granules with the cells' membrane and provides the cell with a control mechanism by which to regulate phagocytosis and degranulation. Other organelles within the cell are few because the neutrophil relies primarily on glycolysis. Thus, there are few mitochondria and a limited amount of smooth and rough endoplasmic reticulum, but a large store of glycogen.

SECTION 1.3 ROLE OF THE NEUTROPHIL IN INFLAMMATION

1.3.1 The inflammatory reaction:

The infiltration of cells at the site of infection represents the principal local defense against the spread of infection by invading micro-organisms. Limiting the spread of infection, and the subsequent removal of pathogens results in an inflammatory reaction. The white blood cells involved in an inflammatory reaction are the polymorphonuclear phagocytes - neutrophils, eosinophils and basophils - and mononuclear phagocytes.

The central role of neutrophils in inflammation has been appreciated since the 1950s. Animals depleted of leukocytes could not mount an inflammatory reaction against immune complexes formed in the wall of venules (Stetson, 1951; Cochrane, Weigle and Dixon, 1959). Similarly, in leukopenic rabbits there was a reduction in all inflammatory parameters quantitated after injection of *Escherichia coli* (Kopaniak and Movat, 1983).

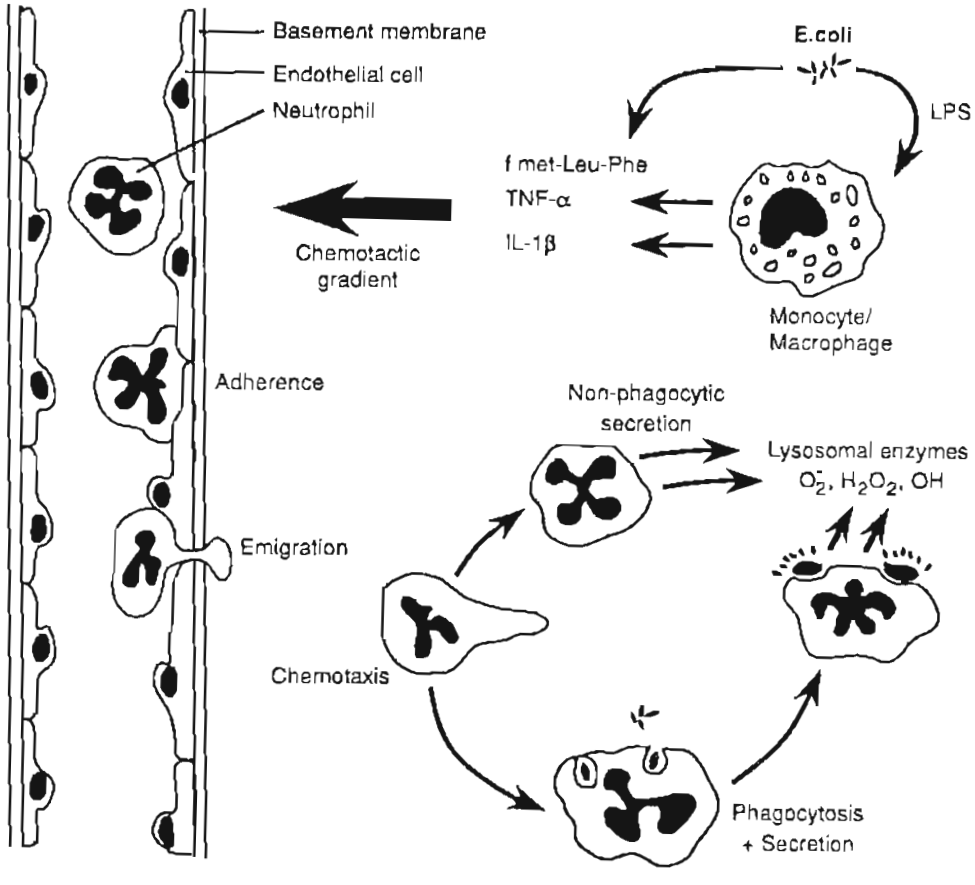
The *in vivo* study of inflammation induced by *E. coli* can be cited as an example of the general phases of inflammation (Movat et al., 1987b). There are five events that can be quantitated, and these are as follows: 1) enhanced blood flow through the dilated microcirculation; 2) enhanced vasopermeability with the exudation of plasma; 3) accumulation of neutrophils; 4) microhemorrhage; and 5) microthrombosis. Thus, neutrophils are the cells that are mobilized in the inflammatory response first. This

migration is thought to be directed by the chemotactic effects of substances generated or released at the site of infection. These include exogenous substances such as bacterial lipopolysaccharide (LPS) and the formylated peptides generated by protein degradation. Endogenous substances produced by monocytes at the site of inflammation include the cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and granulocyte-macrophage colony-stimulating factor (GM-CSF), and the peptide neutrophil activating factor (NAF). These substances have all been reported to be chemotactic for neutrophils (Wang, Bersani and Mantovani, 1987; Cybulsky, Colditz and Movat, 1986; Wang et al., 1987; Thelen et al., 1988). Other endogenous activators of neutrophils include complement, arachidonic acid and its metabolites, such as leukotrienes and prostaglandins and platelet activating factors.

The inflammatory process which develops in response to invasion by *E. coli*, is outlined in Figure 1.2. Essentially, during infection with a gram-negative micro-organism, the release of LPS is thought to induce the production of chemotactic substances, as it is not a chemotaxin itself (Colditz and Movat, 1984). The substances thought to induce neutrophil chemotaxis are the endogenous cytokines IL-1β and TNF-α, GM-CSF, NAF, and the exogenous peptide f Met-Leu-Phe. *In vitro* studies implicate these factors in the increased adhesiveness of neutrophils to endothelial cells (Bevilacqua et al., 1985; Gamble et al., 1985; Gamble et al., 1989; Smith, Gamble and Vadas, unpublished observations; Zimmerman and McIntyre, 1988), and they have all been shown to be chemotaxins (Luger et al., 1983; Wang, Bersani, and Mantovani, 1987; Wang et al., 1987; Peveri et el., 1988; Marasco et al., 1984). *In vivo* studies indicate that IL-1β is the major endogenous mediator of endotoxin-induced inflammation. Thus, shedding of endotoxin stimulates monocytes/macrophages to produce IL-1β and TNF-α, which together with f Met-Leu-Phe induce accumulation of neutrophils at the inflammatory site.

FIGURE 1.2

The inflammatory reaction.


Basement membrane
Endothelial cell
Neutrophil
E.coli
LPS
f met-Leu-Phe
TNF-α
IL-1β
Chemotactic gradient
Monocyte/ Macrophage
Adherence
Non-phagocytic secretion
Lysosomal enzymes
O_2^-, H_2O_2, OH
Emigration
Chemotaxis
Phagocytosis + Secretion

The accumulated neutrophils secrete lysosomal proteases and oxygen metabolites, by phagocytosis of the pathogen, and additional stimulation by both f Met-Leu-Phe and endogenous factors such as those mentioned above. The generation of microbicidal proteases and free oxygen radicals also both act to injure the microcirculation. This causes increased vasopermeability, hemorrhage, and microthrombosis, all of which are side effects of the body's attempt to contain the spread of micro-organisms. The increased blood flow enhances the delivery of neutrophils and therefore enhances microvascular injury. If these events fail to contain the spread of infection, septicemia develops, and eventually the release of such large amounts of endotoxin can cause disseminated vascular coagulation (Movat et al., 1987).

1.3.2 Clinical disorders of neutrophil function:

The importance of the neutrophil in the inflammatory reaction can be appreciated when one considers clinical disorders of neutrophil function. Patients demonstrating an increased susceptibility to infection have been found to possess neutrophils defective in one or more aspects of their normal functions. These patients are particularly susceptible to infection by pyrogenic organisms, demonstrating the importance of neutrophils in the hosts defence against these organisms. The common aspects of infection in these patients is that the infections tend to be frequent and recurrent, but the individual episodes respond well to treatment.

The defects in neutrophil function observed can be attributed both to the cell itself, and to the production of substances necessary for proper cell function. The humoral defects have been well characterized, whereas the molecular basis of many cellular defects in neutrophil function are yet to be defined. Four examples of well-defined cellular defects are chronic granulomatous disease, hereditary myeloperoxidase deficiency, the Chediak-Higashi

syndrome, and the CD-18 deficiency. These will be discussed briefly below to illustrate the importance of neutrophils in the inflammatory process.

1.3.2.1 Chronic Granulomatous Disease:

Chronic Granulomatous Disease (CGD) is a rare genetic disease characterized by severe recurrent infections of the lymph node, skin, lung, liver, and other tissues. The predominant pathogens are *Staphylococcus aureus*, certain gram-negative bacteria and fungi. The condition is due to an inability of the neutrophils and monocytes from these patients to kill certain ingested bacteria and fungi. This defect is associated with impaired metabolic activity in these cells.

The neutrophils of these patients exhibit normal morphology (Quie et al., 1967), chemotaxis (Bridges et al., 1959; Ward and Schlegal, 1969; Snyderman et al., 1975) and phagocytosis (Bridges et al., 1959; Quie et al., 1967). Although they ingest bacteria, the microorganisms remain alive for prolonged periods. The metabolic activity of these cells is low in the following aspects: phagocytosis-induced oxygen consumption (Holmes et al., 1967); H_20_2 formation; superoxide anion formation (Curnutte et al., 1974; Rosen and Klebanoff, 1976); chemiluminescence (Rosen and Klebanoff, 1976); NBT reduction (Baehner and Nathan, 1967,1968); hexose monophosphate shunt activity (Holmes et al., 1967); iodination (Klebanoff and White, 1969); thyroid hormone degradation (Klebanoff and Green, 1973); and estrogen binding (Klebanoff, 1977).

Further studies have elucidated the specific defect in these cells. Segal and Peters (1976) localized an enzyme in the plasma membrane of neutrophils which reduces NBT in the presence of NADH. On studies of CGD patients they found that the NADH dehydrogenase exhibited defective function at low NADH concentrations. In addition, a deficiency of the NADPH oxidase also occurs in CGD patients (Hohn and Lehrer, 1975).

Although these defects have been characterized in a number of patients, there have also been reports of normal enzyme activity in other patients. More recent studies have defined the molecular basis for the disease. Using cell-free activation systems for NADPH oxidase, it has been shown that the membrane fraction is defective in the two cytochrome b-negative forms of CGD (Segal et al. 1983). This has been interpreted as an abnormality in the cytochrome b_{558} which is involved in NADPH oxidase activation.

1.3.2.2 Myeloperoxidase Deficiency:

Myeloperoxidase (MPO) deficiency is characterized by a decrease in the histochemical staining for peroxidase. This deficiency may be hereditary (Lehrer and Cline, 1969) or acquired. In hereditary MPO defieiency, there is a complete absence of MPO from neutrophils and monocytes. Acquired MPO deficiency occurs as a result of another disease. Disease states which have been associated with acquired MPO deficiency include severe bacterial infection (Graham, 1920), acute lethargic encephalitis Economo (Sato and Yoshimatsu, 1925), refractory megaloblastic anemia (Arakawa et al., 1965; Higashi et al., 1965; Lehrer et al., 1972) and leukemia (Catovsky et al., 1972). Acquired MPO deficiency can be permanent or temporary and the MPO levels are either undetectable or reduced.

The selective absence of MPO from the azurophil granules of neutrophils and monocytes occurs in hereditary MPO deficiency (Lehrer and Cline, 1969; Stendahl and Lindgren, 1976; Rosen and Klebanoff, 1976). The absence of MPO is not associated with a reduction in the number of azurophil granules or any other abnormality in the azurophil granules. Eosinophils, basophils and platelets all contain normal amounts of peroxidase, thus this deficiency is only associated with neutrophils and monocytes. The peripheral white blood cell count and differential are normal, as is chemotaxis, phagocytosis and degranulation in patients with hereditary MPO deficiency. Notably, the respiratory burst

of these patients is either normal or elevated, suggesting compensatory microbicidal activity in these cells (Klebanoff and Hamon, 1972).

The deficiency in MPO is associated with a marked reduction in microbicidal activity. In particular, these cells are impaired in their fungicidal and bactericidal activities. Interestingly, the microbicidal defect observed in these patients is not as severe as that associated with CGD. This observation correlates well with the milder clinical course of these patients.

1.3.2.3 Chediak-Higashi Syndrome:

The Chediak-Higashi Syndrome (CHS) is characterized by recurrent and severe pyrogenic infections, partial oculocutaneous albinism, abnormally large granules in all granule-containing cells and a lymphoma-like accelerated phase (Klebanoff and Clark, 1978). The abnormal CHS granules have many of the characteristics of lysosomes, and it is postulated that most of the manifestations of the disease are related either directly or indirectly to the granule abnormality. The recurrent infections associated with CHS are most commonly of the upper and lower respiratory tract, and the skin. Septicemia has also been reported. Interestingly, the causative agents of these infections are predominantly pyrogenic organisms.

1.3.2.4 CD-18 Deficiency:

The structurally and functionally related adhesion glycoproteins Mac-1, LFA-1, and p150,95, each possess a single distinct alpha subunit, but share a common beta subunit (CD-18). Patients possessing a deficiency in the expression of Mac-1, LFA-1, or p150,95 (either individually or in combination) suffer from recurrent or progressive necrotic soft tissue infections, diminished pus formation, impaired wound healing, granulocytosis,

and/or delayed umbilical cord severance (Anderson et al., 1985). The severity of infectious complications among these patients is directly related to the degree of the deficiency in the expression of these molecules. Abnormalities were found in leukocyte mobilization, granulocyte-directed migration, hyperadherence, phagocytosis of C3bi-opsonized particles, and complement- or antibody-dependent cytotoxicity. This syndrome is another illustration of the critical role that granulocytes play in the successful development of an inflammatory reaction.

SECTION 1.4 ASPECTS OF NEUTROPHIL FUNCTION

As mentioned in Section 1.3, the general phases of inflammation involve the activation of a variety of neutrophil functions. The development of a successful inflammatory response is dependent on the expression of these functions, and a defect in any aspect of such functions leads to the development of clinical disorders. Certain aspects of neutrophil function will be discussed in detail below.

1.4.1 Locomotion:

An acute inflammatory response involves the mobilization of neutrophils from the bone marrow followed by migration of these cells from the circulation into the site of infection. This directed migration, termed chemotaxis, is essential for the elaboration of an inflammatory reaction, and the effective removal and containment of invading micro-organisms.

Cells are continuously moving within their environment, and changes in the environment therefore affect cell motility. Random motility is an inherent property of neutrophils, and their exposure to a uniform concentration of chemoattractant results in stimulated random movement. This movement is termed chemokinesis, and differs from chemotaxis in that

the cells do not move directionally. Thus, there are three definable aspects of cell motion random movement; chemokinesis; and chemotaxis.

1.4.1.1 Random movement:

Keller et al. (1977) have defined random movement as follows: 'Motion in which the cell or organism shows a tendency to move along a path that can be represented by a line segment, but that is randomly orientated in relation to the environment'. Although the kinetics of random movement have been proposed as an example of the Brownian random walk, it is clear that neutrophils do not move in this manner. A study of the random movement of neutrophils by Allan and Wilkinson (1978) revealed that if cells were studied over a brief time span (a minute or two for neutrophils) their paths tend to be directional. However, if the cells are studied during longer time intervals, their path appears to be more random, and this is proportional to the time interval involved. This type of movement is termed the 'persistent random walk' and appears to be characteristic of all tissue cells studied.

Alterations in the random movement of cells is dependent on environmental stimuli. Such stimuli can alter either the speed of the cells, their turning behaviour, the direction of movement, or all three aspects of locomotion. The two basic stimuli which can alter cell locomotion are either the chemical or the physical properties of their environment.

1.4.1.2 Chemokinetic movement:

Chemokinesis of neutrophils is a random response to chemical substances in the environment. Although other features of the environment, e.g. adhesiveness or temperature, may alter the speed or turning behaviour of the cells, these aspects would be considered as kinetic rather than chemokinetic. Chemokinesis can be simply defined as

the stimulated random movement of neutrophils, where the speed or turning behaviour of the cells is altered, but the direction of overall movement is not (Keller et al., 1977).

Most of the chemokinesis induced by chemotactic factors on neutrophils is due to a change in cell direction and cell speed, causing the cells to accelerate (Zigmond and Sullivan, 1979). A study of chemokinesis by Wilkinson et al. (1984) indicates that negative chemokinesis can cause cell accumulation at the site where cells move most slowly. This observation could be important in the *in vivo* sense when one considers the effect of migration inhibitory cytokines on local cell accumulation at inflammatory sites.

1.4.1.3 Chemotactic movement:

Whereas chemokinesis is stimulated random movement, chemotaxis is a directional response of neutrophils to substances in their environment. The chemotactic response in neutrophils is characterized by two essential features. Firstly, neutrophils orientate towards the chemotactic source by putting out a leading lamellipodium in the direction facing the gradient source. The lamellipodium is typically ruffled and motile, and devoid of organelles. Behind the lamellipodium is the cell body which includes all the organelles and remains rounded or tapered. Although the chemotactic gradient determines the direction of polarisation, these morphological changes are characteristic of all types of locomotion.

The second characteristic of the chemotactic response is the directional movement of the cells up the gradient following orientation. The major effect of the chemotactic gradient is to narrow the angles of turning and accelerate cell speed (Zigmond, 1974). It appears that cells detect gradients through temporal adaptations (Zigmond and Sullivan, 1979). Thus, movement is due to the capacity of receptors moving forward along the lamellipodium to detect concentration differences at different points in time. A recent report by Walter and

Marasco (1987) supports this theory in that they detect a clustering of formyl peptide receptors over the anterior portion of polarised neutrophils.

1.4.1.4 Contact guidance:

Another aspect of the movement of neutrophils is the influence of the substratum on the direction of movement. This property of cell movement has been termed 'contact guidance' by Weiss (1961) who first proposed it. This concept was expanded in a report by Carter (1965), who proposed that neutrophil motility is controlled by the relative strengths of its peripheral adhesions. Carter coined the term "haptotaxis" to describe this phenomenon. A study by Wilkinson, Shields and Haston (1982) revealed that neutrophils tended to travel up and down parallel fibres of collagen or fibrin in three dimensional fibrous gels. Thus, the cells moved directionally, but movement was bidirectional rather than unidirectional, as for chemotaxis. Contact guidance can alter chemotactic responses by either enhancing or interfering with them (Wilkinson and Lackie, 1983) and, therefore, the choice of *in vitro* assay could well influence the outcome of experiments.

1.4.2 Phagocytosis:

In a temporal sense, the second neutrophil function stimulated by invading micro-organisms is phagocytosis. An essential feature of this process is the establishment of contact between the cells and particulate matter. Therefore, phagocytosis can only occur once the neutrophils have accumulated at the site of infection.

The process of phagocytosis can be separated into several stages. These are as follows: 1) opsonization of the particles by serum factors; 2) recognition and attachment of the opsonized particles to the cell surface; 3) engulfment of these particles; 4) intracellular

killing of micro-organisms; and 5) the digestion of micro-organisms and other ingested matter.

1.4.2.1 Recognition and attachment:

Opsonization is achieved by covering the particles with opsonins such as IgG, C3b, and C3bi. Binding of IgG exposes its Fc portion to the phagocytes, which then bind via their Fc receptors (Fleit, Wright and Unkeless, 1982). The binding of IgG to antigen activates the classical pathway, leading to the generation of C3b and C3bi. IgM has no opsonic capacity, but binding of IgM activates the complement system. C3b and C3bi can also be produced through activation of the alternative pathway. C3b is deposited on the particles, which is then recognized by phagocytes via their C3b receptors (CR1, Fearon, 1980) Furthur enzymatic cleavage exposes C3bi sites which are recognized by the C3bi (CR3) receptor on phagocytes. The recognition of particles opsonized with IgG, C3b, and C3bi is the main mechanism by which neutrophils ingest foreign material.

1.4.2.2 Ingestion:

The mechanism underlying the ingestion of particles by macrophages was studied by Silverstein, Steinman, and Cohn (1977). These studies showed that, following specific binding of the particle, the phagocyte membrane surrounds it. When the tips of the pseudopodia surrounding the particle fuse together, the particle is enclosed in a phagosome. During and after formation of the phagosome, adjacent lysosomes fuse with it and release their contents, thus forming a phagolysosome.

1.4.2.3 Killing of micro-organisms:

During, and after, the process of ingestion, several mechanisms that result in the destruction of the ingested micro-organisms are initiated. These mechanisms centre around two major events: the fusion of the lysosomes with phagosomes and the plasma membrane; and the generation of highly toxic products of oxygen reduction. These microbicidal mechanisms will be discussed further below. The actual killing and digestion of micro-organisms is mediated through the combined action of the granule contents and the oxidizing agents released.

1.4.3 Degranulation:

1.4.3.1 Lysosomal enzymes:

Two aspects of lysosomal mobilisation occur during phagocytosis. The lysosomes either fuse with the phagosome or fuse with the plasma membrane. The release of lysosomal enzymes into the phagosome results in the degradation of the ingested micro-organism, whereas liberation of lysosomal enzymes into the environment also causes damage to the surrounding cells and tissues. Thus degranulation and phagocytosis are interrelated events in the the activation of neutrophils during the inflammatory process.

Fusion of the lysosomes with phagosomes is the mechanism by which microbicidal activity is expressed during phagocytosis. In addition, extracellular organisms are destroyed by the combination of lysosomal fusion with the plasma membrane and the liberation of toxic products of oxygen metabolism.

The lysosymes of neutrophils contain a variety of potentially microbicidal substances which are summarized in Table 1.

Table 1

Constitutents of azurophil and specific granules from human neutrophils.

Azurophil granules	Specific granules
Microbicidal enzymes	
Myeloperoxidase	
Lysosyme	Lysosyme
Neutral proteases	
Elastase	
Cathepsin G	
Protease 3	
Acid hyrolases	
b-glycerophosphatase	Collagenase
b-glumonidase	
N-Acetyl-b-glucosaminidase	
a-Mannosidase	
Cathepsin B	
Cathepsin D	
Other	
Cationic proteins	Vitamin B_{12}-binding proteins
Defensins	Plasminogen activator
Bactericidal permeability increasing protein (BPI)	Histamine
Azurophil-derived bactericidal factors (ADBF)	Receptors for-
f Met-Leu-Phe	CR3 (C3bi)
	Laminin
	Cytochrome b
	Lactoferrin
	Flavoproteins

Firstly, lysosyme is one of a group of enzymes which hydrolyse bacterial cell walls (Baggiolini, 1972). It specifically attacks the β-1-4 glycosidic linkage that joins *N*-acetyl muramic acid and *N*-acetyl glucosamine in the murein backbone of peptidoglycan (Strominger and Ghuysen, 1967). Thus, lysosyme is only active against bacteria, and a wide variety of micro-organisms are sensitive to its action (Salton, 1957). Despite this, there are also a variety of microorganisms such as group A streptococci, staphylococci,

and almost all gram-negative organisms, which resist the action of lysosyme unless treated concomitantly in some other manner (Miller, 1968; Glick, Ranhard and Cole, 1972; Efrati et al., 1976). Because most bacteria of clinical significance are resistant to lysosyme itself, the major function of this enzyme may be to digest microorganisms once they have been phagocytosed, rather than acting as a primary bactericidal agent (Elsbach, 1980).

Neutrophil granules also contain a number of neutral proteases such as serine esterases, elastase (Janoff, 1972) collagenase (Harris and Crane, 1974) and cathepsin G (Barrett, 1974). Elastase is capable of degrading the cell walls of heat-killed staphylococci but is not very effective against live organisms (Janoff and Blondin, 1973; Thorne, Oliver and Barrett, 1976). Cathepsin G is weakly bactericidal against staphylocci and can alter some gram-negative species so that they are readily attacked by lysosyme (Thorne, Oliver and Barrett, 1976). As with lysosyme, the role of the proteases appears to be in post-phagocytic digestion rather than as an antimicrobial (Elsbach, 1980).

Lactoferrin is another component of neutrophil granules with potential microbicidal properties. This protein has a high affinity for iron (Masson, Heremans and Schonne, 1969), and is thought to act in the extracellular environment rather than the phagosome of the neutrophil. This theory is supported by two observations. Klempner et al. (1978) have shown that small quantities of LPS induce the release of about one-third of total neutrophil lactoferrin into the extracellular media. In addition, Leffell and Spitznagel (1975) reported that 86% of the lactoferrin discharged during phagocytosis is secreted into the extracellular media rather than into the phagosome. It is still unknown whether lactoferrin acts concomitantly or not with other neutrophil microbicidal mechanisms.

The cationic proteins of neutrophils were originally isolated by Hirsch (1956) as a complex which he called phagocytin. Zeya and Spitznagel (1966) subsequently demonstrated that phagocytin was a complex of arginine-rich cationic proteins which, when separated into

individual components, could each demonstrate bactericidal activity for different bacterial species. Cationic proteins have also been demonstrated to be antifungal (Drazin and Lehrer, 1977) and active against gram-negative rods (Weiss et al., 1978). All of these proteins are active at neutral pH and rapidly interfere with replication without destroying the structural integrity of the micro-organism.

Another potential microbicidal activity of neutrophils is the increase in the acidity of the phagosome. The pH of phagosomes has been estimated to vary from 3.0 to 6.0 depending on the species studied (Mandell, 1970; Jensen and Bainton, 1973). The cause of the pH change could be due to a number of events. Two possibilities are that it results from the production of lactate during glycolysis, which is necessary for phagocytosis (Kakinuma, 1970), or from the fusing of lysosomes containing acid substances. The change in pH has two advantages. Firstly, some bacteria are killed by organic acids in acid media (Dubos, 1953) and, secondly, the acidification may optimize the activities of other microbicidal enzymes such as the myeloperoxidase/H_2O_2/halide system.

1.4.3.2 Lysosomal fusion with phagosomes:

Formation of the phagolysosome during phagocytosis is limited by the surrounding assembly of contractile proteins (Southwick and Stossel, 1983). As these elements are removed from the phagosome, lysosomes can be seen fusing with the phagosome and releasing their contents (Weissmann et al., 1971; Zucker-Franklin and Hirsch, 1964). Most of the granule fusion occurs with the phagosomal rather than the plasma membrane during phagocytosis. Specific granules fuse with the phagosomes earlier than azurophilic granules (Bainton, 1973; Bentwood and Henson, 1980; Henson, 1971), but this may simply be due to the fact that there are more specific granules present in the cell.

1.4.3.3 Lysosomal fusion with the plasma membrane:

The phagocytosis of particles by neutrophils also induces the fusion of lysosomes with the plasma membrane as a controlled exocytotic process (Hawkins, 1972; Becker and Henson, 1973). The actual molecular events that occur during exocytosis of granules are still unknown. Henson et al. (1988) have defined four events in the fusion process. These are: (i) an intracellular signal causes the translocation of secretory vesicles to the inner surface of the plasma membrane; (ii) repulsive forces are overcome enabling contact of the granule with the plasma membrane; (iii) the membrane bilayers undergo a local destabilization; and (iv) fusion of the two membranes occurs with establishment of the membrane structure, resulting in the exocytosis of granule contents whilst maintaining membrane integrity.

The intracellular signals required for exocytosis have yet to be fully defined, but there is a range of data to support calcium as an essential signal in this process. Lew et al. (1986) have reported that an increase in intracellular calcium up to 200-300nM is the required threshold for exocytosis in ionomycin-treated neutrophils. Although calcium appears to be central to the exocytotic process, a recent report by Nüße and Lindau (1988) indicates that this is a regulatory rather than initiatory step. These workers investigated the dynamics of exocytosis in single human neutrophils using time-resolved patch-clamp capacitance measurements. They reported that GTPγS-stimulated exocytosis was independent of the intracellular-free calcium concentration between 10nM and ~2.5μM. In addition, an elevation of intracellular-free calcium to the micromolar range induced limited fusion in the absence of GTPγS. Such a role for GTP binding proteins in the control of exocytosis is supported by other evidence discussed by Burgoyne (1987). GTP analogs could stimulate secretion in the absence of calcium, even if phospholipase C activation was prevented by pretreatment of the cells with pertussis toxin before permeabilisation.

Since the granules must necessarily interact with the cytoskeleton during translocation to the cell surface, investigators have studied the involvement of the cytoskeleton in neutrophil exocytosis. Because the cytoskeleton appears to play an important role in a number of neutrophil functions, it will be discussed as a separate section.

1.4.4 The neutrophil cytoskeleton:

Neutrophil functions such as locomotion, pinocytosis, phagocytosis and exocytosis all involve reorganization in the distribution of plasma membrane and cytoplasmic components. Such leukocyte motility is dependent on the integrity of the network of proteins comprising the cytoskeleton, because disruption of these components influences the subsequent response.

1.4.4.1 Cytoskeletal structure:

The cytoskeleton of neutrophils is composed of three major structural protein systems and a number of regulatory proteins. Actin is the most abundant component of the cytoskeleton and comprises the microfilaments. Associated with the microfilament system are a number of regulatory proteins which serve to modulate actin assembly as described below. These proteins and their functions are listed in Table 2.

The major concentration of microfilaments and associated proteins in leukocytes, occurs in the peripheral cytoplasm of the neutrophil. They were detected by staining of neutrophils by immunofluorescence, immunoperoxidase and immunogold techniques with antibodies specific for actin and its associated proteins (Oliver, Lalchandani, and Becker, 1977; Valerius et al., 1981; and Yin, Albrecht, and Fattoum, 1980).

Table 2

Microfilament-associated regulatory proteins.

Protein	Function *in vitro*
Actin-binding protein (ABP)	Orthogonal gelation of actin filaments
α-Actinin	Bundling gelation of actin filaments
Profilin	Actin monomer sequestration
Gelsolin	(i) Blockade of the "fast" ends of actin filaments (ii) Severing of actin filaments (iii) Nucleation of actin filament growth
Acumentin	Actin monomer sequestration by blockade of the "slow" ends of actin filaments
Tropomyosin	Stablization of actin filaments
Myosin	Contraction

The other known cytoskeletal protein systems are the microtubule and intermediate filaments. Microtubules are composed of tubulin polymers (Malawista and Bensch, 1967) which radiate from the centriole of the cell. The centrosome determines cell polarity and neutrophils reorient their axis between the nucleus and the leading lamella in response to stimulation (Malech, Root and Gallin, 1977). Intermediate filaments are assemblies of vimentin whose function has yet to be fully defined. Geiger (1987) recently proposed a role for intermediate filaments in the specific interaction between elements of the cytoplasmic matrix and peripheral elements of the nuclear matrix. This proposal is based on several lines of evidence and has yet to be investigated.

Although the function of all three protein systems of the cytoskeleton have yet to be fully defined, it is known from both *in vivo* and *in vitro* studies that these systems can interact (Griffith and Pollard, 1978; Runge et al., 1981; and Schliwa and van Blerkom, 1981).

1.4.4.2 Motor component of the cytoskeleton:

The region of the cell which changes during the movements required for the functions above, is the cytoplasm in the periphery. This cytoplasm appears glassy when viewed under the light microscope, and is termed the hyaline cortex, or the "motor" region of the cell (Southwick and Stossel, 1983). During locomotion, phagocytosis and degranulation, this region expands or contracts and varies in its appearance of fluidity. The principal structure of the hyaline cortex of neutrophils is the microfilaments (Boyles and Bainton, 1979). These filaments are composed of actin, and it is the changes in the degree of polymerisation of this protein which affects the fluidity of the hyaline cortex. The regulation of actin polymerisation occurs via calcium control and a number of regulatory proteins.

Actin is thought to be responsible for the mechanical responses of the cells for two major reasons. Firstly, microfilaments are the prominent component of the hyaline cortex, whereas microtubules and intermediate filaments are generally confined to the cell body (Boyles and Bainton, 1979; Anderson et al., 1982; Malech, Root and Gallin, 1977; Pryzwansky, Schliwa and Porter, 1983; and Schliwa, Pryzwansky and Euteneuer, 1982). Secondly, the cytochalasins, which are known to react with actin filaments, strongly inhibit leukocyte locomotion and phagocytosis, enhance exocytosis, and cause retraction of the neutrophil cortical cytoplasm (Yahara et al., 1982). In contrast, chemicals that affect tubulin assembly, i.e., colchicine, podophyllotoxin, and vinca alkaloids do not have

profound inhibitory effects on neutrophil mechanical functions, as do the cytochalasins (Keller, Naef, and Zimmerman, 1984).

1.4.4.3 Actin assembly:

Actin is a slightly asymmetrical globular protein with a molecular weight of 42,500 (G-actin). In the presence of neutral salts, it spontaneously assembles into long filaments (F-actin) in a manner similar to the condensation of a gas into a liquid, i.e. below a critical concentration, all actin monomers remain dispersed, but when the critical concentration is exceeded, monomers assemble into filaments. The rate-limiting step in this assembly, is the formation of a nucleus composed of two or three monomers. Therefore, the presence of pre-existing nuclei could have an important effect on the kinetics of actin assembly. After nuclei have formed, elongation begins with the binding of each additional monomer to two neighboring monomers, forming a double-helical filament. The association of monomers into filaments is a second-order reaction which is dependent on the concentrations of monomers and of filament ends (Figure 1.3A). Initial growth is rapid at both ends but, elongation at one end of the filament (fast-growing end) is 5 to 10 times faster than at the other end (slow growing end, Pollard and Mooseker, 1981). Actin monomers also dissociate from each filament end, and the dissociation rate is dependent on the concentration of filament ends.

As the filaments grow, the concentration of monomeric actin decreases until the association rate equals the dissociation rate, and a steady state is achieved. The monomer concentration at which these two rates become equal is the critical monomer concentration. When assembly is complete, the critical monomer concentrations of the two filament ends may be different, i.e. the dissociation constant may be higher than the association constant at the slow growing end, and the monomer association rate may exceed the dissociation rate at the fast growing end (Wegner, 1982). These observations suggest the

FIGURE 1.3

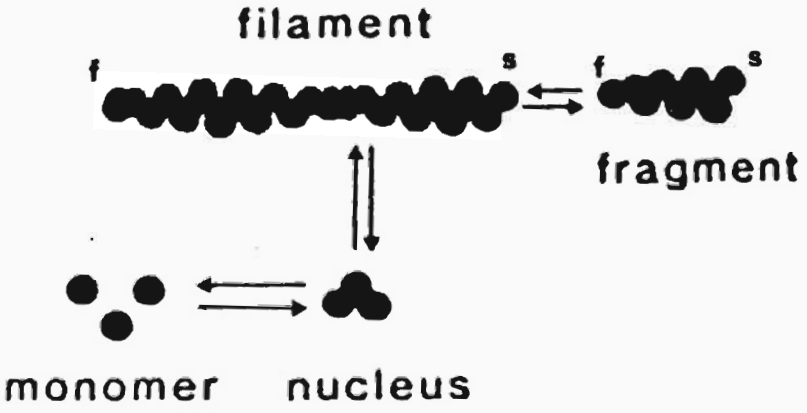
The regulation of actin assembly.

Top. Actin monomers assemble first into nuclei then into filaments. Filaments of actin contain fast-growing (f) and slow-growing (s) ends.

Middle. Profilin binds to actin monomers and prevents their formation into nuclei.

Bottom. Gelsolin and acumentin may control actin assembly by three mechanisms. ***Top)*** At a calcium concentration below μM, gelsolin dissociates from the fast-growing end of actin filaments. This increase in the number of fast-growing ends causes dissociation of acumentin from some of the slow-growing ends, causing annealing of filaments. ***Middle)*** When ionic calcium is in the micromolar range, gelsolin cuts preformed actin filaments and binds tightly to the fast-growing ends. Acumentin binds to the slow-growing ends and inhibits all exchange of actin monomers. ***Bottom)*** Both acumentin and gelsolin can nucleate actin filament growth at micromolar calcium concentrations. This action, combined with ii) above would further inhibit actin polymerisation.

(Adapted from Southwick and Stossel, 1983)


filament
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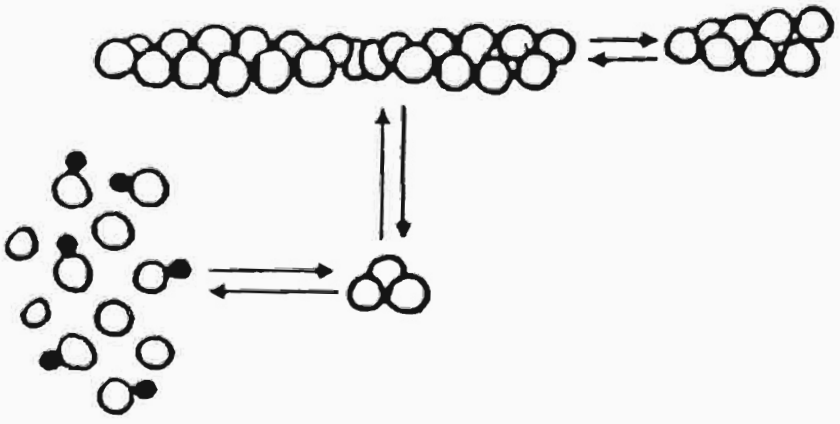
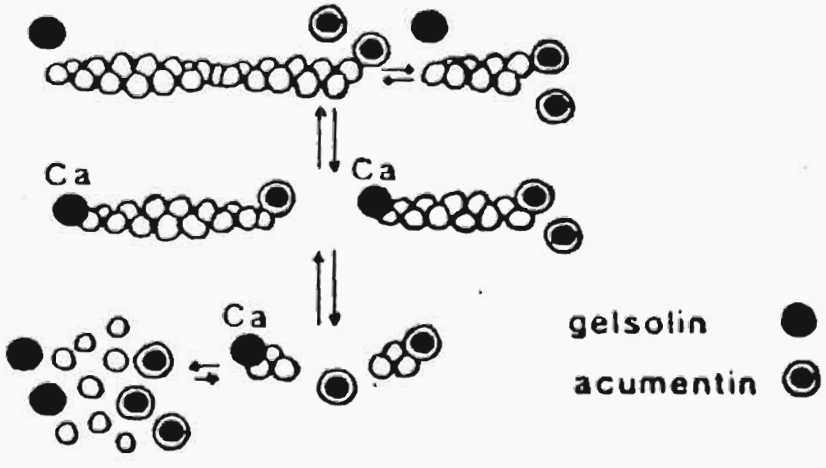
nucleus




Ca
Ca
Ca
gelsolin
acumentin

possibility of cycling of actin monomers through an actin filament from the fast-growing to the slow-growing end (Wegner, 1982). If such a process exists, continuous remodelling of actin filaments would occur.

1.4.4.4 Regulation of actin assembly:

Based on the critical concentration of actin under conditions analogous to those thought to exist in the resting neutrophil, almost all of the actin should be polymerised (Stossel, 1988). This is not the case in a resting neutrophil, as only about half of the total actin content is in filamentous form. Therefore, actin assembly must be regulated to maintain so much actin in monomer form. There are two mechanisms by which this could be done. In one, actin monomers could be sequestered with a sufficiently high affinity to prevent nucleation. In a second model, the growing filament ends could be interfered with by the binding of a molecule at either end. Both mechanisms appear to be active in neutrophils.

Profilin is a basic protein with a molecular weight of 20,000 which sequesters actin monomers (Carlsson et al., 1976). This protein exists in a high affinity state that complexes actin monomers so tightly that denaturing conditions are required to dissociate them *in vitro*. Free profilin can also be isolated, that has a very low affinity for actin monomers. One can therefore propose that the regulation of profilin affinity would in turn modulate actin monomer availability and therefore, polymerisation (Figure 1.3B). Indeed, preliminary evidence suggests that phosphatidylinositol-4,5-bisphosphate (PIP_2) and other lipids of the inositol cycle, lower the affinity of profilin for actin (Lassing and Lindberg, 1985). These molecules may therefore play a role in the regulation of actin assembly by profilin.

Gelsolin binds to the fast-growing end of actin filaments and prevents monomer exchange at this end. It also severs actin-actin bonds in filaments, thereby rupturing them (Janmey et al., 1985). The cytochalasins resemble gelsolin in that they bind to the fast-growing ends of actin filaments and thereby shorten the average actin filament length.

Micromolar concentrations of calcium activate gelsolin to bind to one actin molecule, which exposes another actin binding site with an even higher affinity for actin monomers (Janmey et al., 1986). Severing of a filament can only occur upon binding of the second actin filament. Thus the binding of the first actin blocks filament ends, and the second actin binding causes severing of actin filaments. The former can be removed by calcium chelation with EGTA whereas the severing activity is EGTA-resistant. This second interaction can only be dissociated by PIP_2, which inhibits severing of actin filaments even in the presence of micromolar calcium concentrations (Janmey and Stossel, 1987). Thus, gelsolin is under dual regulation by two known intracellular messengers, and the balance of these mechanisms presumably regulates actin assembly in the cell.

Two other actin associated proteins have been isolated. One is acumentin, which binds to actin with a relatively low affinity (Southwick and Stossel, 1981), and the other is a 42,000 molecular weight calcium-binding protein which binds to the barbed end of actin filaments with a much lower affinity than gelsolin, and is unable to sever filaments. Acumentin is a calcium-insensitive blocking protein which nucleates actin filament growth and binds to the slow-growing end of actin filaments. The mechanism by which acumentin and gelsolin interact to regulate actin assembly is outlined in Figure 1.3C.

1.4.4.5 Actin assembly during pseudopod formation:

Central to functions such as locomotion, adherence, endocytosis and exocytosis, is the formation of pseudopods. The use of cytoskeletal disrupting agents such as cytochalasin B and colchicine have provided clues as to the importance of the cytoskeleton for the expression of the functions mentioned above, but the process of pseudopod formation has not been defined, and the mechanism by which it occurs can at best be speculated upon. Much work has been done to define the components and regulatory events during actin polymerisation, and the second messengers involved in these events are beginning to be defined. Despite this, the actual coupling of receptor-stimulated events to the effector mechanisms ie., actin polymerisation, have yet to be determined.

1.4.5 Oxidative metabolism:

Neutrophils also possess oxygen dependent cytotoxic mechanisms. Oxygen is kinetically inert, but its reactivity can be increased by either reduction or excitation. Ultimately oxygen is reduced to water by the acceptance of four electrons. If partial reduction occurs, intermediates are formed which are highly reactive, i.e. the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^\cdot$, Figure 1.4). The excitation of oxygen occurs when an absorption of energy shifts one of the unpaired electrons of oxygen to an orbital of higher energy with an inversion of spin. The product is a singlet oxygen ($^1O_2$) and can occur in two forms: delta singlet oxygen ($^1\Delta gO_2$) which contains the newly paired electrons in the same orbital, and sigma singlet oxygen ($^1\Sigma g^+O_2$) where the two electrons with opposite spins occupy different orbitals.

1.4.5.1 The respiratory burst:

The importance of the respiratory burst as a microbicidal mechanism has been indicated by an observed decrease in the microbicidal activity of neutrophils in hypoxic conditions (Mandell, 1974; Vel et al., 1984). In addition, patients with chronic granulomatous disease (CGD) possess neutrophils lacking the respiratory burst (Holmes, Page and Good, 1967), and this is associated with defective microbicidal activity (Quie et al., 1967). The respiratory burst of neutrophils can be defined as the increase in oxygen consumption occurring upon stimulation. The enzyme system involved is predominantly thought to be the NADPH oxidase system, which consists of a transmembrane electron transport system in which a reduced pyridine nucleotide (nicotinamide adenine dinucleotide - NADPH) reduces oxygen. This is thought to occur through a series of reactions involving the oxidation and reduction of a flavin, a b-cytochrome and possibly a quinone.

The NADPH oxidase was first described by Rossi and Zatti (1964), and its preference for NADPH as the electron donor is based on the affinity of the enzyme for NADPH rather than NADH (Babior, Curnutte, and Kipnes, 1975; Gabig and Babior, 1979). The enzyme system is embedded in the plasma membrane with only the NADPH binding site of the oxidase extending into the cytoplasm (Babior et al., 1981; Green, Shaefer and Makler, 1980; Nakamura, Baxter and Masters, 1981). Some contribution of electrons from NADH cannot be discounted, as most NADPH oxidase preparations also react with NADH, albeit with a lower affinity.

The involvement of a flavoprotein in the NADPH oxidase system was investigated in reconstitution experiments where the loss of NADPH oxidase activity on extraction of human neutrophils with detergent was prevented by the addition of flavin adenine dinucleotide (FAD) (Babior and Kipnes, 1977; Gabig and Babior, 1979). FAD has been

localized associated with a 65 kilodalton protein from a purified NADPH oxidase preparation from human neutrophils (Markert, Glass and Babior, 1985). This may be the same protein which binds NADPH (Umei, Takeshige, and Minakami, 1986) and is thought to catalyze a two-electron transfer from the NADPH and a one-electron transfer to the b-cytochrome (Kakinuma et al., 1986).

The role of a quinone in the oxidase system is still controversial. A quinone has been detected in neutrophil preparations that was identified as ubiquinone-50 (now termed ubiquinone-10, Crawford and Schneider, 1982; Cunningham et al., 1982). It was enriched in phagolysosomes and in a distinct tertiary granule (Mollinedo and Schneider, 1984). Gabig and Lefker (1985) have recently described a membrane-associated NADPH oxidase complex consisting of a flavoprotein, ubiquinone-10, and a b-cytochrome. Electrons were proposed to flow from NADPH through FAD to ubiquinone-10 in resting cells, and continue through the b-cytochrome to oxygen in stimulated cells.

The involvement of cytochrome b_{559} in the respiratory burst is suggested by a number of findings. Firstly, the low midpoint potential (Cross et al., 1981; Lutter et al., 1985) is close to that reported for the O_2/O_2^- couple (Ilan, Czapski and Meisel, 1976). This is compatible with the b-cytochrome being the terminal component in the NADPH oxidase electron transport chain.

Secondly, several studies have indicated that unstimulated neutrophils possess b-cytochrome in both the plasma membrane and the membrane of the specific granules (Borregard and Tauber, 1984; Borregard et al., 1983; Garcia and Segal, 1984; Higson et al., 1985; Ohno, Seligmann and Gallin, 1985). Furthermore, these reports describe translocation of the cytochrome to the plasma membrane upon stimulation by a variety of

factors. These findings are consistent with a theory suggesting the assembly of the components of the oxidase occurs at the membrane in response to stimulation.

Thirdly, when HL-60 cells are induced to differentiate into relatively mature granulocytes capable of the respiratory burst, a concomitant increase in b-cytochrome levels occurs (Newburger et al., 1984; Roberts et al., 1982).

Finally, the b-cytochrome is absent from the leukocytes of most patients with chronic granulomatous disease, which is an X-linked disease where neutrophils are unable to produce superoxide anion (see above, Ohno et al., 1986; Segal et al., 1978).

1.4.5.2 The mechanism of activation of NADPH oxidase:

The precise pathway of activation of the oxidase system involved in the respiratory burst has not been defined. There are several factors which have been implicated in this process. The major factors include calcium, protein kinase C, and guanosine triphosphate (GTP) binding proteins.

Calcium has been implicated based on the abolition of the respiratory burst by inhibitors of intracellular free calcium mobilization (Edwards and Unger, 1980; Smolen, Korchak and Weissmann, 1981) and calmodulin (Takeshige and Minakami, 1981). Inhibitor studies like these must be considered in the light of their specificity, as they may not be absolutely specific. Although calcium may be required, a change in intracellular free calcium alone is not necessarily the sole requirement. Indeed, stimulation of the respiratory burst by PMA occurs in the absence of an increase in intracellular free calcium (DiVirgilio, Lew and Pozzan, 1984; Sha'afi et al., 1983).

Protein kinase C is another component of the signal transduction mechanism thought to be involved in activation of the neutrophil respiratory burst. It is present in an inactive form in the cytosol, and is activated by 1,2-diacylglycerol then translocated to the plasma membrane (Nishizuka, 1986). This spatial activation is consistent with the translocation of other components of the oxidase system to the membrane (mentioned above) upon stimulation of the neutrophil. Activation of protein kinase C results from the transient binding of diacylglycerol to the enzyme, which increases its affinity for calcium. The requirement for diacylclycerol can be bypassed by substances such as PMA and 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) or by synthetic diacylglycerols, e.g. 1-oleoyl-2-acetylglycerol (OAG) which intercalate into the membrane. Therefore, the phorbol ester-induced stimulation of the respiratory burst (Robinson et al., 1985), and the synergistic effects of suboptimal concentrations of phorbol esters and calcium ionophores (Dale and Penfield, 1984; DiVirgilio, Lew and Pozzan, 1984) implicate protein kinase C in respiratory burst stimulation. In addition, stimulation by OAG, either alone or synergistically with other agents (Cox et al., 1986; Dewald, Payne and Bagglioni, 1984; Fujita et al., 1984; O'Flaherty, Schmitt and Wykle, 1985; Penfield and Dale, 1984) and inhibition of the respiratory burst by protein kinase C inhibitors (Pontremoli et al., 1986; Gennaro, Florio and Romeo, 1985) also lend support to this theory.

Another component of the signal transduction mechanisms described above is the guanosine triphosphate (GTP) binding regulatory proteins. These proteins are described as the coupling mechanism between receptor stimulation and the activation of adenylate cyclase in neutrophils. The GTP-binding protein Ns (or Gs) activates adenylate cyclase whereas the GTP-binding protein Ni (or Gi) inhibits adenylate cyclase (Gilman, 1984). Adenylate cyclase is involved in the formation of cyclic adenosine monophosphate (cAMP), and agents that increase cAMP levels inhibit the respiratory burst (Simchowitz et al., 1980). Chemotactic factor receptors in neutrophils are coupled to GTP-binding

proteins (Goldman et al., 1985; Hyslop, 1984; Koo, Lefkowitz and Snyderman, 1983; Molski et al., 1984; Okajima, Katada and Ui, 1985; Verghese, Smith and Snyderman, 1985; Volpi et al., 1985b), but it has been proposed that these proteins are also required for protein kinase C activation (Becker et al., 1985; Gomperts, Barrowman, and Cockcroft, 1986; Smith et al., 1985). As protein kinase C activation and chemotactic factors stimulate the respiratory burst, it is feasible to suggest a role for the GTP-binding proteins in the activation of the neutrophil respiratory burst.

A rather interesting aspect of neutrophil oxidase stimulation is the localisation of the components of this complex. In stimulated cells, the oxidase complex is localized in the plasma membrane (Dewald et al., 1979; Yamaguchi et al., 1982). In contrast, in unstimulated neutrophils, a high proportion of the b-cytochrome (Borregard, 1985; Borregard and Tauber, 1984; Borregard et al., 1983) and the flavin (Borregard, 1985; Borregard and Tauber, 1984) is located in the granule membranes, and is relocated to the plasma membrane upon stimulation. These observations suggest that the assembly of the oxidase complex at the plasma membrane upon stimulation, may be an important regulatory mechanism during the respiratory burst. Although translocation of oxidase components may not be essential for stimulation of the respiratory burst (Lutter et al., 1985), perhaps a continued supply of components is required for maintenance of activity. Support for this theory is provided by observations that translocation of protein kinase C precedes NADPH oxidase activation by PMA (Gennaro, Florio and Romeo, 1985), suggesting that this may be one of the mechanisms by which the respiratory burst is triggered.

1.4.5.3 Respiratory burst products:

A number of respiratory burst products have potential microbicidal actions. The complete reduction and excitation of oxygen is shown in Figure 1.4. Each product will be dealt with separately.

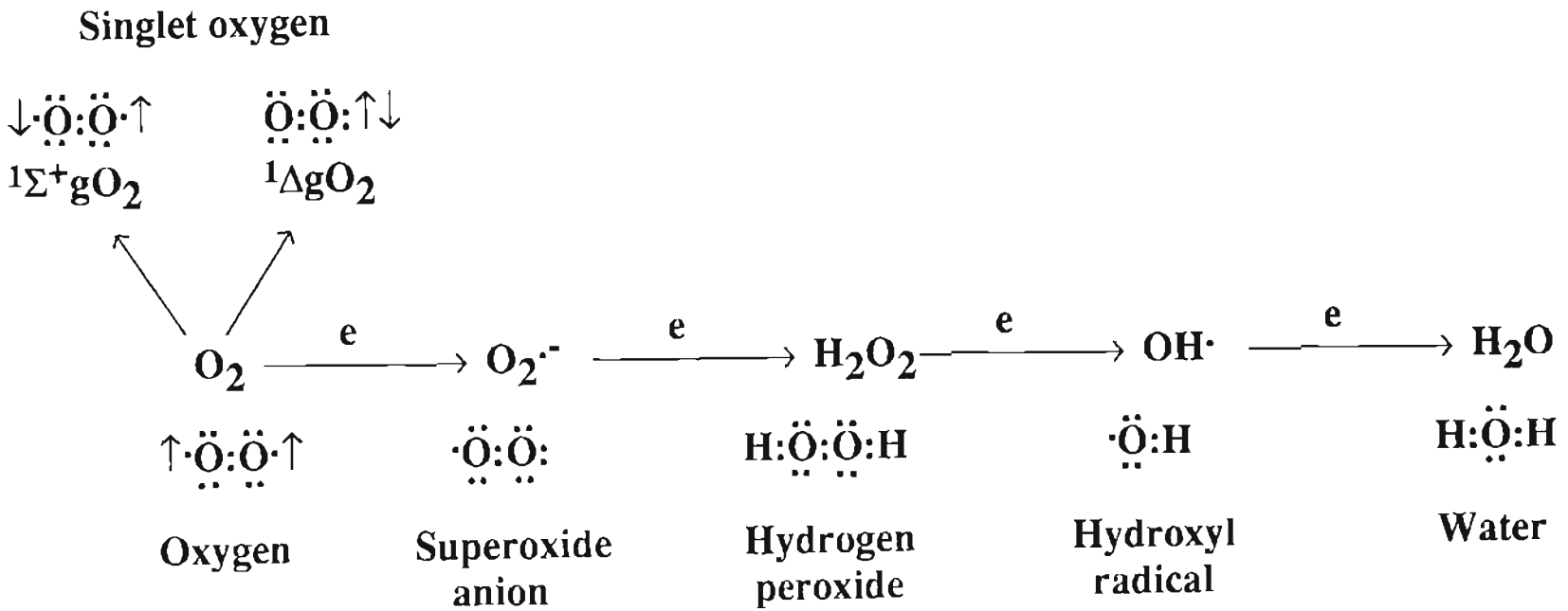
A) Superoxide anion:

The initial product of the respiratory burst formed when oxygen accepts a single electron is O_2^- (Babior, Kipnes and Curnutte, 1973). The O_2^- radical exists almost entirely as O_2^- at neutral or alkaline pH and is predominantly a reductant. The cytochrome C assay is thus based on the reduction of ferricytochrome C by O_2^-, which is converted back to oxygen. When two molecules interact, one is oxidized and the other reduced in a dismutative reaction and oxygen and H_2O_2 are formed. This reaction can either occur spontaneously or be catalyzed by superoxide dismutase (SOD). Three distinct SODs exist which vary in both their intracellular distribution and their metal component (copper-zinc SOD, manganese SOD, iron SOD, Fridovich, 1983). SOD is present in neutrophils (Rest and Spitznagel, 1977), but it is unknown whether it is released into phagosomes. Its presence in phagosomes may not be required as the pH of phagosomes is acidic (Roos et al., 1983) and this pH is optimal for spontaneous dismutation of the superoxide anion.

Whether the superoxide anion is directly toxic to cells or not remains to be demonstrated. The fact that it reacts rather sluggishly with many biologically important compounds suggests that it may not be directly toxic to cells (Bielski and Shiue, 1979; Bielski, Arudi and Sutherland, 1983). This lack of reactivity may be due to the environment that the experiments were performed in, because superoxide anion is considerably

FIGURE 1.4

The reduction and excitation of oxygen.


Singlet oxygen
$^1\Sigma^+gO_2$
$^1\Delta gO_2$
O_2
e
$O_2^{\cdot -}$
e
H_2O_2
e
OH·
e
H_2O
Oxygen
Superoxide anion
Hydrogen peroxide
Hydroxyl radical
Water

more reactive in a nonpolar environment (Sawyer and Valentine, 1981). It is possible that such selective reactivity is important to the cell to ensure that the anion reacts only in the hydrophobic region of the cell membrane. In addition, a decrease in pH induces the formation of HO_2 which is the reactive protonated form of O_2^- (Gebicki and Bielski, 1981). Thus, a second selective mechanism could exist whereby the relatively nonreactive O_2^- form is converted to the reactive HO_2 form in a phagosome where the pH is acidic, rather than in the cytoplasm of the cell.

B) Hydrogen peroxide:

Hydrogen peroxide (H_2O_2) is formed predominantly by the rapid dismutation of O_2^- (Weening, Weever and Roos, 1975). Under some conditions H_2O_2 may also be formed directly from oxygen by divalent reduction without an O_2^- intermediate (Green and Wu, 1986).

The relatively low reactivity of H_2O_2 indicates that it may be toxic at a distance from cells, rather than over shorter distances. H_2O_2 can pass intact through cell membranes (Frimer, Forman and Borg, 1983), and biological fluids. This property of H_2O_2 therefore enables it to be reactive under conditions where other more reactive oxygen species are inactived by scavengers.

The production of H_2O_2 is reported to be the mechanism by which human neutrophils kill the newborn larvae of *Trichinella spiralis* in the presence of immune serum (Bass and Szejda, 1979). Neutrophils are protected by the toxic effects of H_2O_2 by their content of catalase (Roos et al., 1980; Voetman and Roos, 1980) and the components of the glutathione cycle (Roos et al., 1979; Spielberg et al., 1979). These enzymes break down H_2O_2 to oxygen and water, and channel the H_2O_2 into the glutathione cycle. The

glutathione cycle is a sequence of reactions by which the degradation of H_2O_2 is coupled to the increased activity of the hexose monophosphate shunt.

C) Peroxidase/H_2O_2/halide system:

One of the mechanisms by which the toxicity of H_2O_2 can be increased is by the action of peroxidases. Peroxidases alone are not microbicidal, however they can exert an antimicrobial effect indirectly by catalyzing the conversion of a minimally toxic substance to one that is strongly toxic.

Myeloperoxidase (MPO) is present in human neutrophils in very high concentrations (Schultz and Kaminker, 1962). It is an intense green and is responsible for the green colour of pus. It is a complex protein which contains two iron-containing prosthetic groups per molecule (Agner, 1958) that are covalently linked to the heavy subunits by an amide bond (Newton et al., 1965; Wu and Schultz, 1975). It has been proposed that at low concentrations H_2O_2 reacts with only one of the iron atoms, whereas at high concentrations H_2O_2 binds to both and is catalytically degraded to O_2 and H_2O with associated inactivation of the enzyme (Agner, 1963). This double reaction would modulate the peroxidase system activity by both degrading H_2O_2 and inactivating MPO.

MPO forms three distinct complexes on reaction with H_2O_2: compounds I, II and III. Compound I is formed by MPO reacting with H_2O_2 at equimolar concentrations (Odajima and Yamazaki, 1970) or by reacting with hypochlorous acid (HOCl, Harrison, 1976). It is the primary catalytic compound of MPO and is highly unstable. Compound II is formed in the presence of excess H_2O_2 (Odajima and Yamazaki, 1970) and compound III is an oxyperoxidase where oxygen is attached to the heme iron (Yamazaki and Yokota, 1973).

Halides also contribute to increasing the effectiveness of the MPO-mediated activation of H_2O_2. Iodide, bromide, chloride (Klebanoff, 1967; Klebanoff, 1968) or the pseudo-halide thiocyanate (Klebanoff, Clem and Luebke, 1966) are involved in this reaction. Iodide is the most effective halide, but is present in extrememly low amounts in serum, therefore its contribution may be small. The concentration of chloride in biological fluids is considerably greater than that required for the MPO-H_2O_2 system, suggesting it is the primary halide used *in situ*. Two distinct binding sites for chloride on MPO have been proposed. Firstly the substrate binding site which leads to the production of HOCl and, secondly, the inhibitor binding site which leads to the competitive inhibition of H_2O_2 binding to MPO (Harrison, 1976). Toxic products are formed when the halides are oxidized by compound I and possibly by compound III (Winterbourn, Garcia and Segal, 1985). These toxic agents vary with the halide, its concentration, the pH, and other factors. Products include hypohalous acids, halogens, long-lived oxidants such as chloramines or aldehydes, and possibly hydroxyl radicals and singlet oxygen (Klebanoff, 1988).

D) Hydroxyl radical:

Another mechanism by which the toxicity of H_2O_2 can be increased is by reaction with ferrous iron to form $OH^{\cdot}$. When the iron concentration is limited O_2^- reduced the ferric iron to enable the overall reaction to occur as shown below.

i.e. $H_2O_2 + O_2^- \rightarrow O_2 + OH^{\cdot} + OH^-$

Although a direct interaction between H_2O_2 and O_2^- was originally proposed (Haber and Weiss, 1934), trace metal catalysis is accepted as the mechanism for $OH^{\cdot}$ generation by the Haber-Weiss reaction outlined above (Gutteridge, Richmond and Halliwell, 1979; McCord and Day, 1978).

Since stimulated neutrophils produce both O_2^- and H_2O_2 it is feasible that they are also capable of producing OH· by the Haber-Weiss reaction. Although there is a fair amount of evidence to suggest that OH· is formed by stimulated neutrophils, the assays for OH· production are based on indirect measurements. These assays include ethylene formation from thiol ethers (Yang, 1969), methane or formaldehyde formation from dimethylsulphoxide (Repine et al., 1979), CO_2 release from benzoic acid (Sagone et al., 1980), and electron paramagnetic resonance (EPR) spectrometry using a spin trap method (Harbour, Chow and Bolton, 1974; Janzen et al., 1978). The problem with these assays is that although the reactions require the presence of OH·, this component can be substituted by other factors, and therefore, they are not exclusive measures of OH· formation.

There is a large amount of interest in OH· formation because it is an extremely powerful oxidant (Dorfman and Adams, 1973) and is therefore not particularly discriminating in its action. This reactivity poses an availability problem in that the radical can be easily scavenged by compounds in the medium. Despite this, a variety of studies have implicated OH· as a toxic species in cell-free systems which produce O_2^- and H_2O_2 (Badwey, Curnutte and Karnovsky, 1981; Halliwell and Gutteridge, 1986). With regard to the role of OH· in the microbicidal activity of neutrophils, there is evidence suggesting its effect can be inhibited by SOD and catalase bound to latex beads (Johnston et al., 1975) and by some OH· scavengers (Johnston et al., 1975).

E) H_2O_2/Fe^{2+}/iodide antimicrobial system:

H_2O_2 also reacts with ferrous ions and iodide to form both an antibacterial (Klebanoff, 1982) and antifungal system (Levitz and Diamond, 1984). This system is different from the H_2O_2/MPO/halide system in that it is strongly inhibited by OH· scavengers at concentrations that do not affect the MPO-dependent system (Klebanoff, 1982). The pH

optimum, chelator dependent inhibition, and halide requirements of these systems also differ (Klebanoff, 1982). Both systems are inhibited by catalase, unaffected by SOD, and inhibited by azide, although higher azide concentrations are required for inhibition of the Fe^{2+}-dependent system (Klebanoff, 1982).

F) Singlet oxygen:

The excitation of oxygen results in two forms of singlet oxygen: delta singlet oxygen ($^1\Delta gO_2$) and sigma singlet oxygen ($^1\Delta g^+O_2$). Sigma singlet oxygen has a higher energy above ground state but a considerably shorter lifetime (Kearns, 1979). Delta singlet oxygen is considered to be the reactive form of singlet oxygen in solution. It is a strong electrophile and generally forms oxygenated products. These reactions are considered to be toxic by implication from studies with photodynamic dyes. Certain dyes in the presence of light and oxygen are toxic to cells, and their action is thought to be mediated by the formation of singlet oxygen (Foote, 1976).

The formation of singlet oxygen can be via a variety of reactions. The three relevant ones to the neutrophils are as follows:

(i) Oxygen formed during spontaneous dismutation has been proposed to exist in part as singlet oxygen (Khan, 1978).

(ii) The formation of singlet oxygen is a minor product of the Haber-Weiss reaction (Nagano and Fridovich, 1985).

(iii) The interaction of hypochlorite and H_2O_2 are known to form singlet oxygen.

$$OCl^- + H_2O_2 \rightarrow Cl^- + H_2O_2 + {}^1O_2$$

Since HOCl is the primary product formed by the oxidation of chloride by MPO and H_2O_2 (Kasha and Khan, 1970; Nilsson and Kearns, 1974), its reaction with excess H_2O_2 or O_2^- may result in the formation of singlet oxygen.

The formation of singlet oxygen by intact neutrophils has been proposed based on the chemiluminescence of stimulated neutrophils (Allen, Stjernholm and Steele, 19722). The formation of electronically excited states induces chemiluminescence in neutrophils, but the peak of emitted light by stimulated neutrophils is rather broad (Andersen, Brendzel and Lint, 1977). This finding suggests that neutrophil chemiluminescence is due to both singlet oxygen decay and secondary excitations that could be induced by a number of the oxidants formed by stimulated neutrophils (Cheson et al., 1976).

The other method for detecting singlet oxygen production by neutrophils is by chemical traps. As these are also nonspecific assays, a role for singlet oxygen in the microbicidal activity of phagocytes is still to be defined.

SECTION 1.5 CYTOKINES THAT REGULATE NEUTROPHIL FUNCTION

During the inflammatory reaction, neutrophils are activated by both endogenous and exogenous factors. Endogenous factors include complement and lipid metabolites such as platelet-activating factor, arachidonic acid and leukotrienes. Endogenous glycoproteins such as the monocyte- and lymphocyte- derived cytokines TNF-α, IL-1β, GM-CSF, and NAF also influence neutrophil functions. Exogenous substances such as f Met-Leu-Phe and LPS are also neutrophil activators. These substances will be discussed in detail below.

When discussing neutrophil activation it is necessary to define the different responses which can be elicited. Most of the agents mentioned above can trigger a variety of responses from neutrophils. Intrinsic to some of them though is the capacity to change the neutrophil response to another substance. This concept of neutrophil "priming" was first suggested by studies involving GM-CSF (Gasson et al., 1984). These priming agents are primarily incapable of inducing certain responses on their own, but are characterised by their ability to change the response to another agent. Thus, substances such as lipopolysaccharides, platelet-activating factor and the leukotrienes can directly trigger neutrophil responses, but can also "prime" neutrophil responses to another stimulant such as f Met-Leu-Phe. In contrast, the cytokines TNF-α and GM-CSF, whilst not capable of directly activating certain neutrophil responses such as the respiratory burst, do enhance the subsequent response to f Met-Leu-Phe. Therefore, when one considers the interaction of these substances during the inflammatory reaction, these aspects of neutrophil activation should be considered.

1.5.1 Interleukin-1:

Interleukin-1 was originally described as a substance in acute exudate fluid which produced fever when injected into animals or humans (Dinarello, 1984). The active factor in this fluid was localised to a small protein of molecular weight 10-20 kilodaltons which was called "endogenous pyrogen". Endogenous pyrogen was later found to induce more than just fever. It was demonstrated that this protein copurified with "leukocytic endogenous mediator". This substance caused hepatic acute phase protein synthesis, decreases in plasma iron and zinc levels, and produced neutrophilia. Purified endogenous pyrogen stimulated serum amyloid A protein synthesis when injected into mice (McAdam and Dinarello, 1980). A macrophage product called lymphocyte activating factor was also described and found to be of a similar weight to endogenous pyrogen (Gery and Waksman,

1972). Increasingly, evidence suggested that lymphocyte activating factor and endogenous pyrogen were the same substance. Lymphocyte activating factor was changed to interleukin-1, and the protein is now known to account for endogenous pyrogen, lymphocyte activating factor, mononuclear cell factor (Krane et al., 1985) and catabolin (Saklatvala, Sarsfield and Townsend, 1985).

Two IL-1 forms have been cloned; IL-1β from human monocytes (Auron et al., 1984) and IL-1α from a murine macrophage line (Lomedico et al., 1984). When the two IL-1 forms are compared, only five small stretches of amino acid homology exist. Three of these regions could represent an active site of the IL-1 molecule. This possibility could explain the observation that, although these molecules share little structural homology, their biological activities are very similar.

The spectrum of biological activity shared by IL-1α and β appears to be the same (Dinarello, 1986a). Chemically, IL-1β is vulnerable to oxidation, therefore its biological specific activity can be lower than that of IL-1α. Both recombinant human IL-1β and α augment T cell responses to antigens or mitogens, and cause fever in rabbits and other species (Dinarello et al., 1986b). There are some discrepancies between the biological properties reported for the natural IL-1β and its recombinant form. These include the inability of IL-1β to cause neutrophil degranulation *in vitro*. This apparent activity could be due to contaminating proteins. Alternatively the observed activity could be attributed to the ability of IL-1β to augment the biological activity of another protein. Tumor necrosis factor is the most likely contaminant of these early preparations, and synergism between these two proteins could explain the effects formerly observed.

IL-1β possesses multiple biological activities, and these effects are thought to be responsible for the acute phase changes observed *in vivo*. The ability of IL-1β to induce the release of prostaglandins from a variety of cells is thought to mediate these symptoms.

This theory is supported by the reported inhibitory effects of cyclooxygenase inhibitors on these symptoms.

1.5.2 Tumor necrosis factor (TNF-α):

The molecule now known as tumor necrosis factor-alpha (TNF-α) was originally isolated by two separate groups, and functionally defined as two distinct factors. In the late 19th century, Coley described the haemorrhagic necrosis of human tumors by *Streptococcal*- and *Serratia*-derived bacterial broths (Coley, 1906). The bacterial product responsible for the induction of this effect was later isolated by Shear and Andervont (1936), and this substance is now known as lipopolysaccharide. *In vivo* experiments were later reported describing the ability of serum from LPS-treated mice to induce tumor necrosis in another animal. *In vitro* experiments, describing the cytotoxic properties of post-endotoxin serum derived from mice treated with Bacillus Calmette-Guerin (BCG) for a variety of tumor cells supported this observation (Carswell, 1975). This activity was called tumor necrosis factor, isolated by Aggarwal et al. (1985) and the cDNA sequence was reported by several groups (Pennica et al., 1984; Shirai et al., 1985; Wang et al., 1985).

At the same time, Cerami's group started to investigate the cause for cachexia, using trypanosome-induced cachexia as a model system. Cachexia was characterized by severe wasting and hypertriglyceridemia. The hypertriglyceridemia was found to result from systemic suppression of the enzyme lipoprotein lipase (LPL, Rouzer and Cerami, 1980). It was subsequently reported that LPL suppression also occurred in endotoxin-treated mice and that this was transferrable by a serum factor (Kawakami and Cerami, 1981). The factor, cachectin, was found to be produced by macrophages. Cachectin also suppresses LPL expression by adipocytes *in vitro* (Kawakami et al., 1982), and the biosynthesis of acetyl CoA carboxylase and fatty acid synthetase (Pekala et al., 1983). A variety of stimuli

can induce the synthesis and release of cachectin by macrophages, but endotoxin is by far the most potent (Beutler, Milsark and Cerami, 1985).

Beutler et al. purified cachectin and reported it to be a polypeptide of molecular weight 17000 (1985b). Purified cachectin binds to a high-affinity plasma membrane receptor, present on a variety of cultured cells and tissues. The amino-terminal sequence of cachectin was found to be highly homologous to TNF-α (Beutler et al., 1985a), and when TNF-α was cloned, it was found that these two molecules were identical (Pennica et al., 1985).

TNF-α is produced by all types of macrophages thus far tested (Decker and Lohmann-Matthes, 1987). Other cell types reported to produce TNF-α include T-lymphocytes (Cuturi et al., 1987), mast cells (Tharp and Kasper, 1987), and smooth muscle cells (Libby, Warner and Galin, 1987). The amount of TNF-α released by these cells is vanishingly small when compared to the amount produced by stimulated macrophages.

Stimuli capable of inducing macrophage TNF-α release include certain viruses such as Sendai virus (Aderka et al., 1986) and influenza virus (Beutler et al., 1986a), trypanosomal lysates, plasmodial lysates and certain gram-positive organisms. LPS is by far the most potent inducer of TNF-α release, and this effect is augmented by interferon-γ (Beutler et al., 1986c).

A variety of physiologic stimuli modulate TNF-α production. Of these glucocorticoids strongly suppress TNF-α synthesis if administered to macrophages prior to stimulation by LPS (Beutler et al., 1986b). This could account for their protective effect when given to animals prior to an otherwise lethal dose of LPS.

1.5.2.1 Biological effects of TNF-α:

TNF-α has a wide range of biological effects. Receptors for this protein are expressed on virtually all somatic tissues. In addition to a general metabolic effect on a variety of tissues, TNF-α is also an important mediator of the inflammatory response.

Amongst the metabolic effect of TNF-α, the suppression of LPL expression in adipose tissue is probably the best defined. TNF-α is capable of enhancing glycerol release (Kawakami et al., 1987), suppressing adipocyte differentiation (Torti et al., 1985) and suppressing the expression of adipsin (Min and Spiegelman, 1986), glycerolphosphate dehydrogenase and the fatty acid binding protein (Torti et al., 1985).

TNF-α induces a decrease in transmembrane potential in muscle cells, and an increase in glucose transport (Tracey et al., 1986; Lee et al., 1987). These changes in muscle metabolism may explain the wasting observed in cachexia.

TNF-α influences hepatic function in that it induces the release of various acute phase reactants such as complement proteins, alpha-1-antichymotrypsin, and serum amyloid A (Perlmutter et al., 1986). It also suppresses albumin and transferrin synthesis *in vitro*.

The induction of fever by TNF-α can be attributed both to its direct effect on the hypothalamic neurons and its indirect effect of inducing IL-1β production. This explains the biphasic fever generated from TNF-α administration to rabbits (Dinarello et al., 1986c). Bone resorption (Bertolini et al., 1986) and degradation of proteoglycan in cartilage (Saklatvala, 1986) are other metabolic effects of TNF-α. In addition, it induces the production of PGE-2 and collagenase by both sinovial cells and dermal fibroblasts (Dayer, Beutler and Cerami, 1985). These effects suggest TNF-α plays an important role

in local inflammatory reactions involving the bones and joints. This theory is supported by the observations that TNF-α has been detected in sinovial fluid from inflammatory joint diseases (Wollheim et al., 1987) and is produced *in vitro* in response to stimuli that trigger joint inflammation.

Endothelial cells respond to TNF-α in a manner that requires protein synthesis. The increase in adhesiveness of endothelial cells could be due to the increased expression of adhesion molecules (Gamble et al., 1985). Other effects of TNF-α on endothelial cells include the induction of procoagulant factor production (Nawroth and Stern, 1986), decreased expression of thrombo-modulin, endothelial cell rearrangements in tissue explants *in vitro* (Stolpen et al., 1986), and IL-1β production (Nawroth et al., 1986). These effects could account for the ability of TNF-α to induce disseminated intravascular coagulation, hemorrhagic necrosis of tumors and migratory thrombosis.

The expression of IL-2 receptor and HLA-DR antigen on lymphocytes is augmented by TNF-α (Scheurich et al., 1987), which is probably responsible for TNF-α-induced increase in T cell responsiveness to IL-2. TNF-α also influences hematopoietic progenitor cell function (Murphy et al., 1986) and inhibits erythropoiesis (Schneider, Pennington and Talmadge, 1987).

1.5.2.2 Effect of TNF-α on neutrophil function:

The influence of TNF-α on neutrophil function is probably its most important inflammatory effect. Initially, TNF-α was found to affect the capacity of neutrophils to kill antibody-coated tumor cells (Shalaby et al., 1985). Subsequently it was also found to enhance neutrophil adherence to endothelial cells and plastic. This phenomenon was due in part to an increased surface expression of the C3bi receptor and in part to an effect on the endothelial cells (Gamble et al., 1985). Other neutrophil functions influenced by TNF-

α include the respiratory burst and degranulation (Klebanoff et al., 1986). Notably, the effects of TNF-α on neutrophil functions fall into two categories. This cytokine has the capacity to induce both the activation and priming of neutrophils. The priming effects of TNF-α are well documented. TNF-α increases the phagocytosis and degranulation of neutrophils in response to both opsonised and unopsonised zymosan (Klebanoff et al., 1986), and enhances the respiratory burst and inhibits the chemotactic responsiveness of neutrophils to f Met-Leu-Phe (Atkinson et al., 1988b). The mechanism of action of TNF-α on neutrophil activation and priming has yet to be defined. The changes directly activated by TNF-α appear to be associated with changes in the properties of the plasma membrane i.e. adherence, degranulation and receptor modulation. Attempts to define the mechanism of action of TNF-α on neutrophils have been complicated by the fact that direct activation of neutrophils results in a small response, and indirect effects rely on a second stimulus. In addition, the use of inhibitors of signal transduction steps which could be common to both the priming and second stimulus, makes it difficult to dissociate priming and activating signals.

1.5.3 Hemopoietic growth factors:

Hemopoiesis is regulated by a group of growth factors called colony-stimulated factors. They were characterized by their ability to induce the formation of colonies of hematopoietic cells in semisolid matrices (Metcalf, 1984). Granulocyte and monocyte formation is regulated by four such growth factors which are called granulocyte-macrophage CSF (GM-CSF), granulocyte-CSF (G-CSF), macrophage CSF (M-CSF or CSF-1) and multi-CSF (or IL-3). These activities were originally defined from mouse tissue but human analogs have also been identified. An important feature of these factors is that by also stimulating the function of mature myeloid cells, they play a central role in inflammation. Indeed, CSFs have been isolated from the blister fluid of patients with Bullous pemphigoid (Varigos, Morstyn and Vadas, 1982), and from the synovial fluid of

patients with rheumatoid arthiritis (Williamson et al., 1988). Both of these clinical syndromes result from the excessive development of an inflammatory reaction causing tissue damage.

1.5.3.1 Granulocyte-macrophage colony-stimulating factor (GM-CSF):

Human GM-CSF was initially purified from a human T cell leukemia virus (HTLV)-II-infected human T lymphoblastoid cell line known as Mo (Lusis et al., 1981). Purified GM-CSF is a glycoprotein of molecular weight 22000 which has been cloned (Wong et al., 1985) and characterized. Human GM-CSF cDNA has approximately 60% homology with mouse GM-CSF, although it has no effect on mouse bone marrow.

Production of GM-CSF from normal T lymphocytes can be induced by antigen or lectin (Wong et al., 1985), and from endothelial cells by TNF-α, IL-1β or endotoxin (Broudy et al., 1987; Zucali et al., 1986). Certain fibroblastic cells can also produce GM-CSF upon stimulation (Munker et al., 1986).

GM-CSF was originally characterized by its capacity to stimulate the proliferation and differentiation of progenitor cells committed to the granulocyte and macrophage lineages (Metcalf, 1984). Following this, human GM-CSF has also been found to activate a number of neutrophil functions. It inhibits neutrophil migration under agarose (Atkinson et al., 1988a), is chemotactic (Wang et al., 1987), enhances superoxide anion production (Atkinson et al., 1988a), augments antibody-dependent cell-mediated cytotoxicity (ADCC) and increases neutrophil phagocytosis (Lopez et al., 1986). GM-CSF also influences eosinophil leukotriene production and ADCC of Schistosome larvae (Silberstein, 1986). In addition, GM-CSF induces macrophage tumoricidal activity (Grabstein et al., 1986), and causes U937 cells to become functionally more active.

The stimulation of neutrophil function by GM-CSF is not confined to *in vitro* studies. Donahue et al. (1986) demonstrated that GM-CSF administration in monkeys leads to a prominant dose-dependent increase in circulating leukocytes. In addition, the effect of the *in vivo* administration of GM-CSF on neutrophil function has been assessed *in vitro*. Baldwin et al. (1988) found that neutrophil function was restored in cells taken from leukopenic AIDS patients. These studies support a role for GM-CSF in the regulation of neutrophil generation and activation *in vivo*.

1.5.3.2 Granulocyte colony-stimulating factor (G-CSF):

G-CSF is specific for neutrophils and was purified from a bladder carcinoma cell line (5637) and a squamous carcinoma cell line (CHU-2, Nomura et al., 1986; Welte, 1985). This factor has also been called CSF-beta or human pluripotent hematopoietic CSF. The factor has been cloned (Nagata et al., 1986) and characterized. G-CSF has a molecular weight of about 19,600, and stimulates the formation of neutrophil colonies (Souza et al., 1986).

G-CSF activity is limited to the neutrophil and its precursors. It is a potent inducer of human HL-60 promyelocytic leukemia (Nicola and Metcalf, 1984), and myelomonocytic leukemia cell line (WEHI-3B) differentiation. G-CSF stimulates ADCC by human neutrophils and G-CSF receptors are expressed on various murine monomyelocytic cell lines and mature neutrophils.

1.5.3.3. Role of colony stimulating factors in inflammation:

There are two aspects of CSF influence on inflammation. Firstly, because CSFs regulate circulating granulocyte and mononuclear phagocyte numbers, they could regulate the

inflammatory process by controlling cell numbers. Secondly, CSFs serve to modulate the responsiveness of inflammatory cells to a variety of stimuli. In a localized inflammatory response, the activation of T cells by antigen results in, amongst other lymphokines, the release of GM-CSF. GM-CSF may stimulate the local proliferation of mononuclear phagocytes, and facilitate neutrophil chemotaxis towards the site of inflammation. Once cells have been recruited, the presence of CSFs could prime granulocytes and mononuclear phagocytes for increased cytotoxic activity. Monocyte-derived TNF-α and IL-1β could, in turn, induce the release of CSFs from endothelium and other mesenchymal cells. Thus, the development of a local inflammatory response is potentiated by the CSFs.

SECTION 1.6 ENDOGENOUS NEUTROPHIL ACTIVATORS

1.6.1 Complement:

The complement system is a set of proteins that constitutes about 10% of the globulins in the normal serum of humans. Activation of the complement system leads to the generation of biologically active products which mediate inflammation and tissue injury. The inflammatory products of complements include large fragments of C3 (e.g. C3b, C3bi) with opsonic activity as well as low-molecular-weight peptides (from C3 and C5) that exhibit anaphylatoxin activity and directly stimulate leukocytes.

1.6.1.1 C3 opsonins:

Activation of both the classical and alternative complement pathways induces the assembly of C3 convertases. These cleave the alpha chain of native C3 between amino acid residues 77 and 78 to yield two fragments. The smaller of these fragments is C3a, which represents the amino terminal portion of the alpha chain. The larger fragment is C3b, which rapidly undergoes a conformational change which activates a thiolester bond

in the alpha chain (Janatova et al., 1980). The activated thiolester bond is either hydrolyzed or reacts nonspecifically with an amino or hydroxyl group to form an amide or an aldehyde bond (Law, Lichtenberg, and Levine, 1979; Müller-Eberhard, 1988). C3b attaches to surfaces via activation of these thiolester bonds.

Surface-bound C3b activity is regulated by factor H and factor I. Factor H attaches to C3b and renders it susceptiable to factor I, which is a serine proteinase. There are initially two cleavage sites of C3b but the disulphide bonds remain intact, causing the generation of C3bi. C3bi remains surface bound and is recognized by neutrophils.

Heat-labile opsonic activity is attributable to C3b and C3bi which bind to surfaces when generated by the activation of the complement pathway. Binding of these fragments makes them recognizable by neutrophils via receptors for C3b and C3bi on the cell surface. Consequently the opsonization of particles by C3b and C3bi increases both the rate and extent of phagocytosis. In addition, these complement fragments also stimulate the respiratory burst in neutrophils (Schreiber et al., 1982) and induce the secretion of histaminase (Melamed, Arnaout, and Colten, 1982).

1.6.1.2 Chemotactic C5-derived fragments:

Boyden (1962) initially demonstrated that heat-stable chemotactic substances could be generated from rabbit serum incubated with antigen-antibody complexes. This observation was confirmed (Ward, Cochrane, and Müller-Eberhard, 1965) and the major chemoattractant generated was shown to be a low molecular-weight peptide similar to the cleavage product of C5 (Snyderman, Gewurz, and Mergenhagen, 1968). Subsequent studies demonstrated that human C5-derived peptides were chemotactic for neutrophils (Ward and Newman, 1969). The cleavage product C5a is the most potent chemoattractant, inducing chemotaxis at concentrations as low as 0.1nM (Webster et al., 1980). Higher

concentrations (>1-5nM) result in decreased neutrophil migration (Ward and Becker, 1968) which has been proposed as a 'deactivation' mechanism.

Human serum contains a substance known as anaphylatoxin inactivator (carboxypeptidase N, Bokisch and Müller-Eberhard, 1970) which rapidly converts C5a to C5a des Arg. Consequently it is this latter form which is the major species of C5-derived peptides found in human serum following complement activation. Highly purified C5a des Arg is 10 to 20-fold less chemotactic than C5a, but it is now known that C5a des Arg exists as a complex with a heat-stable anionic polypeptide. This "cochemotaxin" enables low concentrations of C5a des Arg to exhibit chemotactic activity (Perez et al., 1981). The fact that the chemotactic profile of C5a des Arg and its cochemotaxin is similar to that exhibited by zymosan-activated serum and highly purified C5a suggests that this complex is responsible for the major chemotactic activity found in complement activated human serum.

The chemotactic ability of C5a and C5a des Arg is mediated by specific surface receptors on neutrophils. The evidence for this is suggested by the ability of C5a and C5a des Arg to desensitize subsequent neutrophil responses to C5a (Webster et al., 1980). This finding implies that these two peptides bind to the same receptor on the neutrophil, although C5a has a higher binding affinity. This observation was confirmed by studies using radiolabelled C5a (Chenoweth and Hugli, 1978) where specific rapid and saturable binding to human neutrophils was demonstrated. A comparison of the concentration-dependent uptake of C5a by neutrophils revealed that nearly identical concentrations of ligand were required for binding and for eliciting chemotaxis (Huey and Hugli, 1985). This report demonstrates that the C5a receptor on human neutrophils is a polypeptide with a molecular weight of 42-48 kilodaltons.

1.6.1.3 C5-derived peptides regulate leukocyte degranulation:

Partially purified C5-derived peptides are degranulating stimuli for cytochalasin B-treated neutrophils (Becker et al., 1974). The release of lysosomal enzymes in response to C5-derived peptides occurs in the absence of phagocytosis and the concomitant release of cytoplasmic enzymes. Highly purified C5a and C5a des Arg also stimulate degranulation from cytochalasin B-treated neutrophils. In addition, C5a and C5a des Arg are also capable of inducing degranulation from adherent neutrophils (Becker et al., 1974). Ultrastructural studies demonstrate the fusion of granules both with each other and with the plasma membrane (Goldstein et al., 1973).

One of the striking features of C5a-treated neutrophils is the immediate appearance of large numbers of cytoplasmic microtubules radiating from the centriole. This observation implies that C5a stimulates microtubule assembly by neutrophils, and this effect is reversible.

The mechanism by which C5a stimulates degranulation and microtubule assembly in neutrophils is unknown. The stimulation of these functions suggests that C5a affects the cell cytoskeleton. Since chemotaxis and lysosomal enzyme release closely parallel neutrophil uptake of C5a (Chenoweth and Hugli, 1978), the same ligand-receptor interaction probably induces both of these functions.

1.6.1.4 C5-derived peptides regulate the respiratory burst:

Goetzl and Austen (1974) were the first to show that C5-derived peptides stimulated the neutrophil respiratory burst. They demonstrated that purified human C5a increased the rate of aerobic glycolysis and hexose monophosphate shunt activity in a concentration-

dependent manner. These observations were confirmed by Goldstein et al. (1975) who demonstrated enhancement of nitroblue tetrazolium dye reduction and superoxide anion production by human neutrophils. This stimulation of the respiratory burst is extremely rapid, reaching maximum values within 2 minutes at 37°C.

1.6.2 Leukotrienes:

Leukotrienes are a group of lipids generated during the metabolism of arachidonic acid. Arachidonic acid is liberated from membrane phospholipids, and can be metabolised by either the 5-lipoxygenase pathway or the cyclooxygenase pathway. In the neutrophil, the 5-lipoxygenase pathway is the major route by which arachidonic acid is metabolised. The 5-lipoxygenase pathway generates products such as a hydroperoxy fatty acid, a hydroxy fatty acid, and hydroxy- and sulfidopeptide leukotrienes. These products are potent inflammatory factors and are known to influence neutrophil activation.

1.6.2.1 The 5-lipoxygenase pathway:

The enzymes of the 5-lipoxygenase pathway are known to exist in few cells including neutrophils, eosinophils, basophils, monocytes, macrophages and mast cells (Lewis and Austen, 1984; MacGlashin et al., 1986). Some lymphocyte subsets are also reported to possess this pathway (Goetzl, 1981). The 5-lipoxygenase has been recovered from supernatants of membrane preparations, indicating that the enzyme is not an intrinsic membrane protein (Soberman et al., 1985). Additional studies suggest that the enzyme is associated with the membrane, because full 5-lipoxygenase activity is only demonstrable when several fractions are added back that were removed during purification (Rouzer and Samuelsson, 1985), and that 5-lipoxygenase activity is also dependent on ionic calcium.

The 5-lipoxygenase pathway is outlined in Figure 1.5. 5-hydroperoxyeicosatetraenoic acid (5-HPETE) is the product of 5-lipoxygenase action on arachidonic acid. It is short-lived and either degraded spontaneously or catalytically via a peroxidase to 5-hydroxyeicosatetraenoic acid (5-HETE). A second catalytic step by the 5-lipoxygenase on 5-HPETE generates the epoxide, leukotriene A_4. It appears that the 5-HPETE generated is not significantly transported out of the cell of origin before its conversion to 5-HETE. Both of the products can be reacylated into lysophospholipids, but only 5-HETE is exported from neutrophils (Williams et al., 1985; Rouzer and Samuelsson, 1985).

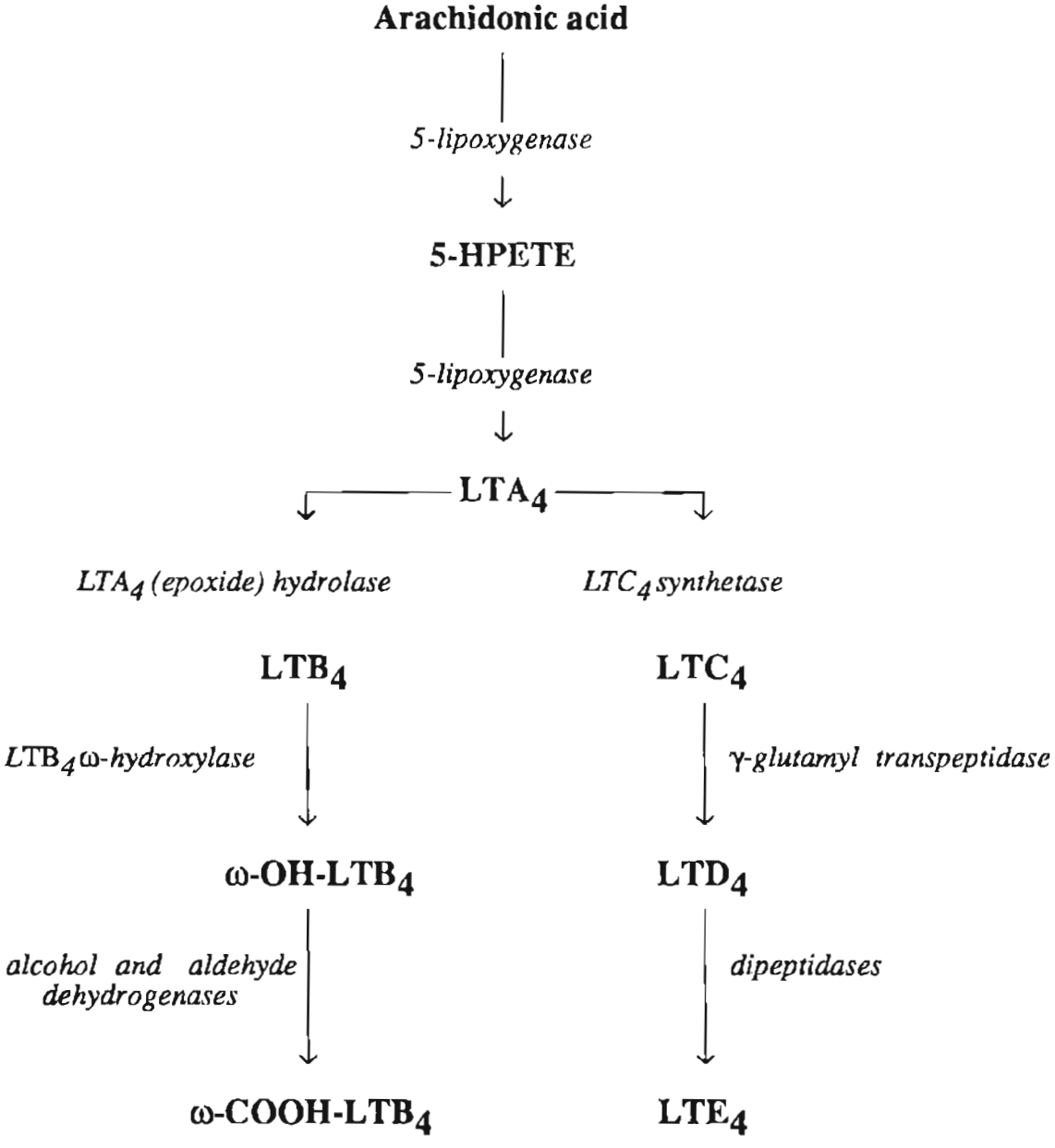
Metabolism of LTA_4 to 5,12-dihydroxyleukotrienes can occur via a specific epoxide hydrolase (Radmark et al., 1984) which converts LTA_4 to LTB_4. The enzyme that converts LTA_4 to LTC_4 is the LTC_4 synthetase which is a microsomal enzyme. The affinities of the LTC_4 synthetase and the LTA_4 hydrolase for LTA_4 differ quite markedly in various cells. In the neutrophil, LTB_4 is preferentially generated in response to A23187 indicating that the LTA_4 hydrolase has a higher affinity.

In neutrophils, LTB_4 is further oxidized and subsequently inactivated. This oxidation occurs by an LTB_4-20-hydroxylase or by an NAD-dependent dehydrogenase (Jubiz et al., 1982; Sumimoto, Takeshige and Minakami, 1985). Since LTB_4 is taken up by human PMNs via a specific receptor, it could be metabolized via this enzyme rather than via the 5-lipoxygenase pathway. LTB_4 which has been generated in response to zymosan activation, is sequestered in human neutrophils and metabolized by ω-oxidation before it is secreted (Williams et al., 1985).

LTC_4 metabolism occurs via two pathways, either peptidolytic or oxidative. LTD_4 is generated by the cleavage of glutamic acid from LTC_4 by a γ-glutamyl transpeptidase. Neutrophils demonstrate minimal generation of LTD_4, but are capable of metabolizing

FIGURE 1.5

Leukotriene synthesis.


Arachidonic acid
5-lipoxygenase
5-HPETE
5-lipoxygenase
LTA4
LTA4 (epoxide) hydrolase
LTC4 synthetase
LTB4
LTC4
LTB4 ω-hydroxylase
γ-glutamyl transpeptidase
ω-OH-LTB4
LTD4
alcohol and aldehyde dehydrogenases
dipeptidases
ω-COOH-LTB4
LTE4

LTD_4 released by other cells to LTE_4 via peptidases contained within their specific granules (Lee et al., 1983).

Oxidation of LTC_4, LTD_4 and LTE_4 only occurs in the extracellular environment of activated neutrophils (Lewis and Austen, 1984) and leads to the generation of the *S*-diastereoisomeric sulfoxides of each, and the 6-*trans*-(C12) diastereoisomers of LTB_4. This oxidation involves the hydrogen peroxide/myeloperoxidase/halide system whereby hypochlorous acid is produced, which attacks the sulfidopeptide leukotriene. The major difference between the peptidolytic and the oxidative pathway, is that the former does not require activated cells and does not inactivate LTC_4.

Activators of LTB_4 biosynthesis in neutrophils includes A23187 (Dessein et al., 1986) and zymosan (Williams et al., 1985). F Met-Leu-Phe does not induce LTB_4 generation in neutrophils, and neither does phorbol myristate acetate (PMA). The 5-lipoxygenase is a calcium dependent enzyme and the inability of f Met-Leu-Phe to stimulate it in view of its ability to induce a calcium flux is puzzling. PMA's inability to stimulate the 5-lipoxygenase is consistent with its inability to induce a calcium flux.

1.6.2.2 Biological effects of 5-lipoxygenase products:

There are two aspects of the possible action of leukotrienes in inflammation. Firstly, 5-lipoxygenase products could be released from the generating cell then interact with a target cell to elicit a biological effect. Alternatively it has been suggested that 5-lipoxygenase products act as intracellular regulators on the cell making them. This possibility has arisen from observations with T cells where tiny intracellular levels of LTB_4 are reported to be sufficient to induce differentiation of a fully competent suppressor cell population (Goetzl,

1981). In addition, a subset of these cells expresses receptors for LTB_4 (Payan and Goetzl, 1984), indicating that the released LTB_4 acts by intercellular regulation as well.

Leukotriene B4 is the main leukotriene produced by neutrophils upon stimulation. This leukotriene induces neutrophil aggregation (Ford-Hutchinson et al., 1980), chemotactic deactivation (Goetzl et al., 1981), and degranulation (Smith, Iden and Bowman, 1984), via specific cell surface receptors. These receptors may either act as an ion channel or as part of a signal transduction process during activation. Uptake of LTB_4 by neutrophils occurs within 2 minutes of exposure and therefore occurs within the same time frame as the initiation of these biological responses (Jubiz et al., 1982).

LTB_4-induced chemotaxis of neutrophils (Goetzl and Pickett, 1981) is receptor-dependent and directional. This observation indicates that intracellular stimulation by LTB_4 is not feasible in this case as directional movement could not occur. As with the f Met-Leu-Phe receptor, the high affinity LTB_4 receptor is thought to mediate neutrophil chemotactic responses, and the low-affinity LTB_4 receptor is thought to trigger the degranulation response (Goldman and Goetzl, 1984).

The 6-*trans*-LTB_4 diastereoisomers cannot activate any of the responses induced by LTB_4, suggesting that they are biologically inactive 5-lipoxygenase products. LTA_4 is known to be released by ionophore-stimulated neutrophils (McGee and Fitzpatrick, 1986). This observation coupled to the fact that 6-*trans*-LTB_4 diastereoisomers are not taken up by neutrophils, suggests that nonenzymatic hydrolysis of LTA_4 to these LTB_4 isomers can occur extracellularly. However, the intracellular regulation of neutrophil activation cannot be discounted because there is no strong evidence to discount their intracellular generation.

1.6.3 Platelet Activating Factors (PAF):

Platelet activating factor (PAF) was originally observed in rabbit buffy coat leukocytes containing IgE-sensitized basophils (Benveniste, Henson, and Cochrane, 1972). In this study, PAF was characterized as a factor released by IgE-sensitized rabbit basophils upon stimulation by antigen, which induced the aggregation of rabbit platelets. It was called "platelet activating factor" because its chemical nature was undefined. Later chemical characterization and finally gas-chromatographic-mass-spectrometric analyses of PAF revealed that rabbit basophil-derived PAF was 1-*O*-hexadecyl/octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine.

PAF is rapidly synthesized by a large range of cells following stimulation. It is not stored within the cell and does not exist in unstimulated cells. The diversity of cells which synthesize PAF suggest that it is involved in the modulation of normal biological processes in addition to acute and chronic inflammatory responses. There is also recent evidence suggesting that multiple, heterogeneous PAF molecules are concurrently produced by stimulated cells (Ludwig and Pinckard, 1987).

1.6.3.1 PAF synthesis and degradation:

Upon cell stimulation, phospholipase A_2 is activated and long-chain fatty acyl residues esterified in the 2 position are hydrolysed from intracellular pools of 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine. The lysophospholipid product, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine, is then acetylated by a specific acetyltransferase to form 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine. This biologically active molecule is degraded via an acetylhydrolase which removes the acetyl group yielding the biologically inactive lyso-PAF (Blank et al., 1981). Lyso-PAF can be reacylated with a long-chain fatty acid via an

acyltransferase (Wykle, Olson and O'Flaherty, 1986), thereby completing the metabolic cycle (Figure 1.6).

A second *de novo* enzymatic pathway exists for PAF synthesis. This pathway is catalysed by a choline phosphotransferase which uses both CDP-choline and 1-*O*-alkyl-2-acetyl-*sn*-glycerol (Renooij and Snyder, 1981). Although this pathway exists in inflammatory cells, it is unlikely that it predominates, because the acetyl-transferase activity significantly increases after cell stimulation, whilst the choline phosphotransferase activity remains unchanged (Alonso et al., 1982).

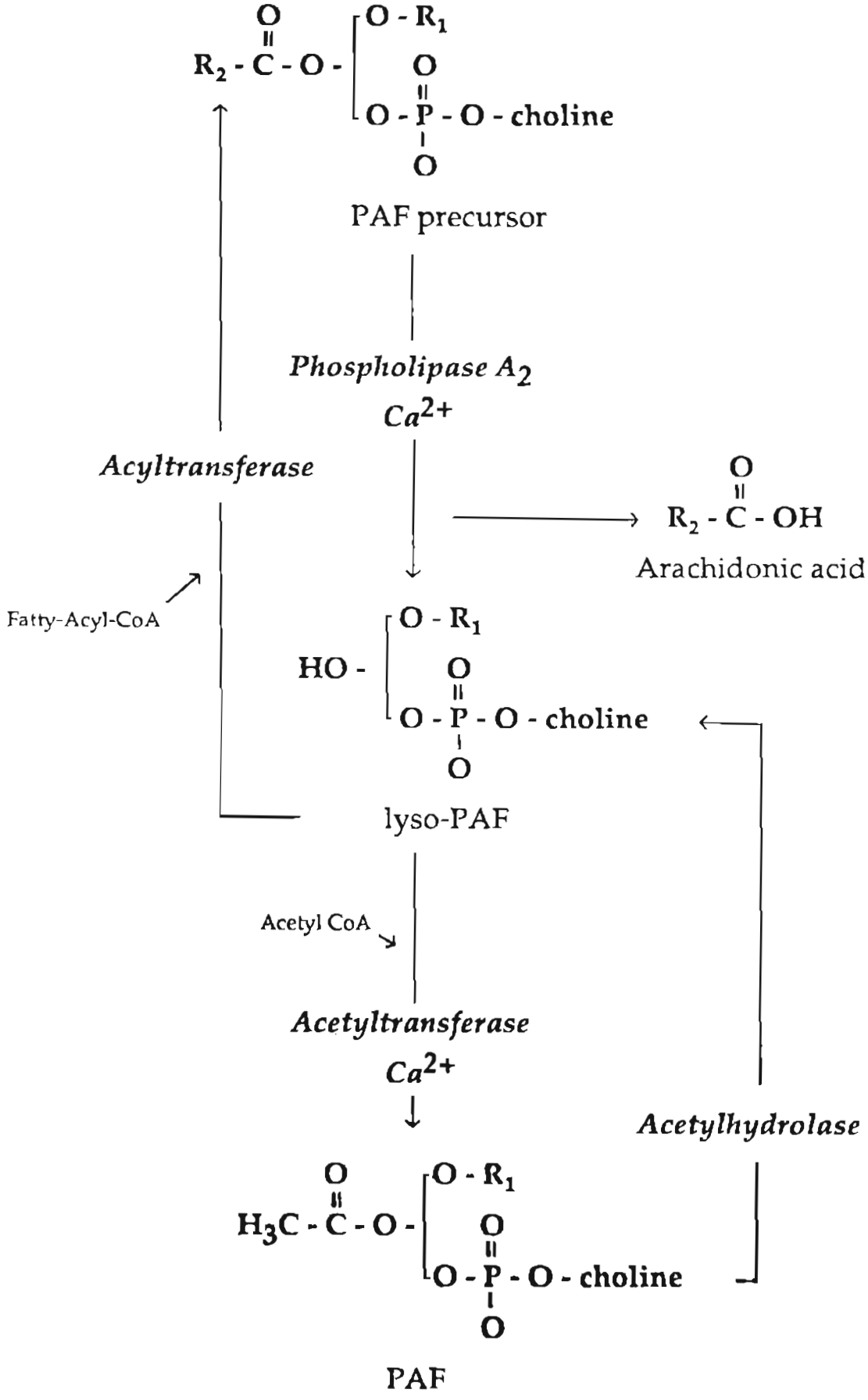
An interesting aspect of PAF's effect on neutrophils is that activation may be modulated through synergistic interactions with various 5-lipoxygenase products. There is actually evidence suggesting that PAF and arachidonic acid metabolites are probably derived from a common precursor, i.e. 1-*O*-alkyl-2-arachidonyl-*sn*-glycero-3-phosphocholine (Chilton et al., 1984). Coupled to this is the observation that choline-containing phosphoglycerides are enriched in arachidonic acid when compared to the diacyl phosphocholine pool (Mueller et al., 1984). In addition, resting neutrophils acylate 80% of exogenously added lyso-PAF with arachidonic acid (Chilton et al., 1983), further strengthening the relationship between the generation of PAF, arachidonic acid and its metabolites. These observations suggest that neutrophils may contain a phospholipase A_2 that is activatable and highly specific for 1-*O*-alkyl-2-arachidonyl-*sn*-glycero-3-phosphocholine which is responsible for the generation of PAF.

A) Phospholipase A_2:

Phospholipase A_2 is essential in PAF biosynthesis, because inhibitors of this enzyme such as mepacrine, bromophenacyl bromide, and hydrocortisone, inhibit both lyso-PAF and PAF formation (Parente and Flower, 1985). An observation that further supports

FIGURE 1.6

PAF synthesis.


PAF precursor
Phospholipase A2
Ca2+
Acyltransferase
Arachidonic acid
Fatty-Acyl-CoA
lyso-PAF
Acetyl CoA
Acetyltransferase
Ca2+
Acetylhydrolase
PAF

the putative link between arachidonic acid metabolism and PAF generation is that certain lipoxygenase products potentiate phospholipase A_2 activity in stimulated neutrophils (Billah, Bryant and Segal, 1985). Thus, the addition of 5-L-HPETE, 5-L-HETE or LTB_4 to stimulated human neutrophils augments both arachidonic acid release and the generation of PAF. Consistent with this is that NDGA, which is a dual lipoxygenase/cyclooxygenase inhibitor, decreases both arachidonic acid release and PAF formation in these cells. Notably, specific cyclooxygenase inhibitors do not inhibit arachidonic acid release or PAF formation (Billah, Bryant and Segal, 1985). One can therefore suggest that the modulation of phospholipase A_2 activity is a critical step in influencing the availability of PAF precursors in the neutrophil.

B) Acetyltransferase:

Acetyltransferase catalyses the transfer of acetate from acetyl CoA to the 2 position of lyso-PAF and other lysophospholipids. (Figure 1.6) The proposal that this enzyme may play the rate-limiting step in PAF generation is suggested by the observation of a temporal relationship between PAF formation and the activity of acetyltransferase. Acetyltransferase activity is localized in the plasma membrane of neutrophils and has a pH optimum between 7.5 and 8.0 (Wykle, Olson and O'Flaherty, 1986).

C) Acetylhydrolase:

Acetylhydrolase catalyses the hydrolysis of the acetyl group from PAF and other related acetylated phosphoglycerides (Figure 1.6). The product of this reaction, lyso-PAF is rapidly reacylated at the 2 position with a long-chain fatty acid (Chilton et al., 1983). Human neutrophil acetyl hydrolase is calcium independent and specific for short-chain fatty acyl residues at the 2 position, and is located predominantly in the cytosol (Wykle,

Olson and O'Flaherty, 1986). The role of intracellular acetyl-hydrolase as a regulatory protein in PAF generation has yet to be defined.

D) Acyltransferase:

Intracellular acyltransferase catalyzes the transfer of a long-chain fatty acid to the 2 position of lyso PAF (Figure 1.6). This enzyme is thought to be responsible for the rapid conversion of exogenously added PAF to 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine to neutrophils (Wykle, Olson and O'Flaherty, 1986). Human neutrophil acyltransferase is primarily located in the plasma membrane and does not require acyl-CoA, ATP or Mg^{2+} (O'Flaherty et al., 1986). Notably the lyso-PAF is predominantly acylated with arachidonic acid (Chilton et al., 1983).

1.6.3.2 PAF synthesis-release coupling:

Considering PAF is such a potent inflammatory agent, the regulation of its synthesis and subsequent release from cells must be tightly controlled. The role of phospholipase A_2 and acetyltransferase are proposed to be important for the regulation of PAF biosynthesis, but little has been done to define the mechanisms regulating PAF release from cells.

The release of newly synthesized PAF is tightly linked to augmented and sustained PAF synthesis (Ludwig, McManus and Pinckard, 1986). Despite this, all of the PAF that is synthesized is not necessarily released, e.g. human endothelial cells (McIntyre, Zimmerman and Prescott, 1986). Indeed, human neutrophils are reported to require the accumulation of a critical intracellular concentration of PAF prior to release (Ludwig et al., 1984; Ludwig et al., 1985), and the release of PAF appears to be related to the degree of neutrophil degranulation. In the absence of extracellular calcium, endogenous calcium stores support up to 40% to 50% of maximal degranulation in f Met-Leu-Phe-stimulated

neutrophils, but PAF synthesis is depressed, and no PAF is released. In the presence of extracellular calcium PAF synthesis and release occurs in a calcium dose-dependent manner. Extracellular calcium not only enhances PAF synthesis but augments neutrophil degranulation. Thus, although PAF release may be related to degranulation, these observations also suggest that there are two mechanisms regulating PAF synthesis and release in stimulated human neutrophils. The first allows the initial synthesis of PAF after cell stimulation and requires only intracellular calcium; the second requires extracellular calcium and modulates both PAF release and sustained PAF synthesis.

The concentration of extracellular albumin also regulates PAF synthesis-release coupling. In the absence of albumin, f Met-Leu-Phe-stimulated neutrophils initially synthesize small amounts of PAF, but no PAF release occurs. Subsequently, further PAF synthesis is abrogated (Ludwig et al., 1985). In the presence of 0.25% albumin total PAF synthesis is increased and the release of 30% to 40% of this PAF occurs within 5 minutes of stimulation. In addition, in the presence of 5% albumin, a sevenfold increase in PAF synthesis and release occurs, which is sustained for 30 minutes. Therefore, the presence of albumin induces a dose-dependent increase in the synthesis and release of PAF.

This property of albumin may reflect its influence on the solubility of PAF in aqueous conditions. If insufficient albumin is present, PAF could by synthesized, expressed on the cell membrane, but remain unreleasable because it has no albumin to bind to. Subsequently, newly synthesized PAF would be reacylated back into the phospholipid pool instead of being released (O'Flaherty et al., 1986). In addition, surface PAF could interact with a PAF receptor which may cause downregulation of subsequent PAF synthesis. In the presence of albumin, newly synthesized PAF would be released onto the albumin before interaction with this receptor. Thus, PAF release and sustained biosynthesis occurs in the presence of albumin.

1.6.3.3 PAF influences neutrophil activation:

It was not until studies assessing the *in vivo* role of PAF were performed that a possible effect on neutrophils was observed by PAF. Within sixty seconds of the intravenous infusion of submicrogram amounts of PAF, neutropenia was observed (McManus et al., 1980). Following this, preliminary *in vitro* studies demonstrated that PAF induced aggregation, chemotaxis and chemokinesis, and secretion of lysosyme and β-glucuronidase from isolated human neutrophils (Pinckard et al., 1980). Subsequent studies have demonstrated that PAF modulates most aspects of neutrophil function and may play an important role in the inflammatory reaction (Table 3).

Table 3
PAF activates neutrophils

Chemotaxis	Pinckard et al., 1980
Chemokinesis	Pinckard et al., 1980
Aggregation	Shaw et al., 1981
Enhanced adherence	Ingraham et al., 1982
Lysosomal enzyme release	Shaw et al. 1981
Leukotriene synthesis	Smith and Bowman, 1982
HETE production	Smith and Bowman, 1982
Superoxide anion production	Shaw et al., 1981
Calcium uptake	Lad et al., 1987
Phosphatidylinositol turnover	Lad et al., 1987

1.6.3.4 Mechanism of activation by PAF:

Extracellular calcium enhances most PAF-induced human neutrophil responses. PAF mobilizes intracellular membrane-associated calcium stores within seconds, as measured by a decrease in the fluorescence of neutrophils preloaded with chlortetracycline (CTC, Lad et al., 1987). Other fluorescent calcium probes such as quin-2 and fura-2 also indicate significant increases in intracellular free calcium concentrations upon stimulation with

PAF. Most of this increase in intracellular free calcium occurs as an influx, because the absence of extracellular calcium significantly reduces this response.

The second messengers involved in PAF-induced calcium mobilisation appear to be both phosphatidyl inositol/phospholipase C dependent and independent. Firstly, PAF induces transient but significant decreases in PIP_2 in human neutrophils (Lad et al., 1987). Secondly, pretreatment with pertussis toxin inhibits PAF-induced PIP_2 turnover and calcium mobilization of intracellular calcium stores in human neutrophils (Lad et al., 1987). In addition, the influx of intracellular calcium is significantly reduced but not completely inhibited by pertussis toxin. Finally, pretreatment with PMA abrogates subsequent PAF-induced increases in intracellular free calcium and degranulation of neutrophils. The reason for this effect has not yet been established, but it has been proposed that the activation of protein kinase C by PMA inactivates the PI/PLC-coupled receptors (Lad et al., 1987).

The above observations have led to the suggestion that two types of PAF receptors exist. The first receptor is associated with an apparent pre-existing calcium channel and is not linked to GTP-binding proteins because it is pertussis toxin resistant. The second receptor is pertussis toxin sensitive and therefore coupled to a GTP-binding protein. This pertussis toxin-sensitive PAF receptor in human neutrophils could modulate (i) PIP_2 turnover and initial mobilization of intracellular calcium stores, (ii) a major portion of extracellular calcium influx, (iii) production of superoxide, secretion of lysosyme, and (iv) neutrophil aggregation and chemotaxis. Evidence to support the existence of a GTP-linked PAF receptor is provided by the observation that GTP analogs decrease the binding affinity of PAF for its receptor (Ng and Wong, 1986).

1.7 EXOGENOUS ACTIVATORS OF NEUTROPHILS

1.7.1 N-formylmethionylleucylphenylalanine (f Met-Leu-Phe):

F Met-Leu-Phe is a synthetic oligopeptide belonging to a large group of formylated peptides characterized by their ability to activate neutrophil functions. The chemotactic activity of formylmethionyl peptides was originally discovered by Schiffmann and coworkers (1975) whilst attempting to define the chemotactic factors in culture filtrates from *E. coli*. Subsequently, the functional activity of approximately 60 synthetic formylpeptides was investigated with respect to their structure (Showell et al., 1976). The most potent of these peptides was shown to be f Met-Leu-Phe, which was later reported to be the major peptide chemotactic factor present in *E. coli* culture filtrates (Marasco et al., 1984).

F Met-Leu-Phe activates a wide range of neutrophil functions and has therefore been intensely studied in an attempt to dissect the individual processes involved in the inflammatory reaction. Interestingly, different concentrations of the same formylpeptide are required to attain the same relative activity for various functions (Becker, 1979). The stimulation of chemotaxis is the most sensitive response, whereas the activation of superoxide production is the least sensitive. These initial observations indicated that the formylpeptide receptor is a multifunctional receptor.

1.7.1.1 The formyl peptide receptor:

The molecular size of the formylpeptide receptor was originally determined by cross-linking radiolabeled hexapeptide (CHO-Nle-Leu-Phe-Nle-^{125}I-Tyr-Lys) to the receptor using dimethyl suberimidate (Niedel, Davis and Cuatrecasas, 1980). Sodium dodecyl

sulfate (SDS)-polyacrylamide gel electrophoresis determined two distinct entities of relative molecular weights 50,000 and 60,000 (Schmitt et al., 1983). The receptor is a glycoprotein (Painter et al., 1982) and contains two *N*-linked oligosaccharide chains. It has also been shown that formylpeptide receptors are present in intracellular pools (Fletcher and Gallin, 1983) as well as on the membrane surface.

Further studies have indicated that the formyl peptide receptor is tightly associated with the membrane (Baldwin, Bennett and Gomperts, 1983). Extraction procedures and papain digest studies have indicated that it is a transmembrane protein to which the oligosaccharide chains are attached on the outside of the cell (Dolmatch and Niedel, 1983). Physicochemical studies have revealed that the receptor exists as a monomer and is either associated with tightly bound endogenous lipid or is an hydrophobic membrane protein (Allen et al., 1986).

The human neutrophil formylpeptide receptor has not yet been purified to homogeneity. There are 50,000-80,000 surface receptors per cell (Allen et al., 1986) and approximately an equal number expressed intracellularly. The most homogenous preparation obtained so far is that prepared by Allen et al. (1986). Using a new photoaffinity label, *N*-formyl-Met-Leu-Phe-N^{ε}-[2-(*p*-azidosalicylamido)ethyl-1,3'-dithiopropionyl]-Lys (FMLPL-SASD), the receptor was isolated to yield a monomer of apparent molecular weight 63,000.

1.7.1.2 Regulation of receptor affinity:

Ligand-receptor interactions have been widely studied using radiolabeled and fluorescently labeled formyl peptides. Zigmond et al. (Sullivan and Zigmond, 1980), have used tritium-labeled *N*-formyl norleucylleucylphenylalanine binding kinetics, internalization and recycling to define the chemotactic receptor cycling involved in locomotion. Sklar et al. (1984) have studied fluoresceinated hexapeptide to study receptor internalization. These

workers have described a slowly dissociating form of ligand-receptor complex which coisolate with detergent-insoluble cytoskeletal residues. This cytoskeletal-associated receptor is thought to represent an early step in the internalization and processing of the receptor and may be part of the mechanism in which activated receptors are turned off (Jesaitis et al., 1984; Jesaitis et al., 1985).

Evidence suggests that the receptors not associated with the cytoskeleton exist in two affinity states (Lane and Snyderman, 1984) and these are modulated by guanine nucleotides (Koo, Lefkowitz and Snyderman, 1983). Marasco et al. (1985) characterized both membrane bound and CHAPS-solubilized receptors. They reported a two-saturable site model fitted better to the data and measured a Kd = 1.25±0.45nM for the high affinity site and a Kd = 19.77±3.28nM for a low affinity site. 35% of the total sites detected were of the higher affinity. Modulation of f Met-Leu-Phe receptors by guanine nucleotides was originally demonstrated by Koo et al. (1983). It was shown that guanine nucleotides did not affect the total receptor number or the receptor affinities, but rather induced a conversion of high affinity receptors to the low affinity state. This effect was reversed on removal of the guanine nucleotides, and the same effect was observed when neutrophil membranes were used. The conversion is specific for guanine nucleotides and exhibits a range of potencies that are typical for guanine nucleotide effects on adenylate cyclase-coupled receptors. The changes occurring in the receptor itself upon affinity modulation, are as yet unknown. It is possible that binding of the ligand to the external section of receptor triggers a conformational change in its cytoplasmic domain. This could alter the cytoplasmic interaction of the receptor with its signal transduction processes.

The relationship between multiple affinity states of the formyl peptide receptor and its influence on various neutrophil functions remains undetermined. Becker (1979) noted that a hierarchy of sensitivity to f Met-Leu-Phe activation exists for a variety of neutrophil functions. Indeed, Lohr and Snyderman (1982), by manipulating the affinity of the formyl

peptide receptor with exogenous agents, postulated that the high affinity receptor triggered chemotaxis and intracellular calcium changes, whereas the low affinity receptor is associated with degranulation and superoxide generation. In contrast, Sklar et al. (1984) have shown that there is a temporal change in formyl peptide receptor affinity that parallels cell activation through the occupied receptor. Recently, evidence suggesting that all formyl peptide receptors may participate in all responses via G proteins has emerged (Sklar et al., 1987). These observations lead to the postulation that the responses with low ED_{50}s require few occupied receptors whereas responses with high ED_{50}s require many receptors to initiate and sustain the responses.

1.7.1.3 Coupling of the receptor to phospholipase C:

To determine whether a GTP-binding protein was associated with formyl peptide receptor activation of neutrophils, pertussis toxin was used. Pertussis toxin catalyzes the covalent modification of both G_i and G_o resulting in the inhibition of function of these proteins. Pertussis toxin-treatment of neutrophils results in the complete inhibition of formyl peptide stimulated function, including arachidonic acid release, degranulation and superoxide generation (Bokoch and Gilman, 1984), chemotaxis (Becker et al., 1985), actin polymerisation (Shefcyk, 1985), and the release of intracellular calcium (Molski et al., 1984). The effect of pertussis toxin on neutrophils could be correlated with the toxin-catalyzed ADP-ribosylation of a 41,000-dalton membrane protein that comigrated with purified $G_i\alpha$ subunit (Bokoch and Gilman,1984).

The coupling of the formyl peptide receptors to the combined processes of calcium release and protein kinase C activation has been implied by the ability of pertussis toxin to attenuate formyl peptide receptor-stimulated breakdown of inositol trisphosphate and bisphosphate (Smith et al., 1985; Volpi et al., 1985b). The formation of inositol triphosphate and diacylglycerol occurs as a result of activation of phospholipase C. The

generation of these second messengers is necessary for calcium release and protein kinase C activation. Evidence to support these observations was provided by Cockcroft and Gomperts (1985) who showed that phospholipase C in neutrophil plasma membranes can be activated by adding GTP analogues in the presence of calcium.

1.7.2 Endotoxin:

Endotoxins are a group of macromolecules derived from the outer cell envelope of gram-negative bacteria. Their central role in inflammatory reactions has been demonstrated from studies where the intravenous administration of endotoxin induces hematologic and hemodynamic changes similar to those seen in patients with sepsis due to gram-negative bacteria. One of the most dramatic effects of endotoxins is the induction of a rapid and pronounced decline in the number of circulating neutrophils. It has recently been proposed that the mediators of this effect are predominantly IL-1β and TNF-α.

Since the neutrophil plays a significant role in host defense against bacterial infection, the mechanisms of interaction of endotoxins with neutrophils has been investigated both *in vivo* and *in vitro*. Lipopolysaccharide (LPS) is responsible for many of the biologic properties of endotoxins, and in particular, the lipid A moiety is important in this activity. Not only do the preparation procedures affect endotoxic activity, but different animal species vary considerably in their sensitivity to endotoxins, with humans being the most sensitive.

1.7.2.1 Endotoxin - neutrophil interactions:

It has recently been proposed by Movat et al. (1987) that many of endotoxins effects *in vivo* are mediated by the generation of inflammatory cytokines such as IL-1β and TNF-α. In addition to this endotoxins can also indirectly activate neutrophils by their ability to

activate serum complement via the classical or alternative pathway, or both (Gewurz, Shin and Mergenhagen, 1968; Morrison and Kline, 1977). Therefore, it is difficult to assess the *in vivo* effects of endotoxin on neutrophil functions.

Direct activation of neutrophil function by endotoxin requires binding to the neutrophil membrane. Evidence from *in vivo* experiments employing intravenous administration of radiolabeled endotoxin (Mathison and Ulevitch, 1979), are difficult to assess. Variables including the species derivation and extraction method, the total dose of injected endotoxin, and interaction of endotoxin with serum constituents complicate the interpretation of such studies.

In vitro studies have shown that washed neutrophils in serum-free medium do bind endotoxin. Adherent human neutrophils bind fluorescein-isothiocyanate-labelled LPS from *S. minnesota* R595 (Wilson et al., 1982), and neutrophils in suspension have been shown to take up endotoxin (Cline et al., 1968). Binding of endotoxin is independent of incubation temperature and unaffected by a variety of metabolic inhibitors. Binding is also independent of divalent cation concentrations in the environment. The nature of the neutrophil endotoxin receptor has yet to be defined, although Springer and Adye (1975) have partially characterized endotoxin binding substances on human neutrophils. The binding activity appears to be a glycerophosphatide(s).

1.7.2.2 Effects on neutrophil functions:

Endotoxin has been reported to promote the adherence of neutrophils to both nylon wool and plastic petri dishes (Spagnuolo and Ellner, 1980; Dahinden, Galanos and Fehr, 1983; Dahinden and Fehr, 1983). The mechanism by which it increases neutrophil adherence is

unknown. *In vivo* the effect of endotoxin may be indirectly via the generation of the chemotactic factor C5a (Gallin, 1980).

Endotoxin also induces neutrophil aggregation *in vivo* (Goodman, Way and Irwin, 1979). Conflicting reports exist as to the *in vitro* ability of endotoxin to induce aggregation. Notably, human neutrophils do not aggregate (Spagnuolo and Ellner, 1980) whereas glycogen-elicited rabbit peritoneal neutrophils do (Thorne, Oliver and Lackie, 1977). This discrepancy could be due to the difference in activation levels of neutrophils from different sources. The collective evidence seems to indicate that endotoxin increases neutrophil adherence without causing aggregation.

Chemotactic responsiveness of neutrophils is decreased following *in vivo* and *in vitro* exposure to endotoxins (Dahinden, Galanos and Fehr, 1983; Territo and Golde, 1976). The inhibition is not due to desensitization because endotoxin has no demonstrable chemotactic activity up to 1mg/ml. The mechanism by which endotoxin inhibits neutrophil locomotion is not defined. It has been suggested that the induction of hyperadherence decreases motility (Dahinden, Galanos and Fehr, 1983) and the constitutents of the specific granules that are discharged in response to endotoxin have been implicated.

Endotoxin induces the degranulation of neutrophils from a variety of sources (Dahinden, Galanos and Fehr, 1983). These observations have reported the release of lysosyme, peroxidase, plasminogen activator and β-glucuronidase. A consideration regarding these studies is that the neutrophil preparations were not pure. Since endotoxin is known to activate monocytes/macrophages and is a potent inducer of IL-1β and TNF-α production from these cells, these studies need to be considered carefully. Dahinden and coworkers (1983), indicate that although suspended neutrophils do not degranulate in response to endotoxin, prior adherence does induce a response. In addition, endotoxin appears to be

capable of promoting the selective release of specific granule constituents from these adherent neutrophils. Endotoxin has also been reported to induce degranulation from human neutrophils in the presence of complement (Cline et al., 1968).

Endotoxin is able to induce a small increase in the neutrophil respiratory burst as measured by nitroblue tetrazolium (NBT) dye reduction and activation of the hexose monophosphate shunt by human neutrophils (Proctor, 1979). The presence of complement markedly increases this effect presumably due to the generation of C5a. Some controversy exists regarding the ability of endotoxins to directly stimulate the oxidative metabolism of neutrophils. A variety of groups have reported that endotoxin enhances hexose monophosphate shunt activity of neutrophils *in vitro* (Wilson et al., 1982; Dahinden and Fehr, 1983; Proctor, 1979). Others have reported its ability to stimulate NBT reduction (Wilson et al., 1982). Since NBT reduction has been reported to occur by neutrophils that were maintained anaerobically, there is some doubt as to whether this assay reflects a true change in oxidative metabolism.

Studies by Dahinden and Fehr (1983) indicate that adherent neutrophils exhibit a significant increase in oxidative metabolic activity following endotoxin challenge. This factor could have contributed to the discrepancies reported above. Interestingly, Guthrie and Johnston (1982) have reported that pretreatment with endotoxin enhances the subsequent generation of superoxide and degranulation in response to fixed immune complexes, opsonized zymosan or PMA. This study has introduced the concept of endotoxin "priming" of neutrophil activation.

1.7.2.3 Priming of neutrophil functions by endotoxin:

Following the initial report by Guthrie and Johnston (1982) demonstrating that endotoxin primes neutrophil responses, other studies have emerged to support this. Guthrie et al. (1984) reported that preincubation of neutrophils with endotoxin enhanced their subsequent response to substances such as f Met-Leu-Phe, phorbol myristate acetate and fixed immune complexes. The molecular basis for this change was investigated. Preincubation with LPS at 0°C prevented priming, but cycloheximide or chelation of extracellular calcium had no effect. The numbers of f Met-Leu-Phe receptors were found to be slightly decreased but no change in binding affinity was observed. Other priming effects of endotoxin include enhanced degranulation and decreased chemotactic responsiveness to f Met-Leu-Phe (Haslett et al., 1985), and enhanced secretion of elastase (Fittschen et al., 1985).

Recently the mechanisms by which endotoxin alters neutrophil responses has been investigated. Worthen et al. (1988) reported that the induction of intracellular platelet-activating factor may contribute to the enhanced superoxide production resulting from endotoxin priming. Priming of the neutrophil respiratory burst does appear to be dependent on intracellular free calcium levels (Forehand et al., 1989). Interestingly, pertussis toxin did not prevent priming, and calcium-dependent protein kinase C activity was unaltered. These observations suggest that the priming target precedes NADPH oxidase activation. In support of these observations, other priming agents such as f Met-Leu-Phe (McPhail, Clayton and Snyderman, 1984), platelet-activating factor (Worthen et al., 1987) and the calcium ionophore, ionomycin (Finkel et al., 1987) induce an increase in basal $[Ca^{2+}]_i$ without stimulating superoxide anion release. Thus, the molecular basis for priming may lie in changes in intracellular free calcium concentrations rather than a direct effect on the effector functions themselves.

CHAPTER 2

MATERIALS AND METHODS

SECTION 2.1 MEDIA, BUFFERS AND CHEMICALS

All buffers were prepared using deionized and filtered water that had been processed by the Milli-RO Water System (Millipore Corporation, Bedford, MA). Unless otherwise stated, solutions were stored sterile at 4°C. General chemicals were of analytical grade.

Medium: RPMI 1640 in the form of powdered medium was purchased from GIBCO Laboratories (Grand Island, N.Y.). After reconstitution the following solutions were added; 20mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, Sigma), 60µg/ml penicillin (David Bull Laboratories, Mulgrave, Victoria), 40µg/ml gentamycin (David Bull Laboratories, Mulgrave, Victoria), 500µM NaOH. The medium was filtered and appropriate sterility tests performed. This medium was used for the isolation of peripheral blood neutrophils from human blood.

Phosphate Buffered Saline (PBS): 8g NaCl, 0.2g KCl, 1.15g Na_2HPO_2 and 0.2g KH_2PO_4 were dissolved in 1 litre of water and the pH adjusted to 7.4 with HCl or NaOH. The pH of this solution was always checked just before use. This buffer was used for the following: 5% Dextran T500; preparation of zymosan activated serum; 3.2% paraformaldehyde; and as washing buffer during staining for filamentous actin.

Hanks Balanced Salt Solution: 0.04g KCl, 0.006g KH_2PO_4, 0.8g NaCl, 0.012g Na_2HPO_4, 0.018g $CaCl_2.2H_20$, 0.02g $MgSO_4.7H_20$, 0.1g glucose and 2.1g $NaHCO_3$ were dissolved in 1 litre of water and the pH adjusted to 7.4 with HCl or NaOH. The pH of this solution was always checked before use. This buffer was used in the assay for f Met-Leu-[^{3}H]Phe binding.

N-formylmethionylleucylphenylalanine: Purchased from Sigma and dissolved in ethanol at 10^{-3}M. This solution was stored at -70°C.

Cytochalasin B: Purchased from Sigma. Prepared from *Helminthsporium dematioideum.* 1mg/ml cytochalasin B was dissolved in dimethylsulphonic acid and stored at 4°C.

Calcium Ionophore (A23187): Purchased from Behring Diagnostics, La Jolla, CA. 100mM A23187 was dissolved in dimethyl sulphoxide and stored at -20°C.

SECTION 2.2 PURIFICATION OF HUMAN NEUTROPHILS

Neutrophils were obtained from the peripheral blood of healthy volunteers. Blood was collected into a sterile syringe containing heparin sodium (David Bull Laboratories, Mulgrave, Victoria) and sedimented with 5% dextran (Dextran T500, Pharmacia) in phosphate buffered saline (PBS, 2ml:10ml blood). After density gradient centrifugation at 450g on Lymphoprep (Nyegaard, Oslo), erythrocytes were lysed by hypotonic shock using 0.2% sodium chloride followed by restoring isotonicity with 1.6% sodium chloride.

When a large number of neutrophils was required, they were prepared from Red Cross packed cells. The blood donation was centrifuged at 4000g for 5 min in a Sorvall freestanding centrifuge. The fraction remaining after the plasma was removed, was diluted 1:2 in RPMI 1640, and neutrophils prepared as described above.

After preparation, neutrophils were resuspended to 10^7/ml in RPMI 1640 containing 20mM HEPES buffer and antibiotics. This medium was supplemented with either 1:100 Nutridoma-HU (Boehringer Mannheim Biochemicals, Mannheim, West Germany) or 0.1% fatty acid free bovine serum albumin (Fraction V, Sigma, St. Louis, MO), and assays were carried out in this medium unless otherwise stated. The lipopolysaccharide content of this medium was determined to be less than 1.9pg/ml using the limulus amebocyte lysate assay (Levin and Bang, 1968).

SECTION 2.3 CYTOKINES

2.3.1 Recombinant human tumor necrosis factor-α (rH TNF-α):

The rH TNF-α used were lot numbers 3056-55 and 4260-1, containing approximately 5×10^7 units/mg and 1.2×10^8 units/mg respectively. Both were generously provided by Genentech, South San Fransisco, California. The molecule was produced in *Escherichia coli* (Pennica et al., 1985) and purified to approximately 99.8%. The lipopolysaccharide content of this solution was determined to be less than 0.125EU/ml as measured by the manufacturers, using the limulus amebocyte lysate assay. Dilutions from stock material were made weekly into RPMI 1640 containing 20mM HEPES antibiotics, and 1% bovine serum albumin (BSA, Commonwealth Serum Laboratories, Melbourne, Victoria) and stored at 4^oC. The final concentration of LPS present in incubation suspensions was less than 0.1EU/ml.

2.3.2 Recombinant human granulocyte-macrophage colony-stimulating factor (rH GM-CSF):

The rH GM-CSF was lot number C01-P0004 containing approximately 4.7×10^6 units/mg (when tested on Chronic myeloid leukaemic cells), was 97.2% pure and was generously provided by Genetics Institute, Cambridge, Massachusetts. The protein was obtained from the supernatant of COS cells and purified as described (Wong et al., 1985). Dilutions from stock material were made into RPMI 1640 and 2% FCS and kept sterile at -70^oC. The lipopolysaccharide content of this solution was determined to be less than 0.4EU/ml as measured by the limulus amebocyte lysate assay (Levin and Bang, 1968).

2.3.3 Recombinant human interleukin-1β:

The rH interleukin 1-β was provided by Immunex, Seattle, WA, and contained 10^8U/mg when assayed by thymocyte mitogenesis (Kronheim et al., 1985). Stock material was stored at -70°C and diluted into RPMI 1640 + 0.1% BSA for use. The lipopolysaccharide content of this solution was determined to be less than 0.4EU/ml.

SECTION 2.4 FUNCTIONAL ASSAYS

2.4.1 Superoxide Assay:

Purified neutrophils (10^7/ml) were incubated with medium or different concentrations of cytokines for various times at 37°C. After this, 150µl of cells (10^6) were added to a mixture of 100µl freshly prepared cytochrome C (type VI, 12.4 mg/ml, Sigma), 100µl f Met-Leu-Phe (Sigma) and made up to 1 ml with medium. The mixtures were then incubated at 37°C for 15 minutes, after which the cells were rapidly cooled, pelleted at 4°C and the supernatants transferred to plastic disposable cuvettes. Superoxide production was measured by the reduction of cytochrome C using an extinction coefficient of 21.1 (van Gelder and Slater, 1962) at 550nm (Weening, Weever and Roos, 1975).

In experiments where the rate of superoxide anion production was measured, cells were preincubated with 100u/ml rH TNF-α for 45 minutes at 37°C. 150µl of cells were then added to disposable plastic cuvettes containing 100µl f Met-Leu-Phe (10^{-7}M final), 100µl freshly prepared cytochrome C and made up to 1ml with medium. This suspension was incubated in a thermostatically controlled cuvette holder at 37°C for 10 minutes. The change in OD550nm was continuously monitored and recorded at 30 second intervals in a DU-50 Spectrophotometer (Beckman Instruments, Berkeley, CA). The optical density of

the negative controls decreased slightly during these experiments due to the settling of cells.

In control experiments the release of superoxide anion was confirmed by allowing the reaction to f Met-Leu-Phe to proceed in the presence of freshly prepared superoxide dismutase (56µg/ml final, Sigma). The release of superoxide anion was completely abolished in the presence of superoxide dismutase. To ensure the change in OD550nm was not limited by cytochrome C concentration, maximum reduction was determined by adding a few grains of sodium dithionite (BDH Chemicals Aust., Kilsyth, Victoria) to the final mix.

2.4.2 Chemotaxis assay:

The chemotactic response of neutrophils to a gradient of f Met-Leu-Phe was tested under agarose as described (Nelson, Quie and Simmons, 1975). Briefly, 5ml of 0.5% agarose (type II, Sigma) dissolved in RPMI 1640 and 2% FCS with 20mM HEPES, and 375µg/ml sodium bicarbonate (Flow Laboratories, North Ryde, N.S.W.), was set in a plastic petri dish (50mm, Kayline, Adelaide, South Australia) and 2.4mm diameter wells were formed 2.4mm apart in a horizontal line from the centre to the edge of the plate. Neutrophils at 2.5 x 10^7/ml in medium were preincubated 45 minutes at 37°C in the presence or absence of 100u/ml rH TNF-α. This period of incubation was chosen to ensure maximal stimulation by rH TNF-α. 5µl of cells was then added to the centre well, 5µl of medium added to the inner well and 5µl of 10^{-7}M f Met-Leu-Phe was added to the outer well. The petri dish was incubated for 2 hours at 37°C in 5% CO_2. The cells were fixed with methanol at 4°C overnight, followed by 40% formalin for 30 min at 25°C before the agarose was removed.

Three parameters of locomotion were measured: (a) random movement was quantified by placing medium in the inner and outer wells of the chemotaxis plate; (b) chemokinesis was

measured by placing the chemotactic stimulus in both the cell-containing and the outer well of the chemotaxis plate; and (c) chemotaxis was measured by placing the chemotactic stimulus in the outer well only. Locomotion was visualized and quantified using an ocular micrometer, and results are expressed in arbitrary units as either random movement, chemokinetic movement or chemotactic movement.

2.4.3 f Met-Leu-[^{3}H]Phe binding to neutrophils:

Binding of f Met-Leu-[^{3}H]Phe to purified neutrophils was measured at 4^{o}C in siliconized 12 x 75mm glass test tubes in a total volume of 105µl. Cells from the same donor at a concentration of 10^7/ml were incubated in the presence or absence of rH TNF-α for 60 minutes at 37^{o}C in RPMI 1640 + 1:100 Nutridoma then washed in cold incubation buffer (Hanks buffered saline with 1.6mM CaCl, 10mM sodium azide, 5µg/ml cytochalasin B (Sigma) and 0.1% BSA (Commonwealth Serum Laboratories) and resuspended to 10^7/ml. Sodium azide and cytochalasin B were included in the incubation buffer to prevent ligand internalisation which may occur at 4^{o}C (Sullivan and Zigmond, 1980). 100µl cells were added to triplicate tubes containing 5µl of f Met-Leu-[^{3}H]Phe at various concentrations (100 nM-0.5 nM) in incubation buffer, or to duplicate tubes containing, in addition, unlabelled f Met-Leu-Phe (100µM). Bound f Met-Leu-[^{3}H]Phe was measured after 30 minutes by filtration through glass fiber filters (GF/B, Whatman, Maidstone, England) using Hanks buffered saline, and quantified by liquid scintillation counting.

The binding data were analysed using LIGAND, a weighted nonlinear least squares regression analysis computer programme (McPherson, 1985). After subtracting the non-specific binding from total binding, the specific binding data were fitted to one or more of the appropriate equations from a one-saturable-site model, a two-saturable-sites model, and the Hill equation (Munson and Rodbard, 1980; Colquhoun, 1979; DeLean and Rodbard, 1979). The weighted sums of squared deviations (WSSDs) generated by LIGAND for one

saturable site, two saturable sites, and the Hill equation were compared using the F-test (McPherson, 1985).

2.4.4 High-performance liquid chromatography of f Met-Leu-[^{3}H]Phe:

To ensure that impurities were not present in the commercially available f Met-Leu-[^{3}H]Phe used (50.9 to 55.3 Ci/mmol, New England Nuclear, Boston, MA), the preparation was analysed by high-performance liquid chromatography. The system consisted of a Beckman 334 gradient system with a variable wavelength detector. Spectrophotometric detection was at 220nm. The sample was run through a reverse phase, C18, 5µm particle size Ultrasphere column (Beckman Instruments, Berkeley, CA). Chromatography was run under the following conditions: flow rate 1.0ml/min, temperature 25^{o}C, gradient elution over 20 minutes from 20% to 60% CH_3CN in 0.1% phosphoric acid. Under these conditions, f Met-Leu-[^{3}H]Phe appeared as a single peak at 16 minutes, corresponding to the retention time of unlabelled f Met-Leu-Phe. One-minute fractions were collected and radioactive content determined in a Tricarb 2000CA liquid scintillation counter. Greater than 95.5% of radioactivity corresponded to the 16 minute peak in all preparations tested.

2.4.5 Lysosyme assay:

Neutrophils at 10^7/ml in RPMI 1640 + 1:100 Nutridoma were incubated in the presence or absence of various concentrations of rH TNF-α for 60 minutes at 37^{o}C. Following this, they were incubated with 5µg/ml cytochalasin B for 10 minutes at 37^{o}C then either stimulated in the presence or absence of f Met-Leu-Phe for 15 minutes at 37^{o}C. The cells were spun at 450g for 5 minutes then the supernatants were tested for lysosyme content.

The amount of lysosyme in neutrophil supernatants was detected by the reduction in optical density at 450nm of a suspension of *Micrococcus lysodeiktus* according to the

method of Klass et al. (1977). Briefly, a suspension of *M. lysodeiktus* (lyophilised, Sigma) at an OD450nm of 0.65-0.7 was equilibrated for 30 minutes at 37°C (buffer used was 0.066M KH_2PO_4 with 0.066M K_2HPO_4, pH 6.6). A standard curve was established with 400, 200, 100 and 50u/ml lysosyme (hen egg white, Sigma) by monitoring the reduction in OD450nm every 30 seconds for 3 minutes at 37°C in a thermostatted cuvette holder attached to a DU-50 Spectrophotometer (Beckman Instruments). The change in OD450nm for unknown supernatants was then calculated off the standard curve. Results are expressed as u/ml lysosyme per 10^6 neutrophils.

2.4.6 Lactate dehydrogenase assay:

The lactate dehydrogenase content of neutrophil supernatants was detected spectrophotometrically by the change in OD at 340nm due to the conversion of NAD^+ to NADH (Henry, Canon and Winkleman, 1974). The conversion is based on the following reaction:

$$\text{pyruvate} + \text{NADH} \xrightarrow[\text{LDH}]{} \text{lactate} + \text{NAD}^+$$

Supernatants were obtained from neutrophils as for the degranulation assay. The same supernatants were tested for both lysosyme and lactate dehydrogenase. Briefly, 32mgs NAD (Grade II, 98% free acid, Boehringer Mannheim) were dissolved in 1ml lactate-AMP buffer (24ml D/L lactic acid [88%, AJAX Chemicals, Sydney, New South Wales], and 89g 2-amino-2-methyl-propan-1-ol [AMP, BDH Chemicals] per litre, pH 9.0). One hundred microlitres of this solution was added to 5µl of the sample supernatant and 20µl of lactate-AMP buffer. This solution was incubated for 2 minutes at 37°C, and the change in OD340nm monitored every 10 seconds using a COBAS BIO centrifugal analyser (Roche Diagnostics, Basle, Switzerland). Results are expressed as units per litre.

2.4.7 Preparation of zymosan activated serum:

Zymosan activated serum was prepared according to the method of Kajdacsy-Balla (1976). Blood was collected into a cold sterile syringe then spun immediately at 200g for 12 minutes at 4°C to remove white blood cells and red blood cells. The platelet rich plasma was allowed to clot at room temperature then the serum left overnight at 4°C with 1M epsilon-aminocaproic acid (Sigma).

To inactivate the zymosan, 10mg/ml zymosan (Sigma) in PBS was boiled for 30-60 minutes then washed 3 times in PBS. The zymosan was resuspended to 10mg/ml in the serum and incubated in a shaking water bath at 37°C for 60-90 minutes. The zymosan was removed by centrifugation, then centrifuging the supernatant. The zymosan activated serum was then stored at -70°C.

2.4.8 Staining for CR3:

Neutrophils were suspended to 5 x 10^6 cells/ml in RPMI + 1:100 Nutridoma. They were incubated in the presence of 100u/ml rH TNF-α, 10^{-7}M f Met-Leu-Phe or medium for 60 minutes at 37°C. Following this, they were washed and resuspended in cold medium containing 10µg anti-Mo1 (Mouse IgM, Coulter Electronics, Hialeah, Florida) + 10mM sodium azide. Cells were stained for 30 minutes on ice then washed twice in cold medium and resuspended in cold medium containing 1:40 sheep anti-mouse immunoglobulin (FITC-conjugated, Silenus Laboratories, Hawthorne, Victoria). Cells were incubated on ice for 30 minutes then washed twice in cold medium. Finally cells were resuspended in 1ml cold FACS FIX (5mM sodium azide, 1% formaldehyde, 2% glucose in PBS) and analysed by flow cytometry (Coulter Epics V, Coulter Electronics). Results are expressed as the mean log fluorescence. This is defined as the sum of the number of cells in each

channel, multiplied by the fluorescence intensity of each channel, then divided by the total number of cells counted.

SECTION 2.5 BIOCHEMICAL ASSAYS

2.5.1 Actin polymerization:

Neutrophils were suspended to 5 x 10^6 cells/ml in RPMI 1640 + 0.1% BSA. Cells were incubated in the presence or absence of rH TNF-α or f Met-Leu-Phe for various times at 37°C, then fixed by adding 10^6 cells to 1ml ice cold paraformaldehyde (3.2% in PBS). In experiments where the effect of rH TNF-α on the response to f Met-Leu-Phe was studied, cells were preincubated for 30 minutes in the presence or absence of rH TNF-α, then stimulated for 10 seconds with f Met-Leu-Phe. The reaction was terminated by fixation.

After fixing, the cells were washed twice in PBS then resuspended in 50µl PBS containing 0.6µg/ml NBD-phallacidin (Molecular Probes, Eugene, OR) and 100µg/ml L-alpha-lysophosphatidylcholine. After staining for 30 minutes at room temperature, they were washed once more then resuspended in 1ml FACS FIX and analysed by flow cytometry. For experiments where the cells were photographed, they were resuspended in 100µl immunofluorescent mountant (3:7, glycine saline buffer [1.4g glycine, 0.07g NaOH, 1.7g NaCl, 0.1g NaN_3 per 100ml water, pH 8.6]:glycerol, v/v) and examined microscopically (Olympus BHS microscope with an epifluorescent system).

2.5.2 Arachidonic acid release:

Purified neutrophils (10^7/ml) were incubated in RPMI 1640 + 0.1% fatty acid free BSA (Sigma) with 2 x 10^6µCi/ml arachidonic acid, [5,6,8,9,11,12,14,15 - $^3H(N)$] (stored at -20°C, New England Nuclear) for 60 minutes at 37°C. The cells were then washed three

times in cold medium and resuspended to 10^7/ml. Aliquots of cells (3 x 10^6 cells) were incubated for various times at 37°C in the presence or absence of various stimuli. An aliquot of neutrophils was incubated with 4μM A23187 (Behring Diagnostics, La Jolla, CA) for 10 minutes at 37°C as a positive control. The cells were placed on ice to terminate the incubation then spun at 400g for 5 minutes at 4°C. An aliquot of the supernatant was transferred into a scintillation vial with 10ml Aqueous Counting Scintillant II (Amershan, Arlington Heights, IL) and radioactivity measured using a Packard Tri-Carb 2000CA liquid scintillation counter. Results are expressed as dpm per 2 x 10^6 neutrophils.

2.5.3 Reverse-phase HPLC analysis of [^{3}H]-arachidonic acid-labelled neutrophil supernatants:

Neutrophil supernatants were prepared as described above. The supernatants were removed into fresh tubes and extracted in an ice cold solution of 80% methanol (final concentration) overnight. The precipitate was spun out at 1000g for 2 min at 4°C, and the methanol supernatants dried down in a Speed Vac (Savant Instruments Inc., Farmingdale, NY). Samples were stored under argon at -20°C until analysed by high performance liquid chromatography (HPLC). The samples were extracted into 80% ethanol, evaporated to dryness using a Speed Vac rotary evaporator (Savant Instruments, Hicksville, NY) and reconstituted in the reverse phase-HPLC mobile phase. The arachidonic acid (AA) metabolites were separated by reverse phase-HPLC on a Beckman C_{18}, 5μm Ultrasphere ODS 4.6mm x 25cm column (Beckman Instruments Inc., Berkeley, CA) with a Waters HPLC system (two model 510 pumps, model 680 gradient controller and a model U6K injector). The buffer system consisted of a stepwise gradient of methanol (Mallinkrodt, Melbourne, Australia) and 0.01% acetic acid in distilled deionized water, pH 5.6, adjusted with ammonia, modified from Henke et al. (1988) (30 min at 55%, 30 min at 65%, 20 min at 73% and 15 min at 100%) at a flow rate of 0.8ml/min. This system separated

prostaglandin (PG), leukotriene (LT), hydroxyeicosatetraenoic acid (HETE) groups and arachidonic acid. All solvents were of HPLC grade and were filtered and degassed before use. Fractions were collected into plastic minivials (Packard Instrument Co., Downers Grove, IL), and radioactivity measured by liquid scintillation counting. The HPLC system was calibrated using a series of tritiated PG, LT and HETES. 6-keto-PGF_{100} and Thromboxane B_2 were from Amersham, and PGD_2, PGF_{200}, LTB_4, 15HETE, 12HETE, 5HETE, arachidonic acid were from New England Nuclear, Boston, MA. The unlabelled 20-0H LTB_4 standard used was a gift from Dr. B. Spur (Institut Henri Beaufour, Paris, France) and synthesized according to Soberman et al. (1988) The retention time of this compound was determined by monitoring UV absorbance at 270nm. The recovery of radioactivity from chromatography was $96.5 \pm 18.8\%$.

2.5.4 Extraction of neutrophil lipids:

Cellular lipids from neutrophils loaded and stimulated as described above, were extracted by a slight modification of the method of Wertz and Mueller (1978). The cells were resuspended to 10^7/ml and precipitated in 5% trichloroacetic acid (TCA, Merck, Schuchardt, FRG) for 20 minutes at 4°C. The TCA insoluble material was then dissolved in 5ml chloroform/methanol (2:1, v/v) + 50µg/ml butylated hydroxytoluene (BHT, Sigma). The aqueous phase was partitioned with 1ml 0.04% $CaCl_2$. Following this, the organic phase was further extracted with 1ml solvent and 1ml $CaCl_2$. The lipid extract was dried under a stream of nitrogen at 25°C and stored under argon at -20°C until analysis by thin layer chromatography.

2.5.5 Thin layer chromatography:

Total lipids from 10^7 cells were dissolved in chloroform:methanol (2:1, v/v) + 50µg/ml BHT for application to aluminium backed thin layer chromatography plates (silica gel 60, 0.2mm thick, Merck).

To separate the ^{3}H-arachidonic acid from the cell lipids, a solvent system consisting of Hexane:diethylether:formic acid (80:20:2, v/v, room temperature) was used. The lipids used for standards were stored at -20°C in chloroform:methanol (2:1, v/v) + 50µg/ml BHT under argon. The plates were sprayed with En^{3}hance (New England Nuclear, Boston, MA.) and autoradiographed on Hyperfilm-^{3}H (Amersham, Sweden). Fractions were then identified by visualisation with I_2 vapour. After evaporation of the I_2 from the plate, the fractions were scraped into scintillation vials and quantified by liquid scintillation counting. Results are expressed as a percentage of incorporation into total cellular lipids.

SECTION 2.6 STATISTICS

Statistical analyses of the functional and biochemical data was performed using the unpaired two-tailed students t test. Where significant changes have been found, the level of significance is defined in adjacent parentheses.

Statistical tests used for the analysis of Scatchard binding studies were the F ratio test, and the Hill equation (see above).

CHAPTER 3

REGULATION OF THE NEUTROPHIL RESPIRATORY BURST AND MIGRATION BY RECOMBINANT HUMAN TUMOR NECROSIS FACTOR-ALPHA.

SECTION 3.1 INTRODUCTION

The process of inflammation *in vivo* involves the mobilisation of phagocytes towards the site of infection. This localisation involves the active locomotion of granulocytes and mononuclear phagocytes from the blood stream into the infected site (reviewed by Wilkinson, 1980). Having migrated to the focus of infection, the phagocytes' microbicidal functions are activated. These functions centre around two major events: 1) degranulation, which involves the fusion of cytoplasmic granules of the phagocyte with the plasma membrane, and 2) the respiratory burst, which results from the generation of highly toxic products of oxygen reduction. The activation of these two functions combined, results in the killing and digestion of micro-organisms at the site of infection (reviewed by Root and Cohen, 1981).

The events described above are generally regulated by substances generated or released at the infected site. There are two types of stimuli responsible for neutrophil activation - exogenous substances released from bacteria, and endogenous factors released or produced by cells at the site of infection. The two exogenous substances that have been well studied are bacterial lipopolysaccharide (LPS) and *N*-formylmethionylleucylphenylalanine (f Met-Leu-Phe). LPS plays a dual role in that it directly activates neutrophils (Wilson, 1985), and stimulates macrophages to produce inflammatory cytokines (Beutler et al., 1986c). F Met-Leu-Phe has been identified as the major stimulatory formylated peptide for neutrophils (Marasco et al., 1984), that is released at the site of infection.

Endogenous stimuli are factors produced or released by the body. For example, the binding of IgM to bacteria activates the complement cascade thus setting up a gradient of C5a (Gallin, Clark, and Frank, 1975). This molecule is both chemotactic for neutrophils (Snyderman et al., 1968), and activates neutrophil functions such as degranulation (Chenoweth and Hugli, 1978; Webster et al., 1980) and superoxide anion generation

(Goldstein, Feit, and Weissmann, 1975; Goldstein et al., 1975). Other endogenous factors are the inflammatory cytokines produced at the site of infection. As mentioned above, LPS induces the production of tumor necrosis factor-alpha (TNF-α) and interleukin-1β (IL-1β) from activated macrophages (Beutler et al., 1986c; Lachman, 1983). Recently, another factor produced by human monocytes has been implicated in inflammation. This factor is known as neutrophil activating factor (NAF) and has been demonstrated to stimulate neutrophil functions such as superoxide production, degranulation, shape change, chemotaxis and calcium flux (Thelen et al., 1988; Peveri et al., 1988).

The role of these two cytokines in *Escherichia coli* induced inflammation has been well studied by Movat et al. (1987b). They propose the following model: the LPS from the bacteria leads to stimulation of the production of IL-1β and TNF-α; these molecules, together with f Met-Leu-Phe induce the accumulation of neutrophils at the site of infection; phagocytic and nonphagocytic stimulation of the neutrophils causes degranulation and the generation of oxygen radicals; besides killing the micro-organisms, these events also cause tissue injury which results in increased vasopermeability, hemorrhage, and thrombosis. This *in vivo* model therefore predicts that the production of TNF-α and the generation of f Met-Leu-Phe combine to induce the accumulation of neutrophils at the site of infection, resulting in the stimulation of their microbicidal activity and the progression of the inflammatory response.

Tumor necrosis factor-alpha has been shown to activate various neutrophil functions. Shalaby et al. (1985) demonstrated that recombinant human TNF-α (rH TNF-α) enhanced antibody dependent cell killing by neutrophils. Following this, reports emerged supporting a role for rH TNF-α in stimulating neutrophil functions. For example, rH TNF-α was reported to stimulate neutrophil adherence (Gamble et al., 1985) and to stimulate the iodination, hydrogen peroxide production and lysosyme release by neutrophils (Klebanoff et al., 1986). Because TNF-α is produced by macrophages at the site of inflammation, and

was previously reported to activate neutrophil functions *in vitro*, I sought to determine whether rH TNF-α influenced other neutrophil responses *in vitro*. Two functions were chosen which had not been previously studied.

Firstly, because degranulation and the respiratory burst are the two major microbicidal mechanisms in neutrophils, and Klebanoff et al. (1986) had defined an effect on degranulation, the influence of rH TNF-α on the respiratory burst was studied. During the respiratory burst, molecular oxygen undergoes a single electron reduction to superoxide (0_2^-). This reaction is catalysed by an NADPH oxidase. 0_2^- is reduced further by spontaneous dismutation to hydrogen peroxide. The reduction of ferricytochrome c by 0_2^- was used to quantitate the respiratory burst.

The second function studied was the influence of rH TNF-α on cell locomotion, because neutrophils must migrate from the bloodstream to the site of infection in order to mount an inflammatory repsonse. There are three aspects of neutrophil locomotion - random, chemokinetic and chemotactic (see Introduction), therefore the method chosen to study this function was the migration under agarose technique described by Nelson, Quie and Simmons (1975). This assay allows for the quantitation of all three types of locomotion as well as direct visualisation of neutrophil migratory patterns.

This chapter deals with two major questions. Firstly, TNF-α is produced at the site of infection, therefore can it stimulate neutrophil functions such as superoxide production and chemotaxis? Secondly, because f Met-Leu-Phe is also present at the site of infection, can rH TNF-α modulate these neutrophil functions in response to this peptide?

SECTION 3.2 RESULTS

3.2.1. rH TNF-α enhances the production of superoxide anion in response to f Met-Leu-Phe:

To determine whether rH TNF-α could influence the respiratory burst of neutrophils, cells were incubated in the presence or absence of 100u/ml of rH TNF-α for 30 minutes at 37°C, then stimulated in the presence or absence of 10^{-7}M f Met-Leu-Phe for 15 minutes at 37°C. Figure 3.1 represents the mean superoxide response from 17 separate experiments. Cells that were incubated in the presence of rH TNF-α only, exhibited a small but insignificant increase in their respiratory burst ($p>0.05$). When neutrophils were stimulated with 10^{-7}M f Met-Leu-Phe, they produced approximately 12 nmoles $O_2^-/10^6$ cells, and this response doubled upon preincubation with rH TNF-α ($p<0.001$). These experiments therefore illustrate the ability of rH TNF-α to enhance the response of neutrophils to an inflammatory stimulus such as f Met-Leu-Phe, without directly activating the respiratory burst.

It became evident throughout these experiments that the volunteers exhibited a wide range of responses to both f Met-Leu-Phe and rH TNF-α. Figures 3.2A and 3.2B are scatter diagrams representing 17 separate experiments. Despite this variability in responses, only one out of seventeen volunteers exhibited a significant response to rH TNF-α alone ($p<0.01$). In contrast, when cells were incubated with rH TNF-α then f Met-Leu-Phe, fourteen out of the seventeen individuals tested elicited a significantly enhanced response when compared to f Met-Leu-Phe alone (3 donors, $p>0.05$; 4 donors, $p<0.05$; 2 donors, $p<0.01$; 8 donors, $p<0.001$).

To determine whether rH TNF-α and f Met-Leu-Phe could prime the respiratory burst to each other, the following experiments were performed. Neutrophils were preincubated

FIGURE 3.1

rH TNF-α enhances the production of superoxide anion in response to f Met-Leu-Phe.

Neutrophils were incubated with 100u/ml rH TNF-α for 30 minutes at 37°C then stimulated with 10^{-7}M f Met-Leu-Phe for 15 minutes at 37°C. Numbers represent the mean ± S.E.M. from 17 separate experiments where each point was determined in duplicate or triplicate.

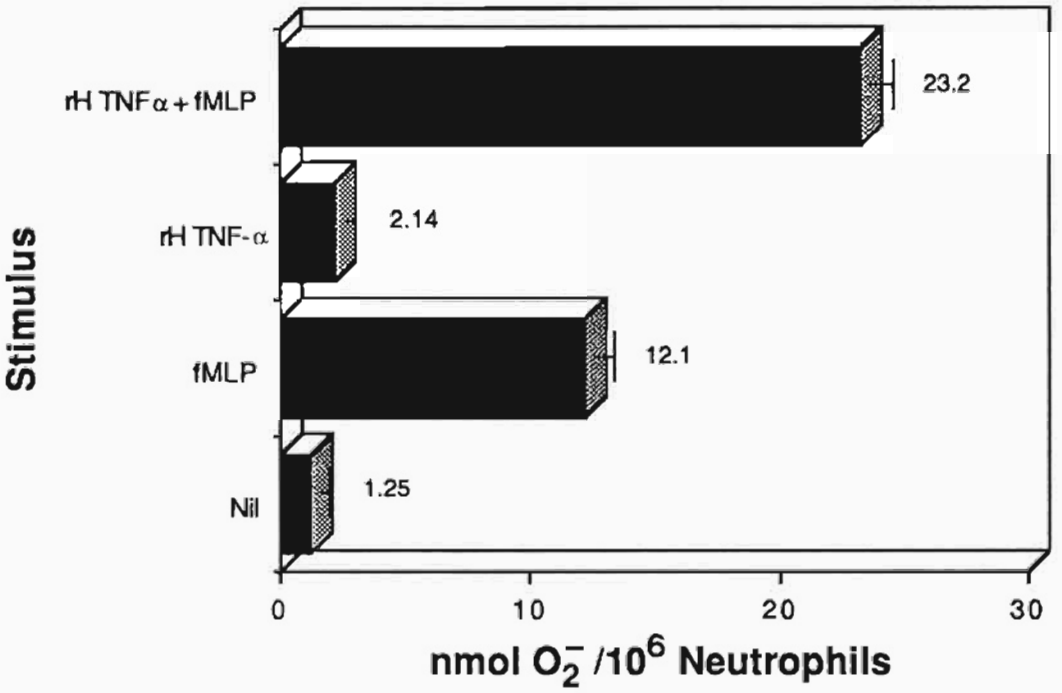
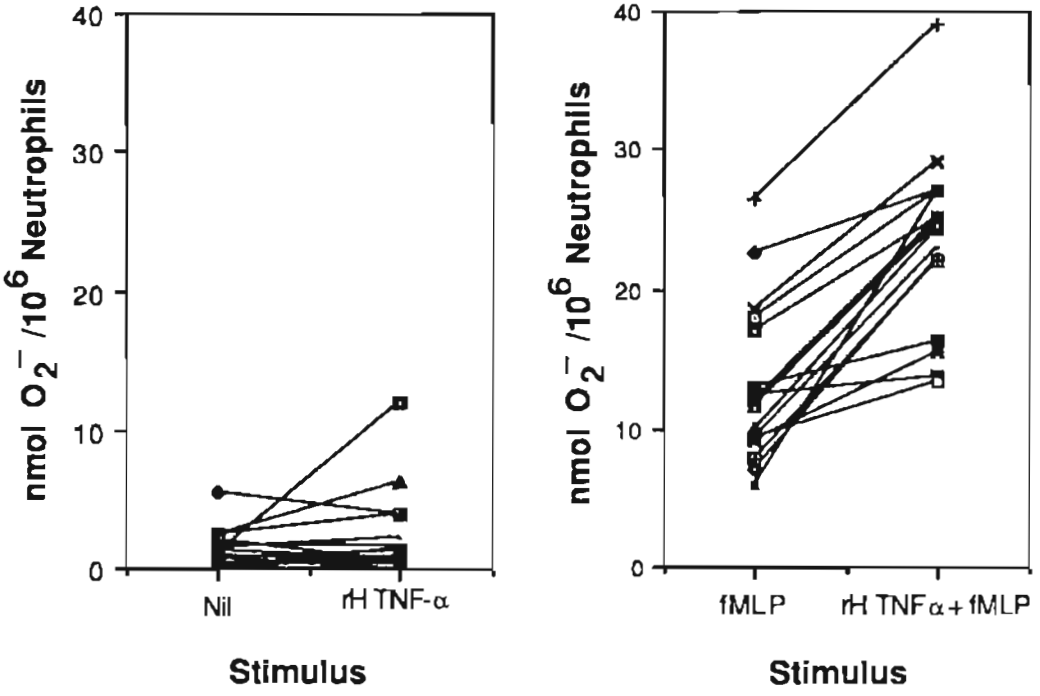

rH TNFα + fMLP
rH TNF-α
fMLP
Nil
23.2
2.14
12.1
1.25
Stimulus
0
10
20
30
nmol O_2^- /10^6 Neutrophils

FIGURE 3.2

The degree of enhancement by rH TNF-α varies between donors.

Neutrophils were incubated with 100u/ml rH TNF-α for 30 minutes at 37°C *(left)* then stimulated with 10^{-7}M f Met-Leu-Phe for 15 minutes at 37°C *(right)*. Each symbol represents the mean of an individual experiment.


40
30
20
10
0
nmol O_2^- /10^6 Neutrophils
Nil
rH TNF-α
Stimulus
40
30
20
10
0
nmol O_2^- /10^6 Neutrophils
fMLP
rH TNFα + fMLP
Stimulus

with either medium, 100u/ml rH TNF-α or 10^{-7}M f Met-Leu-Phe. They were then stimulated with either 10^{-7}M f Met-Leu-Phe or 100u/ml rH TNF-α. As previously shown, the response to f Met-Leu-Phe (8 ± 1.8 nmol $O_2^-/10^6$ neutrophils) was enhanced by preincubation with rH TNF-α (14.5 ± 0.2 nmol $O_2^-/10^6$ neutrophils). In contrast, preincubation of cells with f Met-Leu-Phe induced direct superoxide release, but this was unaffected by subsequent incubation with rH TNF-α (5.2 ± 1 nmol $O_2^-/10^6$ neutrophils). This data therefore indicates that the cells must be exposed to rH TNF-α before f Met-Leu-Phe to elicit an enhanced response.

A number of controls were performed to determine the specificity of the effect observed. Firstly, it is possible that other substances cause the reduction of cytochrome C, e.g. ascorbate (McCord, Crapo and Fridovich, 1977), therefore to determine whether rH TNF-α was enhancing the production of superoxide anion itself in response to f Met-Leu-Phe, neutrophils were incubated in the presence or absence of superoxide dismutase. This enzyme specifically catalyses the decomposition of superoxide anion, and therefore prevents the reduction of ferricytochrome c. Figure 3.3A illustrates that when neutrophils are incubated with rH TNF-α then f Met-Leu-Phe, they predominantly produce superoxide anion, because the O_2^--mediated reduction of ferricytochrome c is completely abolished in the presence of superoxide dismutase.

Secondly, there was the possibility of contamination by LPS. It is widely known that small amounts of LPS are able to stimulate neutrophil function (Guthrie et al., 1984). To determine whether LPS was involved in the enhancement of neutrophil function by rH TNF-α, the rH TNF-α was boiled and then used in the superoxide assay. Figure 3.3B represents cells that were incubated for 30 minutes in the presence of either medium or rH TNF-α, or in the presence of their boiled equivalents. The neutrophils were then stimulated with various doses of f Met-Leu-Phe for 15 minutes at 37°C. There was no enhancement of the neutrophil respiratory burst in response to f Met-Leu-Phe when the

FIGURE 3.3A

rH TNF-α-induced enhancement of the neutrophil respiratory burst is specific for superoxide anion.

Neutrophils were incubated with 100u/ml rH TNF-α for 30 minutes at 37°C. They were then stimulated with 10^{-7}M f Met-Leu-Phe in the presence or absence of 280u/ml superoxide dismutase. Numbers represent the mean ± S.E.M. from one experiment where each point was determined in triplicate.

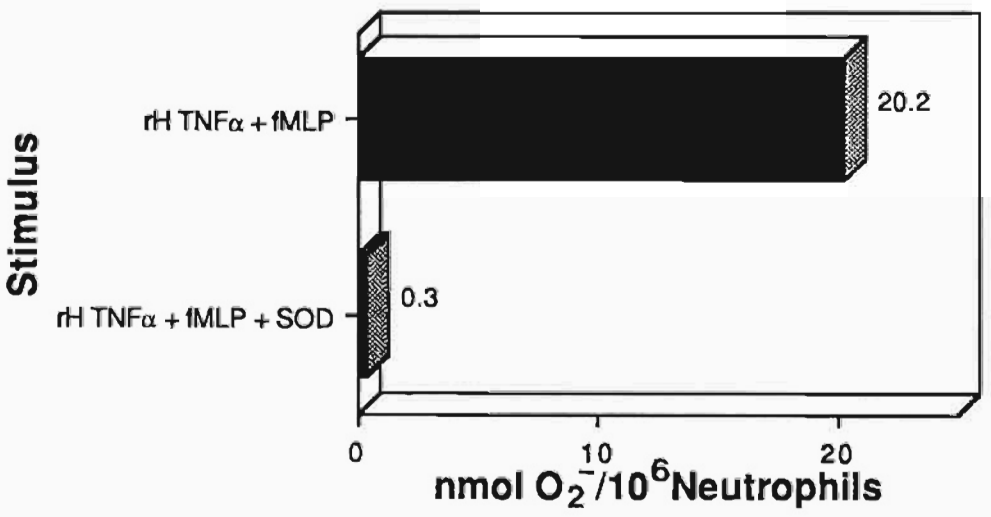
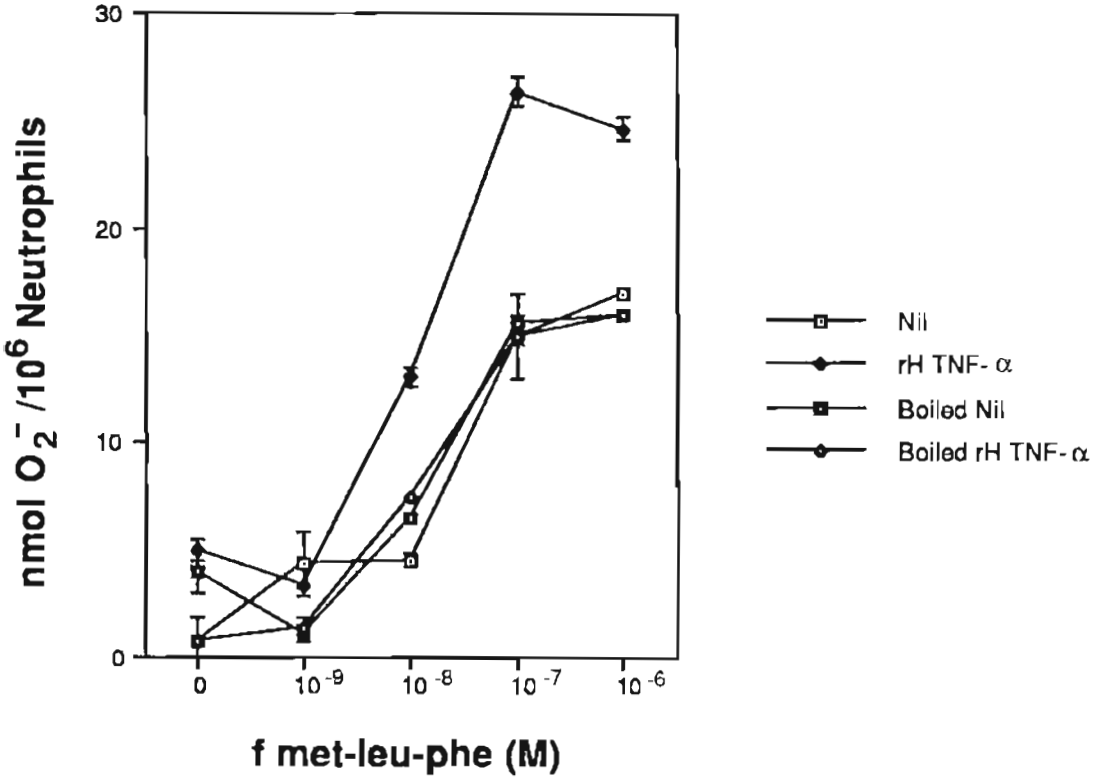

Stimulus
rH TNFα + fMLP
rH TNFα + fMLP + SOD
20.2
0.3
0
10
20
nmol O_2^-/10^6Neutrophils

FIGURE 3.3B

Boiling of rH TNF-α abolishes the enhancement of superoxide anion production.

Neutrophils were incubated with 100u/ml rH TNF-α or with 100u/ml boiled rH TNF-α for 30 minutes at 37°C. They were then stimulated with various concentrations of f Met-Leu-Phe for 15 minutes at 37°C. Numbers represent the mean ± S.E.M. of triplicates from one experiment.


30
20
10
0
nmol O_2^- /10^6 Neutrophils
0
10^{-9}
10^{-8}
10^{-7}
10^{-6}
f met-leu-phe (M)
Nil
rH TNF- α
Boiled Nil
Boiled rH TNF- α

cells were incubated with boiled rH TNF-α. As LPS is not destroyed by boiling one can conclude that the increase in superoxide production is indeed caused by stimulation of the neutrophils by rH TNF-α. In addition, the concentration of LPS in the medium used was quantitated by the limulus amebocyte lysate assay, and was found to be less than 0.1EU/ml.

3.2.2. rH TNF-α induced enhancement of superoxide production is sustained after washing the cells:

To determine whether neutrophils require the continuous presence of rH TNF-α for an enhanced response to f Met-Leu-Phe to occur, washing experiments were performed. Neutrophils were incubated in medium or with 100u/ml rH TNF-α for 30 minutes at 37°C, then washed before stimulation with 10^{-7}M f Met-Leu-Phe for 15 minutes at 37°C. The enhanced superoxide response induced by rH TNF-α (24 ± 3 nmol/10^6 neutrophils) was not affected by the removal of rH TNF-α from the suspension (33 ± 0.5 nmol/10^6 neutrophils). This data indicates that the rH TNF-α induced enhancement of neutrophil superoxide production in response to f Met-Leu-Phe is not reversible by washing.

3.2.3. rH TNF-α enhances the response to zymosan activated serum:

The observed effect on neutrophil superoxide anion production by rH TNF-α raises the question of whether this phenomenon is confined to the f Met-Leu-Phe response. To investigate this, the influence of rH TNF-α on the neutrophil response to zymosan activated serum (crude C5a) was studied. C5a is a complement fragment which, like f Met-Leu-Phe, is chemotactic for neutrophils (Snyderman et al., 1969) and activates a number of neutrophil functions (Chenoweth and Hugli, 1981; Webster et al., 1980; Goldstein, Feit and Weissmann, 1975; Goldstein et al., 1975). The difference between this ligand and f Met-Leu-Phe is that this molecule is host-derived rather than bacterially

derived. In Figure 3.4 neutrophils were preincubated in the presence or absence of 100u/ml rH TNF-α for 30 minutes at 37°C, then stimulated with various dilutions of zymosan activated serum (ZAS) for 15 minutes at 37°C. At all dilutions tested rH TNF-α enhanced the production of superoxide anion in response to ZAS. These data indicate that rH TNF-α can modulate the neutrophil respiratory burst to C5a as well as f Met-Leu-Phe.

3.2.4. rH TNF-α does not influence the response to phorbol myristate acetate:

The phorbol ester, phorbol 12-myristate 13-acetate (PMA) is widely used as a potent stimulator of superoxide production in neutrophils. It traverses the plasma membrane and binds to its intracellular receptor, protein kinase C (Kikkawa et al., 1983; Uchida and Filburn, 1984). To determine whether rH TNF-α influences the response to a ligand such as PMA which does not have a plasma membrane receptor, cells were preincubated in the presence or absence of 100u/ml rH TNF-α for 30 minutes at 37°C, then stimulated with various concentrations of PMA for 15 minutes at 37°C. Figure 3.5 represents the influence of rH TNF-α on neutrophil responsiveness to various concentrations of PMA. At no concentration of PMA tested did rH TNF-α alter the amount of superoxide anion produced by neutrophils. This phenomenon was observed within three separate experiments as well as when the data were pooled.

3.2.5. rH TNF-α is not chemotactic and inhibits the chemotaxis of neutrophils under agarose in response to f Met-Leu-Phe:

Because neutrophils exhibit three types of locomotion, the migration under agarose technique described by Nelson, Quie and Simmons (1975) was used. In this assay, cells are placed in the centre well of a set of three wells punched out of agarose. For random movement, medium is placed in the inner and outer wells. To measure chemokinesis, the

FIGURE 3.4

rH TNF-α enhances the production of superoxide in response to zymosan activated serum.

Neutrophils were incubated in the presence or absence of 100u/ml rH TNF-α for 30 minutes at 37°C, then stimulated with various concentrations of ZAS for 15 minutes at 37°C. Numbers represent the mean ± S.E.M. of triplicates from a representative experiment.

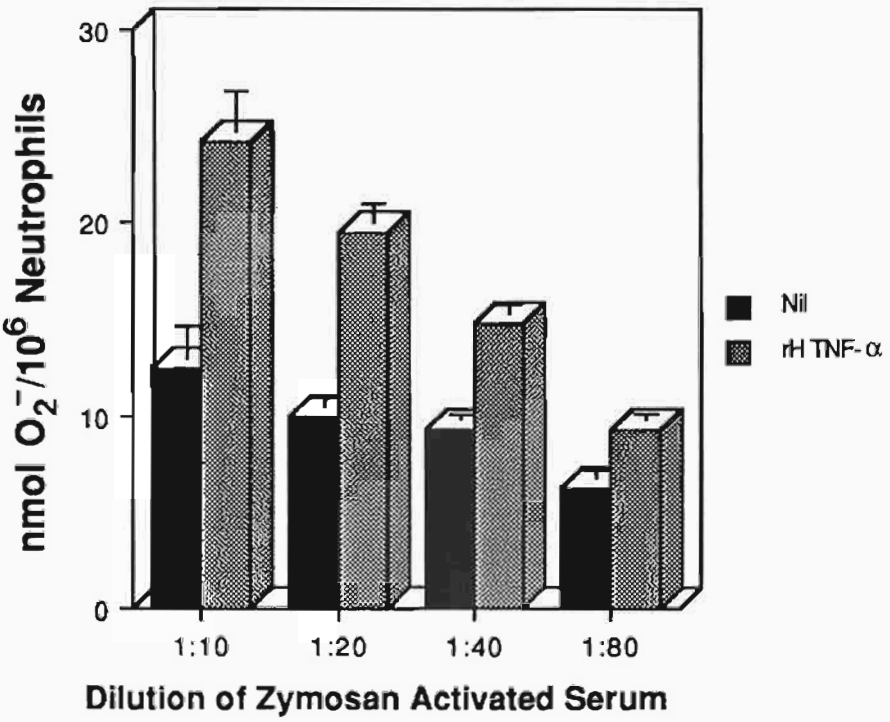
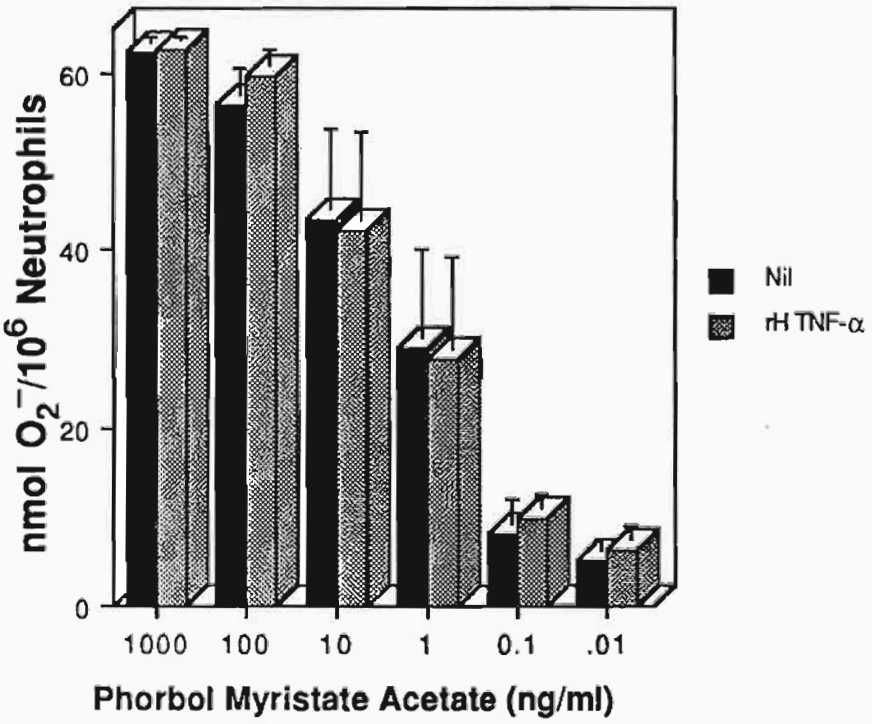

30
20
10
0
nmol $O_2^-/10^6$ Neutrophils
1:10
1:20
1:40
1:80
Dilution of Zymosan Activated Serum
Nil
rH TNF-α

FIGURE 3.5

rH TNF-α does not alter the response of neutrophils to phorbol myristate acetate.

Neutrophils were incubated with 100u/ml rH TNF-α for 30 minutes at 37°C then stimulated with various concentrations of PMA for 15 minutes at 37°C. Numbers represent the mean ± S.E.M. from 3 separate experiments where each point was determined in triplicate or duplicate.


nmol $O_2^-/10^6$ Neutrophils
60
40
20
0
1000
100
10
1
0.1
.01
Phorbol Myristate Acetate (ng/ml)
Nil
rH TNF-α

chemotactic stimulus is placed in the cell-containing well and the outer well thus placing the cells in a uniform environment of stimulus. To measure chemotaxis, the stimulus is placed only in the outer well thus allowing the cells to migrate up a concentration gradient.

Subsequent to the observations presented here, conflicting reports emerged as to whether rH TNF-α is chemotactic for neutrophils. Ming, Bersani and Mantovani (1987) and Figari, Mori and Palladino Jr. (1987) reported that rH TNF-α is directly chemotactic for neutrophils and monocytes. In contrast, Mrowietz, Schroder and Christopher (1988) reported that rH TNF-α does not induce a chemotactic response from neutrophils or monocytes. To investigate whether rH TNF-α could influence the chemotactic response of neutrophils, it was first necessary to establish whether rH TNF-α was directly chemotactic to neutrophils in the assay system used.

To investigate this, neutrophils were placed in the centre well of the chemotaxis dish and various concentrations of rH TNF-α were placed in the outer wells. In addition, 10^{-7} M f Met-Leu-Phe was assayed as a positive control. Figure 3.6 represents the mean of four experiments to test rH TNF-α for chemotactic activity. At no dose tested was rH TNF-α chemotactic for neutrophils, whereas f Met-Leu-Phe, as expected, exhibited very strong chemotactic activity.

To determine whether rH TNF-α could influence the chemotactic migration of neutrophils in response to f Met-Leu-Phe, cells were preincubated with 100u/ml of rH TNF-α for 30 minutes at 37°C then washed and placed in the chemotaxis dishes. Figure 3.7 represents the chemotactic migration of these cells towards 10^{-7} M f Met-Leu-Phe. This figure illustrates the ability of rH TNF-α to inhibit the chemotactic migration of neutrophils towards f Met-Leu-Phe.

FIGURE 3.6

rH TNF-α is not a chemoattractant for neutrophils.

Unstimulated neutrophils were tested for their chemotactic responsiveness to either medium, various concentrations of rH TNF-α or 10^{-7}M f Met-Leu-Phe. Numbers represent the mean ± S.E.M. of 4 experiments where each point was determined in quadruplicate.

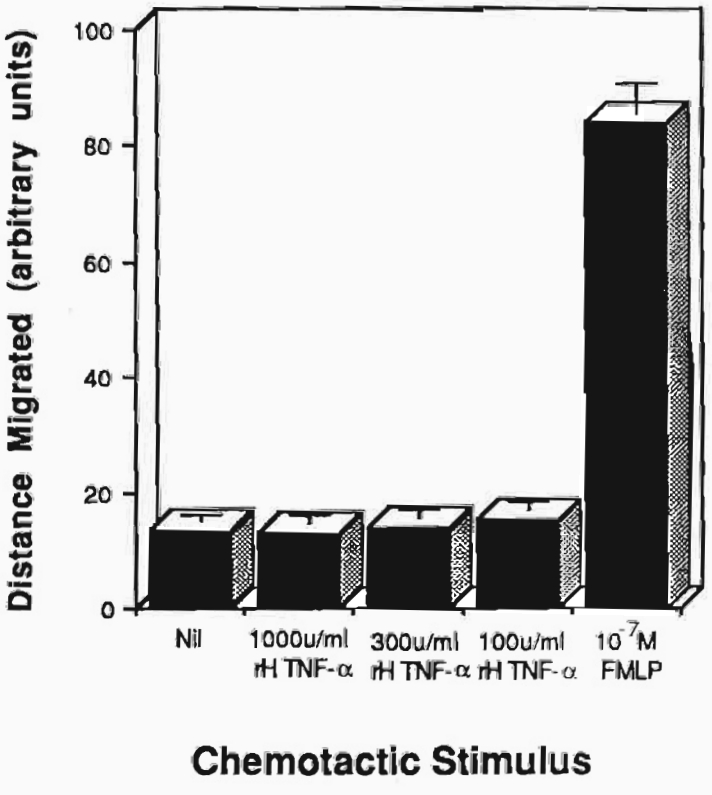
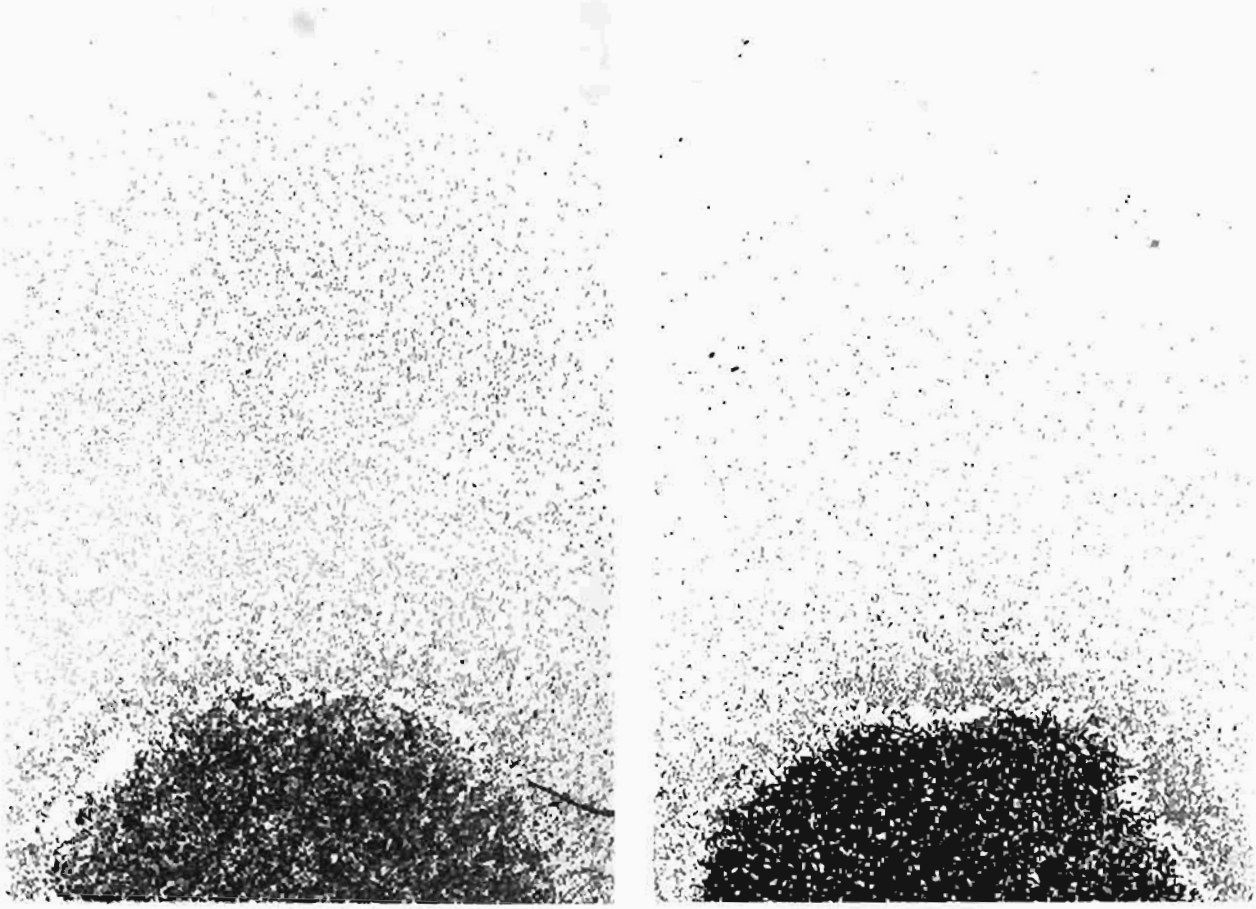

Distance Migrated (arbitrary units)
100
80
60
40
20
0
Nil
1000u/ml rH TNF-α
300u/ml rH TNF-α
100u/ml rH TNF-α
10⁻⁷M FMLP
Chemotactic Stimulus

FIGURE 3.7

rH TNF-α inhibits the migration of neutrophils towards a gradient of f Met-Leu-Phe.

Neutrophils were incubated with (right) or without (left) 100u/ml rH TNF-α for 30 minutes at 37^{o}C, then washed and placed in the centre well of the chemotaxis dishes (bottom). Their chemotactic response towards a source of 10^{-7}M f Met-Leu-Phe (top) was then assayed.



3.2.6. rH TNF-α inhibits neutrophil migration in a dose- and time-dependent manner:

To determine the optimal dose at which rH TNF-α inhibits neutrophil chemotaxis in response to f Met-Leu-Phe, cells were incubated with various doses of rH TNF-α for 30 minutes at 37°C, then washed and placed in the chemotaxis assay. Figure 3.8 represents the dose-dependent inhibition of neutrophil chemotaxis towards 10^{-7}M f Met-Leu-Phe. At the highest concentration of rH TNF-α tested, the inhibition was 50% that of the unstimulated response to f Met-Leu-Phe. Over the 6 experiments performed to test different doses of rH TNF-α, the inhibition of migration at 1000u/ml varied from between 10% to 60% of that shown by unstimulated neutrophils.

To further characterize the inhibition of neutrophil migration by rH TNF-α, the influence of this cytokine on both the random and the chemokinetic migration of neutrophils was studied. Figure 3.9 represents the mean of two separate experiments where the cells were preincubated with 100u/ml rH TNF-α for various times at 37°C, then washed and placed in the chemotaxis assay. It is clear that rH TNF-α inhibits all three aspects of neutrophil migration under agarose in response to f Met-Leu-Phe. The inhibition of neutrophil motility induced by rH TNF-α is detectable by 5 minutes and virtually maximal by 15 minutes, after which it reaches a plateau. This data indicates that rH TNF-α inhibits the random, chemokinetic and chemotactic migration of neutrophils in response to f Met-Leu-Phe, and that this effect exhibits similar kinetics for all three aspects of migration.

3.2.7. rH TNF-α inhibits neutrophil chemotaxis towards zymosan activated serum:

To define whether rH TNF-α could inhibit neutrophil chemotaxis towards another receptor-dependent stimulus, its effect on the response to ZAS was investigated.

FIGURE 3.8

Dose-dependent inhibition of neutrophil migration by rH TNF-α.

Neutrophils were incubated with various concentrations of rH TNF-α then washed and placed in the chemotaxis plates. Their chemotactic response towards a source of 10^{-7}M f Met-Leu-Phe was then assayed. Numbers represent the mean ± S.E.M. of a representative experiment where each point was determined in triplicate.

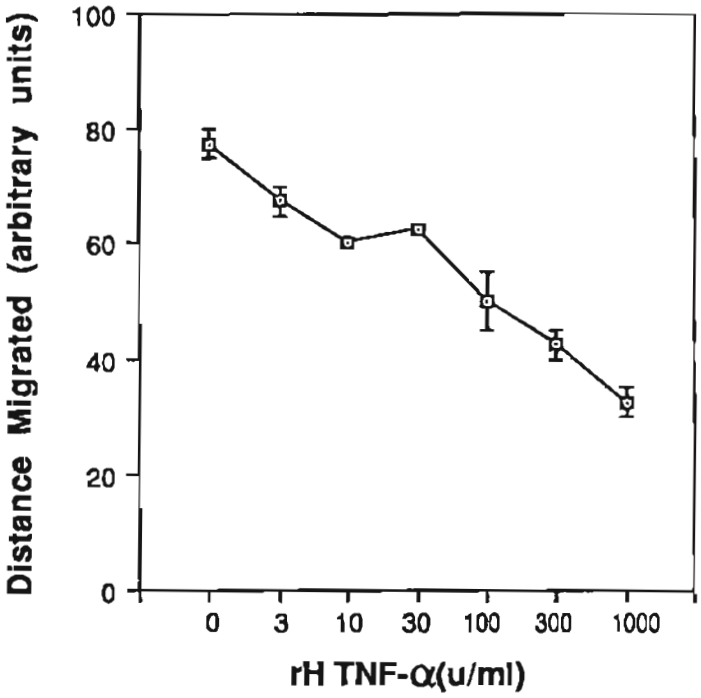
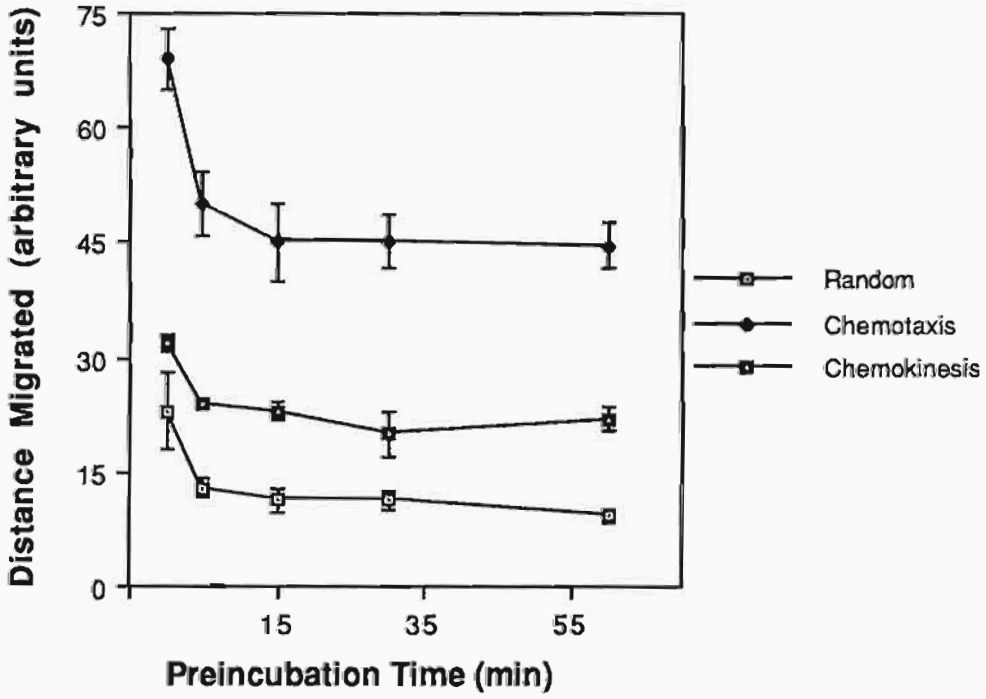

Distance Migrated (arbitrary units)
100
80
60
40
20
0
0
3
10
30
100
300
1000
rH TNF-α(u/ml)

FIGURE 3.9

Time dependent inhibition of neutrophil migration by rH TNF-α.

Neutrophils were incubated with 100u/ml rH TNF-α for various times at 37°C then washed and placed in the chemotaxis assay. Migration in the absence of stimulus (random), with f Met-Leu-Phe in both cell-containing and adjacent medium-containing wells (chemokinesis), and with f Met-Leu-Phe only in non-cell-containing wells (chemotaxis) was assayed. Numbers represent the mean ± S.E.M. from 2 experiments where each point was determined in duplicate.


Distance Migrated (arbitrary units)
75
60
45
30
15
0
15
35
55
Preincubation Time (min)
Random
Chemotaxis
Chemokinesis

In Figure 3.10, the cells were preincubated in the presence or absence of 100u/ml rH TNF-α then washed and placed in the chemotaxis assay. Their response to either 10^{-7}M f Met-Leu-Phe or to half dilution ZAS was tested. Preincubation with rH TNF-α inhibited the responses by 27% and 19% respectively. Therefore, as with superoxide production, rH TNF-α is able to inhibit the chemotactic response of neutrophils to another receptor-dependent stimulus such as ZAS.

SECTION 3.3 DISCUSSION

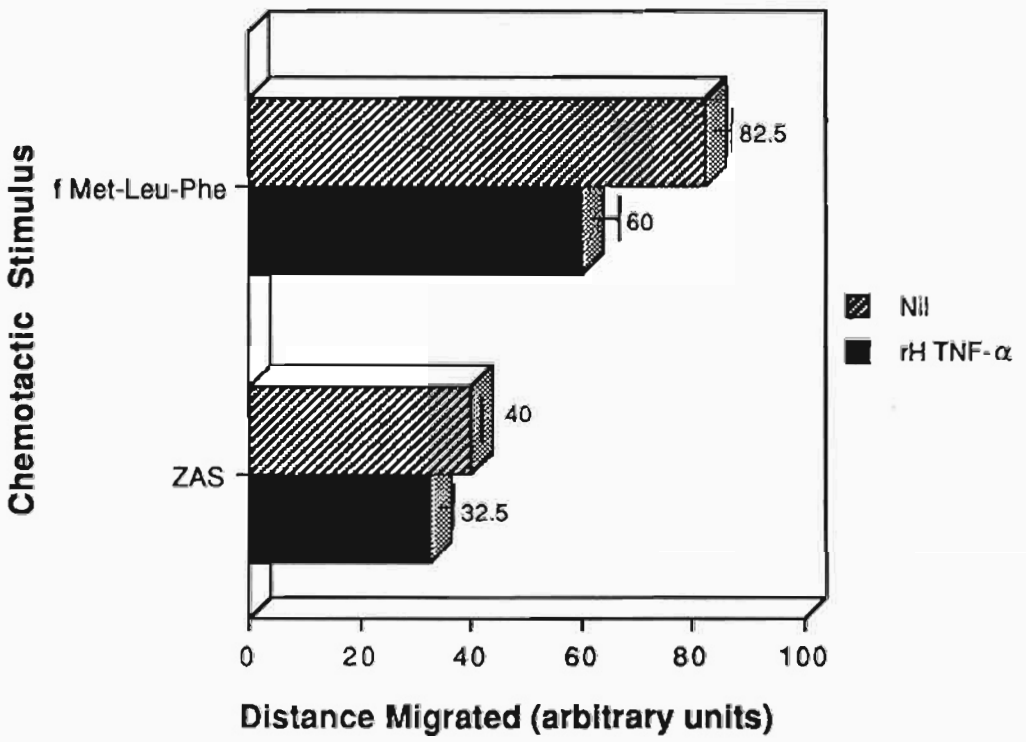
The data presented in this chapter indicates that rH TNF-α can influence neutrophil functions in both a positive and negative manner. Whilst not able to induce superoxide production by itself, rH TNF-α has the capacity to enhance the response of neutrophils to inflammatory stimuli such as f Met-Leu-Phe and zymosan activated serum. In direct contrast, rH TNF-α inhibits the chemotactic migration of neutrophils to the same stimuli.

It was shown here that rH TNF-α is unable to induce the production of superoxide anion directly, but does enhance the response to f Met-Leu-Phe. These data are in contrast to that reported by Klebanoff et al. (1986) who reported that rH TNF-α is a weak but direct stimulus of the neutrophil respiratory burst. The reason for this discrepancy probably lies in the type of assays used in these studies. I have measured the neutrophil respiratory burst by assaying for the specific production of superoxide anion by the reduction of ferricytochrome c. This assay only detects oxidative species. In contrast, Klebanoff et al. assayed for the respiratory burst using iodination. This method is based on the release of myeloperoxidase (MPO) by degranulation, and the formation of H_20_2 to form a complex, designated compound 1. This complex oxidizes iodide to a species that forms a covalent linkage with tyrosine residues of proteins, unsaturated lipids and other constituents (Turk et al., 1983). Therefore, iodination is a measure of both degranulation and the respiratory burst. As the data presented in Chapter 6 indicates that rH TNF-α can directly stimulate

FIGURE 3.10

rH TNF-α also inhibits neutrophil migration towards zymosan activated serum.

Neutrophils were incubated with or without 100u/ml rH TNF-α for 30 minutes at 37°C, then washed and placed in the chemotaxis assay. Chemotactic migration towards either 10^{-7}M f Met-Leu-Phe or 1:10 ZAS was assayed. Numbers represent the mean ± S.E.M. of triplicates from a representative experiment.


Chemotactic Stimulus
f Met-Leu-Phe
ZAS
82.5
60
40
32.5
Nil
rH TNF-α
0
20
40
60
80
100
Distance Migrated (arbitrary units)

neutrophil degranulation, this and the longer incubation period employed by Klebanoff et al. could account for the differences observed.

Two other reports present data indicating that rH TNF-α can directly elicit the production of superoxide anion from neutrophils. Shalaby et al. (1987) performed their superoxide assay in microtitre plates with 5 x 10^5 cells/well. Tsujimoto et al. (1986) performed their superoxide assay in suspension but did not specify whether polypropylene test tubes were used. These assay conditions, combined with the observation that rH TNF-α is a potent stimulant of neutrophil adherence (Gamble et al., 1985) could well predispose the neutrophils towards a higher level of activation than when incubated in suspension in polypropylene test tubes. Indeed, a report by Nathan (1987) involves the study of neutrophil activation by adherence. Nathan shows that neutrophils release large amount of H_2O_2 when incubated on polystyrene tissue culture plates. In addition, rH TNF-α is shown to increase this adherence-stimulated response by approximately 20%. Therefore, adherence could act as a priming event in neutrophil activation, and elicit responses to a factor such as rH TNF-α which is normally incapable of direct activation.

Another study that supports this theory was performed by Berkow et al. (1987). This group incubated their neutrophils in suspension in a shaking water bath in order to keep them suspended. They were unable to detect the production of superoxide anion in response to rH TNF-α but, did report the widely accepted phenomenon of enhancement in response to f Met-Leu-Phe. Therefore, despite the conflicting reports as to the direct effect of rH TNF-α on neutrophil superoxide production, the enhancement of this response to f Met-Leu-Phe has been reported by various groups during the course of my work, and my data supports these observations.

The other aspect of neutrophil function presented in this chapter is neutrophil migration. This function was assayed by visualizing migration under agarose as opposed to the

Boyden chamber technique. The data presented here indicates that rH TNF-α can inhibit both random and f Met-Leu-Phe stimulated movement. During the course of this work, Shalaby et al. (1987) reported that rH TNF-α inhibited random neutrophil migration under agarose. This is in agreement with the data presented here. Also in agreement, Mrowietz, Schroder and Christopher (1988) have recently reported that rH TNF-α is not chemotactic when assayed by the migration under agarose technique.

Finally, the ability of rH TNF-α to modulate neutrophil superoxide generation and chemotaxis occurs in the presence of a second stimulus which specifically binds to a membrane receptor. The data presented in this chapter indicates that rH TNF-α affects neutrophil responsiveness to f Met-Leu-Phe and ZAS. The modulation of neutrophil functional receptors has been previously reported and has been correlated with a change in the associated functional responses. For example, Gamble et al. (1985) reported that rH TNF-α induced an increase in the surface expression of CR3, the receptor for C3bi, and that this may be partly responsible for the increase in adhesion observed. Other antigens termed granulocyte functional antigens I and II (GFA-1, GFA-2) which are involved in antibody-dependent cell-mediated cytotoxicity (Lopez and Vadas, 1984; Lopez et al., 1985) are also upregulated. For example, CSF-α was reported to regulate GFA-1 (Lopez and Vadas, 1984), and f Met-Leu-Phe and lipopolysaccharide regulate both antigens (Vadas, Lopez and Williamson, 1985). In both cases the change in receptor expression was correlated with a change in associated neutrophil functions. These reports, combined with the data presented in this chapter, imply that rH TNF-α could regulate neutrophil responses to f Met-Leu-Phe by the modulation of its cell surface receptor.

CHAPTER 4

RECOMBINANT HUMAN TUMOR NECROSIS FACTOR-ALPHA REGULATES f MET-LEU-PHE RECEPTOR AFFINITY AND FUNCTION ON HUMAN NEUTROPHILS

SECTION 4.1 INTRODUCTION

In cases of bacterial infection, the inflammatory response is thought to be promoted by agents such as f Met-Leu-Phe (Becker, 1979). Not only do neutrophils migrate in response to a gradient of f Met-Leu-Phe but, at higher doses, f Met-Leu-Phe also activates various toxic mechanisms, such as the capacity to generate superoxide anion.

Formyl peptide binding to neutrophils and its effect on various neutrophil functions has been previously studied (Showell et al., 1976; Freer et al., 1980; Freer et al., 1982). A correlation between the ED_{50}s for various neutrophil functions and the Kd for ligand binding has been observed (Kreutzer et al., 1978). These observations are consistent with the theory that high-affinity receptors play a role in chemotaxis whereas the low-affinity component plays a role in secretory responses and superoxide anion production.

In Chapter 3, data was presented that shows rH TNF-α has the capacity to enhance neutrophil superoxide generation, but inhibits neutrophil chemotaxis in response to f Met-Leu-Phe. Since both TNF-α and f Met-Leu-Phe appear to be involved in stimulating inflammatory responses, it was possible that rH TNF-α modulates neutrophil responses to f Met-Leu-Phe by altering the expression of f Met-Leu-Phe receptors on human neutrophils.

SECTION 4.2 RESULTS

4.2.1 rH TNF-α alters the production of superoxide anion by neutrophils stimulated with f Met-Leu-Phe:

Previous experiments (Chapter 3) showed that rH TNF-α enhanced the production of superoxide anion by neutrophils in response to f Met-Leu-Phe. To determine the optimal

concentrations of cytokine required for this effect, a titration of rH TNF-α was done. Neutrophils were preincubated with various concentrations of rH TNF-α for 45 minutes then stimulated with 10^{-7}M f Met-Leu-Phe. This showed that rH TNF-α enhanced superoxide anion production by neutrophils stimulated with 10^{-7}M f Met-Leu-Phe in a dose-dependent manner (Figure 4.1). Very little or no reduction of cytochrome c was detected with neutrophils preincubated with rH TNF-α without the addition of f Met-Leu-Phe. While it was observed that the optimal concentration of rH TNF-α required for maximal superoxide anion production by neutrophils varied between donors, 100u/ml was the most consistent concentration of rH TNF-α to induce maximal levels of superoxide anion production after stimulation with f Met-Leu-Phe, and was therefore used in subsequent experiments.

In parallel experiments, a titration of f Met-Leu-Phe in the presence or absence of rH TNF-α was performed. Neutrophils were preincubated in the presence or absence of 100u/ml rH TNF-α for 45 minutes then stimulated with various concentrations of f Met-Leu-Phe. This showed that neutrophils responded with an increased production of superoxide anion at concentrations of f Met-Leu-Phe from 10^{-6}M to 10^{-8}M (Figure 4.2). In this individual a significant response to f Met-Leu-Phe alone was observed. The response to f Met-Leu-Phe differed between individuals and therefore influenced the degree of enhancement observed by preincubation with rH TNF-α (See Chapter3, Figure 3.2). Over 9 experiments, preincubation with rH TNF-α enhanced the response to f Met-Leu-Phe from 0.5-fold to 18-fold. The most consistently optimal concentration of f Met-Leu-Phe for enhanced responsiveness of neutrophils to rH TNF-α was 10^{-7}M. No shift in the titration curve of f Met-Leu-Phe was observed with neutrophils stimulated with rH TNF-α, rather there was an increase in the total amount of superoxide anion at each concentration of f Met-Leu-Phe tested.

FIGURE 4.1

rH TNF-α stimulates the production of superoxide anion by neutrophils in the presence of 10^{-7}M f Met-Leu-Phe.

Neutrophils were preincubated with various concentrations of rH TNF-α for 45 minutes, then stimulated with 10^{-7}M f Met-Leu-Phe. Each point represents the mean of triplicate determinations from a single experiment representative of 9 others. The bars represent the S.E.M. when this is bigger than the symbols.

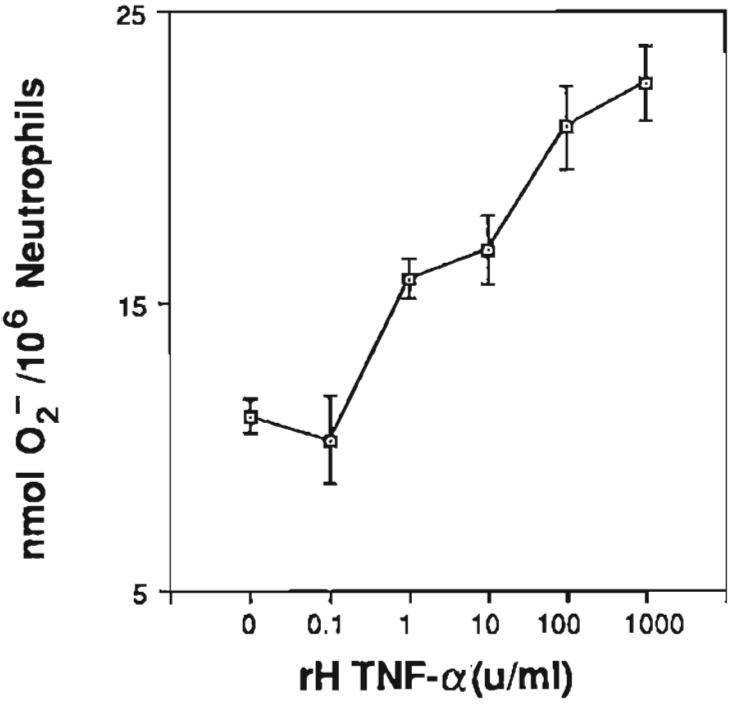
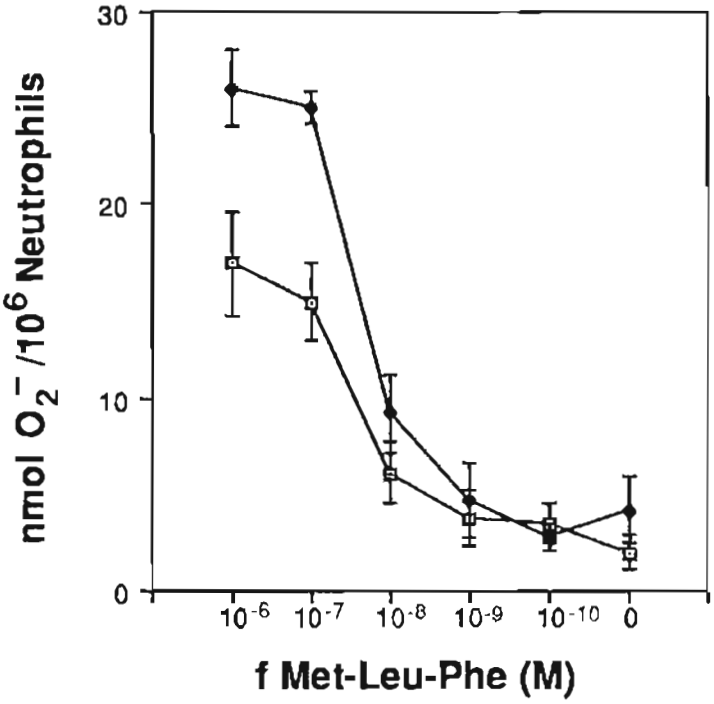

25
15
5
nmol O_2^- /10^6 Neutrophils
0
0.1
1
10
100
1000
rH TNF-α(u/ml)

FIGURE 4.2

rH TNF-α stimulates the production of superoxide anion by neutrophils in response to various concentrations of f Met-Leu-Phe.

Neutrophils were preincubated in the presence or absence of 100u/ml rH TNF-α for 45 minutes, then stimulated with various concentrations of f Met-Leu-Phe. Open squares show the values for unstimulated neutrophils and closed diamonds show the values for rH TNF-α-stimulated neutrophils. Each point represents the mean of triplicate determinations from a single experiment representative of 5 others. The bars represent the S.E.M. when this is larger than the symbols.


30
20
10
0
nmol O_2^- /10^6 Neutrophils
10^{-6} 10^{-7} 10^{-8} 10^{-9} 10^{-10} 0
f Met-Leu-Phe (M)

To determine whether the increase in superoxide anion production from neutrophils stimulated rH TNF-α was associated with a change in the rate of superoxide anion production, neutrophils were incubated with cytochrome c and f Met-Leu-Phe over a 10 minute period. Figure 4.3 shows a typical experiment where preincubating neutrophils with 100u/ml rH TNF-α enhanced the rate of production of superoxide in their subsequent response to f Met-Leu-Phe. The levels reached a plateau by 5 minutes. This effect was consistent over 10 experiments where the mean rate of change in OD550nm per minute over five minutes for rH TNF-α-stimulated neutrophils was 0.094 ± 0.005 (n=10), whereas the mean rate for unstimulated neutrophils was 0.031 ± 0.008 (n=10). Both unstimulated neutrophils and neutrophils preincubated with rH TNF-α exhibited an average change in OD550nm per minute over five minutes of 0.001 ± 0.001 (n=10).

Time course experiments performed by preincubating neutrophils for various times with rH TNF-α showed that activation had occurred by 5 minutes and had reached optimal levels by 15 minutes (Figure 4.4). The amount of superoxide anion released by rH TNF-α-stimulated neutrophils remained constant for up to 60 minutes preincubation. Unstimulated neutrophils became progressively more responsive to f Met-Leu-Phe with time, a phenomenon which was observed over 6 experiments. At every time point tested, cells incubated with rH TNF-α alone released very little superoxide anion.

4.2.2 rH TNF-α inhibits the chemotactic migration of neutrophils in response to a gradient of f Met-Leu-Phe:

To determine whether rH TNF-α influences a second f Met-Leu-Phe mediated response, the chemotaxis of neutrophils towards a gradient of f Met-Leu-Phe was studied. The neutrophils were preincubated with rH TNF-α then washed and placed in the agarose-containing dishes in the presence or absence of a gradient of f Met-Leu-Phe. It was shown that preincubating neutrophils with rH TNF-α inhibited random, chemokinetic and

FIGURE 4.3

rH TNF-α increases the rate of superoxide anion production by neutrophils in response to f Met-Leu-Phe.

Following preincubation in the presence (closed squares) or absence (open squares) of 100u/ml rH TNF-α, cells were incubated over 10 minutes in a thermostatically controlled cuvette holder at 37°C, in the presence of cytochrome c. The OD550nm was continuously monitored every 30 seconds.

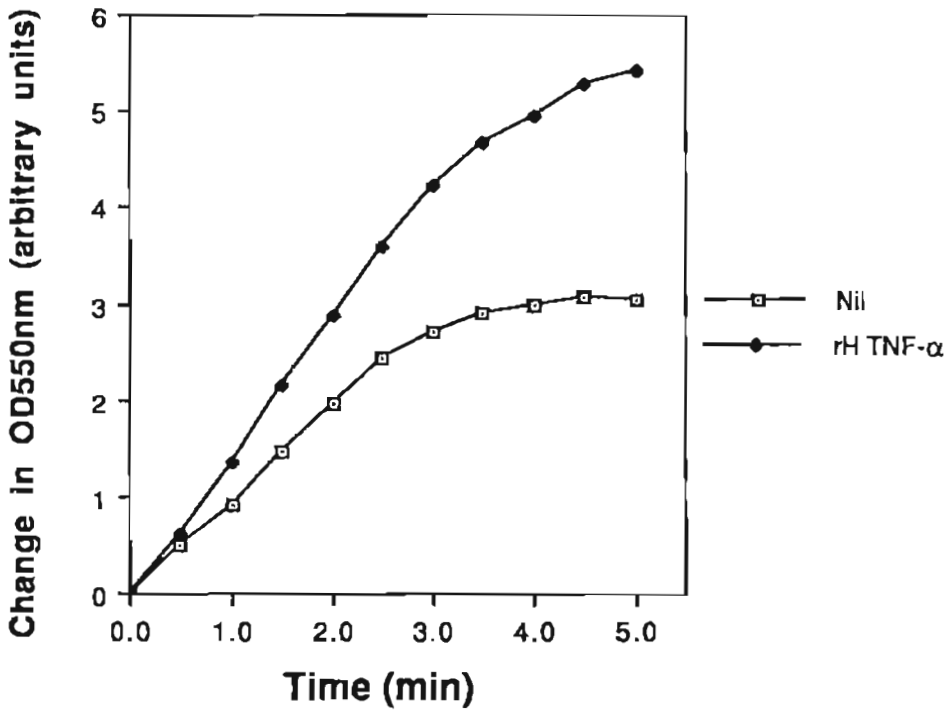
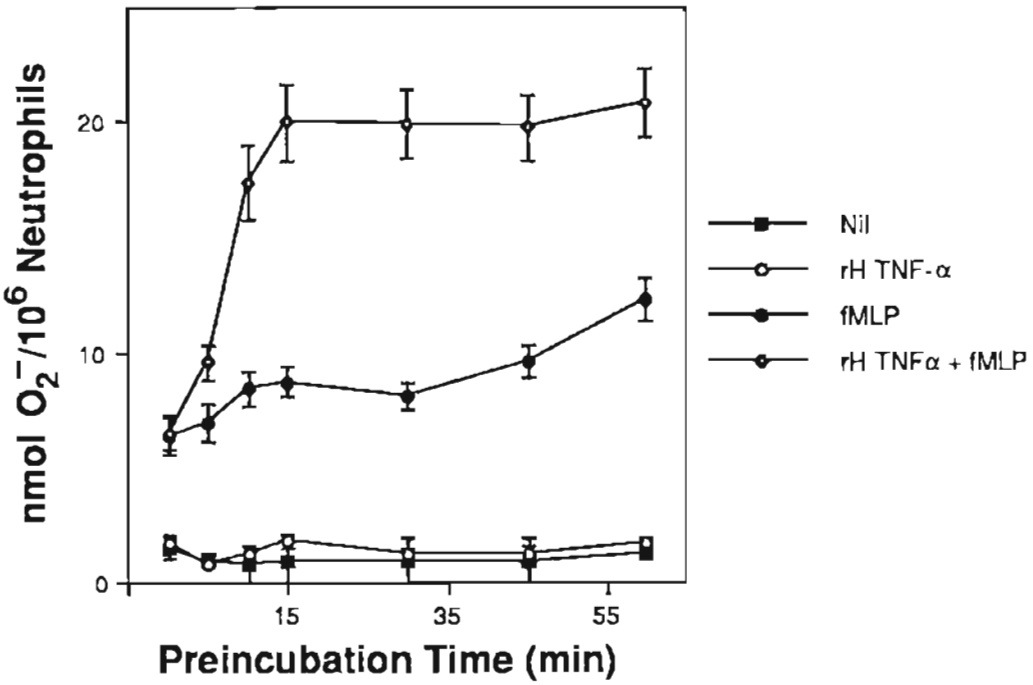

Change in OD550nm (arbitrary units)
6
5
4
3
2
1
0
0.0
1.0
2.0
3.0
4.0
5.0
Time (min)
Nil
rH TNF-α

FIGURE 4.4

Time-dependent stimulation by rH TNF-α of superoxide anion production by neutrophils.

Cells were incubated in the presence or absence of 100u/ml rH TNF-α for various times, then with or without 10^{-7}M f Met-Leu-Phe for 5 minutes. Each point represents the mean of eighteen determinations (triplicates from 6 different experiments). Bars represent the S.E.M. when this is larger than the symbols.


nmol $O_2^-/10^6$ Neutrophils
20
10
0
15
35
55
Preincubation Time (min)
Nil
rH TNF-α
fMLP
rH TNFα + fMLP

chemotactic migration (Figure 4.5). As with superoxide anion release the response varied between different individuals.

To ensure the inhibition of chemotaxis in rH TNF-α-stimulated cells was not due to oxidant toxicity, chemotaxis was allowed to proceed in the presence of 100μg/ml superoxide dismutase and 70μg/ml catalase. In the absence of these enzymes, chemotaxis was reduced from 82.5 ± 2.5 in unstimulated cells to 45 ± 5 in rH TNF-α-stimulated cells. In the presence of superoxide dismutase and catalase chemotaxis was reduced from 80 ± 5 to 42.5 ± 5 in rH TNF-α-stimulated cells. The degree of inhibition of random and chemokinetic migration was similarly unaffected.

4.2.3 Binding of f Met-Leu-[^{3}H]Phe to neutrophils:

Neutrophils incubated with 100u/ml rH TNF-α for 60 minutes exhibited saturable binding of f Met-Leu-[^{3}H]Phe as did unstimulated neutrophils.

Statistical analysis of the binding data indicated that the two-saturable-sites model was preferable to the one-saturable-site model for neutrophils incubated in medium for 60 minutes at 37°C. Figure 4.6A represents the Scatchard analysis of this same data generated by the program LIGAND. Unstimulated neutrophils exhibit a curvilinear Scatchard plot (Figure 4.6A), whereas neutrophils from the same donor that had been incubated with rH TNF-α for 60 minutes fitted better to a one-saturable-site model, and the Scatchard plot for these neutrophils was linear (Figure 4.6B).

A summary of the analysis of the binding models from four separate experiments, both for unstimulated and rH TNF-α-stimulated neutrophils is shown in Table 1. The characteristics of the two-saturable-sites model were a high affinity site with a Kd value of 2 ± 0.7, and a low affinity site with a Kd value of 180 ± 50nM. One to nine percent of

FIGURE 4.5

Inhibition by rH TNF-α of the random, chemokinetic and chemotactic migration of neutrophils towards a gradient of f Met-Leu-Phe.

Cells were incubated in the presence or absence of 100u/ml rH TNF-α for 60 minutes, then washed and assayed for their chemotactic response to f Met-Leu-Phe. Each point represents the mean of eight determinations (duplicates of four different experiments). Bars represent the S.E.M.

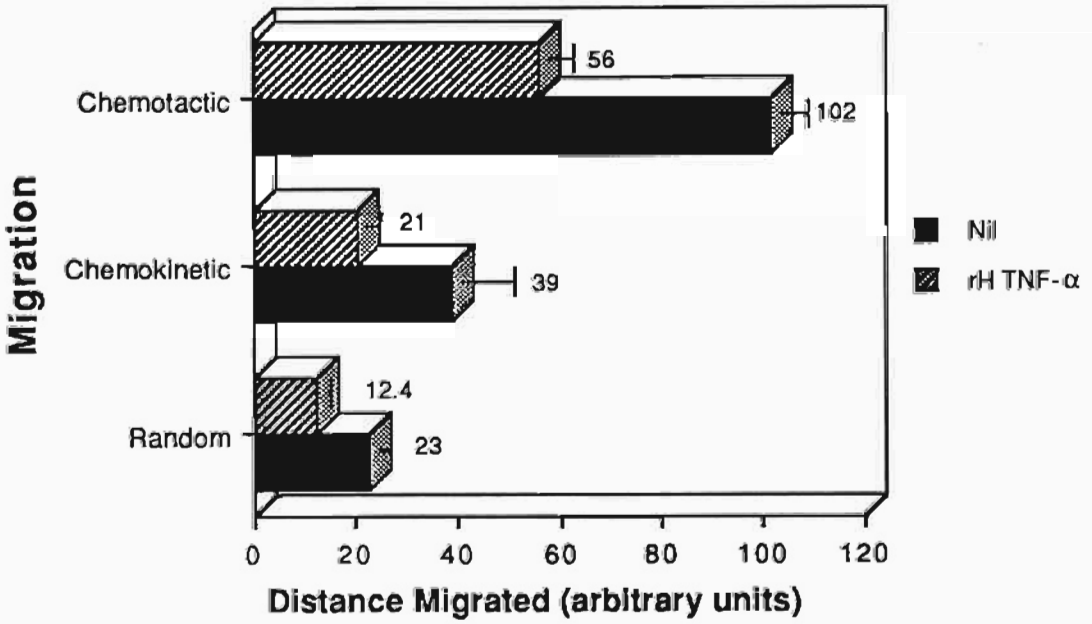
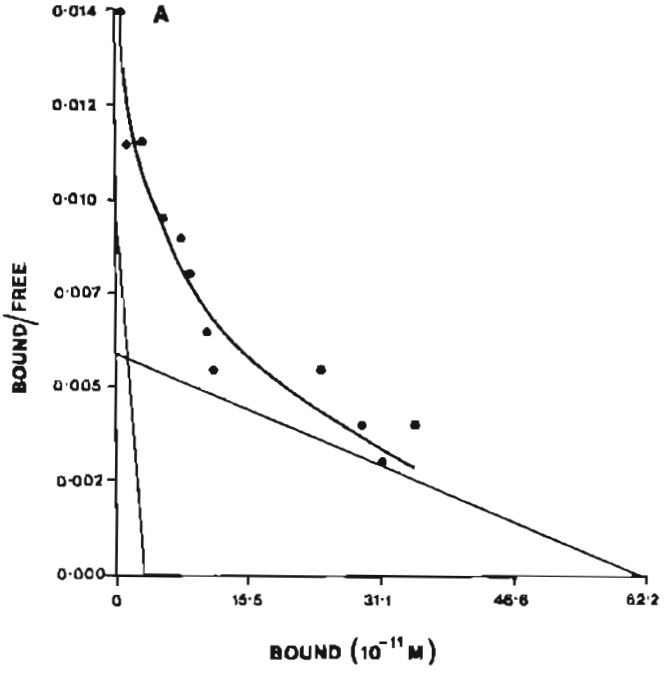
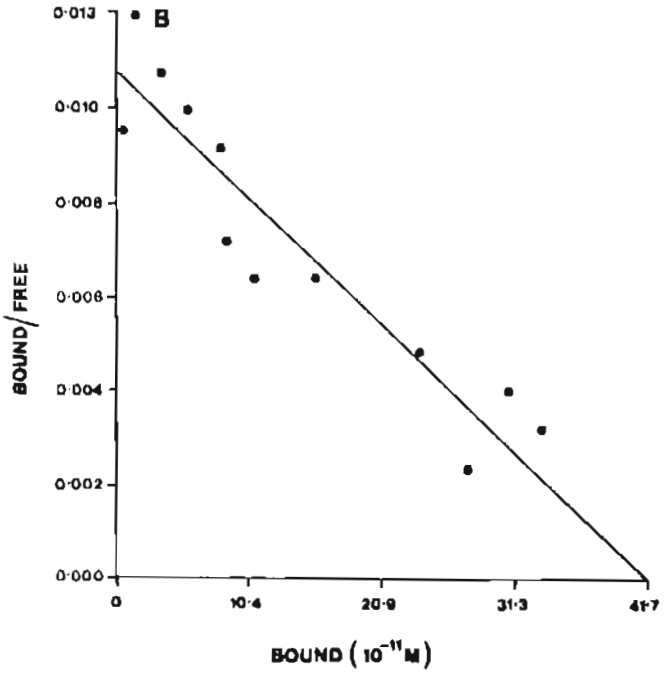

Migration
Chemotactic
Chemokinetic
Random
56
102
21
39
12.4
23
0
20
40
60
80
100
120
Distance Migrated (arbitrary units)
Nil
rH TNF-α

FIGURE 4.6

Preincubation with rH TNF-α modulates f Met-Leu-[3H]Phe binding to neutrophils.

Scatchard analysis of f Met-Leu-[^{3}H]Phe equilibrium binding to unstimulated neutrophils (A) and neutrophils stimulated with 100u/ml rH TNF-α (B).


A
0·014
0·012
0·010
0·007
0·005
0·002
0·000
BOUND/FREE
0
15·5
31·1
46·6
62·2
BOUND (10^{-11} M)


B
0·010
0·008
0·006
0·004
0·002
0·000
BOUND/FREE
0
10·4
20·9
31·3
41·7
BOUND (10^{-11} M)

the total number of sites detected were of the higher affinity. In addition, these same data when applied to the Hill equation were characterized by a Hill coefficient of 0.94 ± 0.03, suggesting the curvilinearity of the Scatchard plot is not due to site-site interactions. Although the affinity was altered by rH TNF-α stimulation, the total number of receptors remained significantly unchanged as determined by the student's two-tailed t test (data not shown).

TABLE 1

rH TNF-α (100u/ml) alters the affinity of f Met-Leu-Phe receptors on human neutrophils

Binding Parameters	Unstimulated Neutrophils	Neutrophils + rH TNF-α
Kd_1 (nM)	2 ± 0.7*	40 ± 10[3]
No. sites/cell	2000 ± 700†	$33{,}000 \pm 8000$
Kd_2 (nM)	180 ± 50	N/A
No. sites/cell	$40{,}000 \pm 9500$	N/A
Significance	$p<0.01$‖	$p>0.05$
	(1)	(2)

* Arithmetic means ± standard error of computer-estimated values from four separate experiments each with a curvilinear Scatchard plot for normal neutrophils and a linear Scatchard plot for rH TNF-α-stimulated neutrophils.

† The number of sites per cell was compared to those on normal neutrophils from four separate experiments and no significant change was found ($p>0.1$).

[3] The Kd of the low affinity and of the high affinity population was found to be significantly different from the Kd of the homogeneous receptor population of rH TNF-α-stimulated neutrophils ($p<0.05$ for both).

‖ Significance, indicated by results of F-ratio tests, pertains to the statistical difference of the data relative to those for the one saturable site model (1) and the two saturable sites model (2).

SECTION 4.3 DISCUSSION

In this Chapter it was shown that rH TNF-α modulates the affinity but not the total number of f Met-Leu-Phe receptors on human neutrophils. This may explain in part the observation that rH TNF-α enhances superoxide anion production by neutrophils in response to f Met-Leu-Phe but inhibits their chemotaxis towards a gradient of f Met-Leu-Phe.

The study of the equilibrium binding of f Met-Leu[^{3}H]Phe to unstimulated neutrophils revealed a curvilinear plot when analysed as described by Scatchard (1949). A nonlinear Scatchard plot is consistent with a heterologous population of f Met-Leu-Phe receptors, and the binding of formyl peptides to the receptors of human (Williams et al., 1977; Koo, Lefkowitz and Snyderman, 1982; Mehta and Spilberg, 1983), rabbit (Mackin, Huang and Becker, 1982) and rat neutrophils (Marasco, Feltner and Ward, 1985) has been shown previously to result in curvilinear Scatchard plots. In general, such investigations have been interpreted as due to the presence of two binding sites consisting of a high affinity and low affinity component (Koo, Lefkowitz and Snyderman, 1982; Mackin, Huang and Becker, 1982). Several possible explanations for the curvilinearity of the Scatchard analysis of f Met-Leu-Phe receptors on normal neutrophils include the possibility that two interconvertible states of the same receptor exist (Koo, Lefkowitz and Snyderman, 1983; Lohr and Snyderman, 1982; Yuli, Tomonaga and Snyderman, 1982); the two sites may be independent (Mackin et al., 1983); and the existence of negative cooperativity (Marasco, Feltner and Ward, 1985; Seligmann, Fletcher and Gallin, 1982).

LIGAND analysis of the binding data reveals a high affinity component consisting of approximately 2000 sites per cell with a Kd of 2nM. The low affinity component consisted of approximately 40,000 sites per cell with a Kd of 180nM. When these data were fitted to the Hill equation (Colquhoun, 1979; DeLean and Rodbard, 1979), a Hill

coefficient of 0.94 was observed suggesting a two-site binding model was more appropriate than negative site-site interactions. Despite the various interpretations placed on a curvilinear Scatchard plot, the observations thus far suggest the existence of two components of the f Met-Leu-Phe receptor population on normal resting neutrophils in agreement with several other groups (Koo, Lefkowitz and Snyderman, 1982; Mackin, Huang and Becker, 1982).

The stimulation of cells with 100u/ml rH TNF-α caused a conversion of the characteristics of the Scatchard plot. The resulting plot was linear indicating a homogenous population of receptors. The affinity of this receptor population was significantly different from those characteristic of unstimulated neutrophils. This suggests either that the high affinity receptors are internalised (Niedel, Wilkinson and Cuatrecasas, 1979) and lower affinity ones expressed from the intracellular pool (Fletcher and Gallin, 1983) or that the formyl peptide receptor exists in an interconvertible state as is the case for interleukin-2 receptors (Robb and Rusk, 1986; Robb, 1986). Such regulation of the expression of functionally important molecules is an interesting phenomenon as bacterial products such as lipopolysaccharide (Vadas, Lopez and Williamson, 1985) and the complement component C5a (Yancey et al., 1985) exert a similar effect. Thus the regulation of functional molecules on the cell surface may be an important mechanism by which TNF-α activates cells *in vivo*.

It was found that rH TNF-α activates a number of parameters of superoxide anion production by neutrophils in response to f Met-Leu-Phe. Thus it appears that cells pre-exposed to rH TNF-α are primed and their subsequent response to a bacterial product such as f Met-Leu-Phe is both more efficient and of greater magnitude. This phenomenon caused by a natural cytokine is likely to be physiologically significant as it could enhance the capacity of neutrophils to respond to bacterial products during the course of an inflammatory reaction. In titration experiments designed to reveal whether rH TNF-α

influenced the f Met-Leu-Phe concentration required to stimulate neutrophils, rH TNF-α activated neutrophils to release more superoxide anion. This suggests that rH TNF-α may be activating a previously unresponsive population of cells or that the f Met-Leu-Phe receptors are being modulated.

A notable characteristic of the activation of neutrophils by rH TNF-α is that no detectable release of superoxide anion occurs in the presence of rH TNF-α alone. This would be advantageous *in vivo* as the neutrophils would not be activated to produce superoxide anion, which would cause tissue damage, until they meet the appropriate target. The inability of rH TNF-α to activate mature neutrophils alone is not surprising when one considers that it was observed that rH TNF-α does not translocate protein kinase C in mature neutrophils (unpublished observations), which is a mediator in response to f Met-Leu-Phe (Naccache et al., 1985) and phorbol myristate acetate (Volpi et al., 1985a).

In contrast to stimulation of superoxide anion release, rH TNF-α inhibits both random and f Met-Leu-Phe stimulated motility. The inhibition of random neutrophil migration is less than the inhibition of f Met-Leu-Phe-induced movement. This suggests some specificity in the effect of rH TNF-α but also illustrates that receptor modulation is not the sole cause for the changes observed. These data further suggest that rH TNF-α may cause a fundamental change in neutrophils resulting in a generalised decrease in motility. The possibilities include an influence on actin polymerisation, and the modulation of other receptors. Those possibilities will be the subject of future investigations.

The loss of f Met-Leu-Phe stimulated motility together with the disappearance of the high affinity formyl peptide receptors nevertheless supports previous data suggesting that high affinity receptors mediate chemotaxis whereas low affinity receptors mediate superoxide anion release (Becker, 1979; Kreutzer et al., 1978). Although receptor modulation may explain the functional observations, the change in low affinity receptors observed here

cannot be directly equated with the changes in cell function for two reasons. Firstly, stimulus-response coupling is a complex process which depends on receptor occupancy rather than total receptor numbers. Secondly, the intracellular transduction mechanisms are known to amplify the initial signal from the receptor, and it is not known what the magnitude of this effect is.

Although the mechanism of action is unknown, previous studies have associated the formyl peptide receptor with a family of proteins that regulate adenylate cyclase known as the guanine nucleotide binding proteins or G proteins (Gilman, 1984). Specifically, the formyl peptide receptor is coupled to a pertussis toxin sensitive G protein termed Ni. There is evidence to suggest that the G proteins modulate the affinity of the formyl peptide receptor on human (Koo, Lefkowitz and Snyderman,1983; Lad, Olson and Smiley, 1985; Goldman et al., 1985), rabbit (Molski et al., 1984; Volpi et al., 1985b) and guinea pig (Okajima, Katada and Ui, 1985) neutrophils. Considering this and the recent finding that some cytokines interact with G proteins (Evans, Beckner and Farrar, 1987), it is feasible that this is one mechanism by which rH TNF-α modulates the affinity of f Met-Leu-Phe receptors, although the TNF-α receptor may not necessarily be coupled to the same type of G protein. It is possible that a third G protein may be involved in the transduction signal from the TNF-α receptor to the f Met-Leu-Phe receptor.

In addition, the high affinity and low affinity receptors may not be distinct molecules - rather they may represent a single interconvertible molecule, thus the change in affinity but not numbers presented in this paper can be interpreted as supportive of this theory. Although both the respiratory burst and chemotaxis are sensitive to pertussis toxin, new G proteins are still being discovered and it is feasible that other pertussis toxin sensitive G proteins exist. Thus the loss of high affinity receptors induced by rH TNF-α is consistent with the loss of chemotactic responsiveness, whereas the increased capacity to generate

superoxide anion may also be partially explained by the change in f Met-Leu-Phe receptor affinity.

These findings are particularly relevant to *in vivo* situations where the functional activation and immobilisation of neutrophils to sites of inflammation are desirable properties, and suggest that TNF-α may play an important role during the inflammatory reaction. It is interesting that the effects of rH TNF-α reported in this chapter are shared by another biologically important compound, granulocyte-macrophage colony-stimulating factor (Atkinson et al., 1988a). This compound has also been implicated in the processes of inflammation and these findings support the relevance of these observations to *in vivo* situations.

CHAPTER 5

RECOMBINANT HUMAN GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR REGULATES F MET-LEU-PHE RECEPTORS ON HUMAN NEUTROPHILS

SECTION 5.1 INTRODUCTION

Not only monocyte-derived cytokines such as TNF-α, but also T cell-derived cytokines such as GM-CSF have been shown to influence neutrophil function. GM-CSF belongs to a family of cytokines known as colony stimulating factors (CSF), named originally because of their important role in the proliferation and differentiation of progenitor cells (Metcalf, 1984), but more recently some of these factors have been shown also to functionally activate mature cells in a lineage-specific fashion (Vadas, Nicola and Metcalf, 1983; Lopez et al., 1983; Metcalf et al., 1986).

It has been shown that purified recombinant human GM-CSF can activate several functions of mature neutrophils and eosinophils. For example, rH GM-CSF stimulated phagocytosis of serum opsonized yeast, neutrophil-mediated iodination in the presence of zymosan, and the cytotoxic activity of these cells against antibody-coated targets (Lopez et al., 1986). In addition, rH GM-CSF also enhanced N-formylmethionyl-leucylphenylalanine- (f Met-Leu-Phe) stimulated functions of neutrophils such as degranulation and superoxide anion production (Lopez et al., 1986). Because this cytokine has been reported to influence neutrophil functions in a manner that is similar to rH TNF-α, it's influence on f Met-Leu-Phe receptor expression was studied.

The mechanism by which rH GM-CSF enhances the effect of f Met-Leu-Phe is not known, but one possibility is the regulation of f Met-Leu-Phe receptor expression. This may simply involve a change in the number of receptors expressed on the cell surface. For example, rH GM-CSF has been shown to increase the surface expression of the receptor for C3bi (Lopez et al., 1986) and of the p150,95 molecule which are members of a family of leukocyte adhesion molecules (Arnaout et al., 1986). Alternatively, cell surface receptor changes may involve more subtle mechanisms such as phosphorylation of the receptor resulting in a change in the affinity or function of the receptor. For example,

f Met-Leu-Phe triggers the phosphorylation of the C3b receptor on neutrophils (Changelian and Fearon, 1986). It is also known that the formyl peptide receptors on neutrophils are regulated both in their affinity and number (Gallin, Wright and Schiffman, 1978; Fletcher and Gallin, 1980) by secretory stimuli, and also by f Met-Leu-Phe itself (Seligmann, Fletcher and Gallin, 1982).

It is shown here that rH GM-CSF alters the affinity but not the numbers of f Met-Leu-Phe receptors and that this regulation of receptor expression is consistent with the biological effects of rH GM-CSF on human neutrophils.

SECTION 5.2 RESULTS

5.2.1 rH GM-CSF alters the production of superoxide anion by neutrophils stimulated with f Met-Leu-Phe:

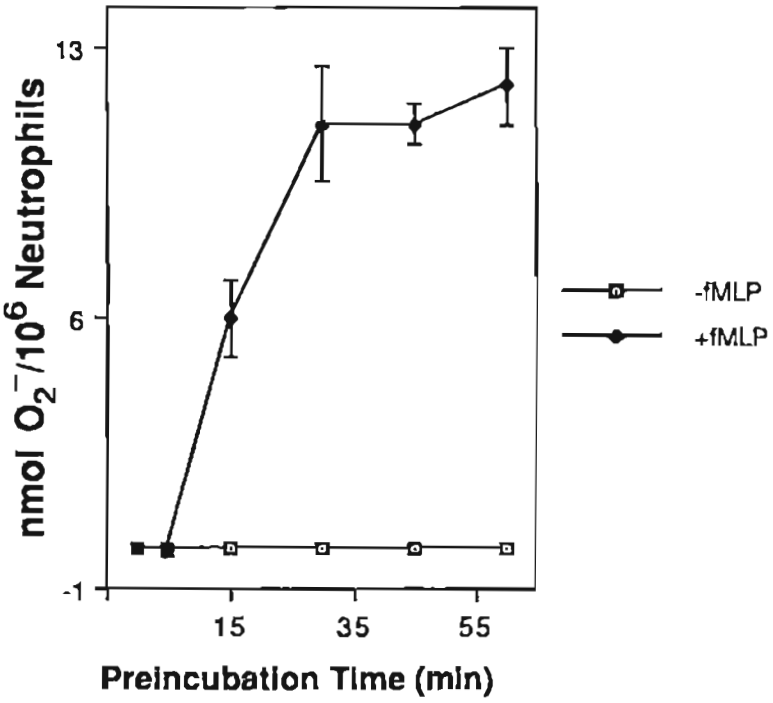
Preliminary experiments showed that rH GM-CSF did not stimulate superoxide anion production by itself, but enhanced the production of superoxide anion by neutrophils in response to f Met-Leu-Phe, and 100 ng/ml (10^{-11}M) was the optimal concentration (data not shown).

Time-course experiments performed by preincubating neutrophils for various times with 100 ng/ml rH GM-CSF to determine the time necessary for rH GM-CSF to prime neutrophils, showed that activation had occurred by 5 minutes and had reached optimal levels by 30 minutes (Figure 5.1). Cells that were preincubated in the presence or absence of rH GM-CSF and not stimulated with f Met-Leu-Phe, exhibited no detectable production of superoxide anion. The amount of superoxide released in response to f Met-Leu-Phe was constant for up to 60 minutes preincubation with rH GM-CSF. The amount of superoxide released in response to f Met-Leu-Phe from cells that have been treated

FIGURE 5.1

Time-dependent stimulation by rH GM-CSF of superoxide anion production by neutrophils.

Neutrophils were incubated in the presence or absence of 100 ng/ml rH GM-CSF for various times at 37^{o}C, then for 5 minutes with (closed symbols) or without (open symbols) 10^{-7}M f Met-Leu-Phe at 37^{o}C. Each point represents the difference between unstimulated and rH GM-CSF-treated neutrophils. Each point represents the mean of triplicate determinations $\pm$ S.E.M.


13
6
-1
nmol $O_2^-/10^6$ Neutrophils
15
35
55
Preincubation Time (min)
-fMLP
+fMLP

with rH GM-CSF for 60 minutes represents a 40% increase over untreated neutrophils.

To determine whether the increase in superoxide anion production from neutrophils preincubated with rH GM-CSF was associated with a change in rate of superoxide anion production, neutrophils were incubated with f Met-Leu-Phe in the presence of cytochrome c over a 10 minute period. Preincubating neutrophils with 100 ng/ml rH GM-CSF enhanced the rate of production of superoxide anion in their subsequent response to f Met-Leu-Phe. The levels reached a plateau at 5 minutes. This effect was consistent over 7 experiments where the mean rate of change in OD550nm per minute over 5 minutes for rH GM-CSF-treated cells was 0.086 $\pm$ 0.007 whereas the mean rate of change for unstimulated neutrophils was 0.015 $\pm$ 0.006.

5.2.2 rH GM-CSF inhibits the chemotactic migration of neutrophils in response to a gradient of f Met-Leu-Phe:

To determine whether rH GM-CSF influences a different f Met-Leu-Phe-mediated neutrophil response, the chemotaxis of neutrophils towards a gradient of f Met-Leu-Phe was studied. The neutrophils were preincubated with rH GM-CSF, then washed and placed in the agarose-containing dishes in the presence or absence of a gradient of f Met-Leu-Phe.

To determine the optimal concentration of rH GM-CSF required for this effect, a titration of the factor was performed. This showed that rH GM-CSF inhibited chemotaxis towards a gradient of f Met-Leu-Phe in a dose-dependent manner (Figure 5.2). The inhibition of chemotaxis was also found to be dependent on the time of preincubation with rH GM-CSF (Figure 5.3). As with superoxide anion release, the inhibition of neutrophil migration was evident by 5 minutes and reached a plateau at 30 minutes.

FIGURE 5.2

Dose-dependent inhibition by rH GM-CSF of neutrophil migration towards a gradient of f Met-Leu-Phe.

Following incubation with various concentrations of rH GM-CSF for 60 minutes at 37°C, the cells were washed and assayed in the presence or absence of a gradient caused by 10^{-7}M f Met-Leu-Phe for 2 hours at 37°C. Each point represents the mean of four determinations ± S.E.M. Results are expressed as distance migrated in arbitrary units.

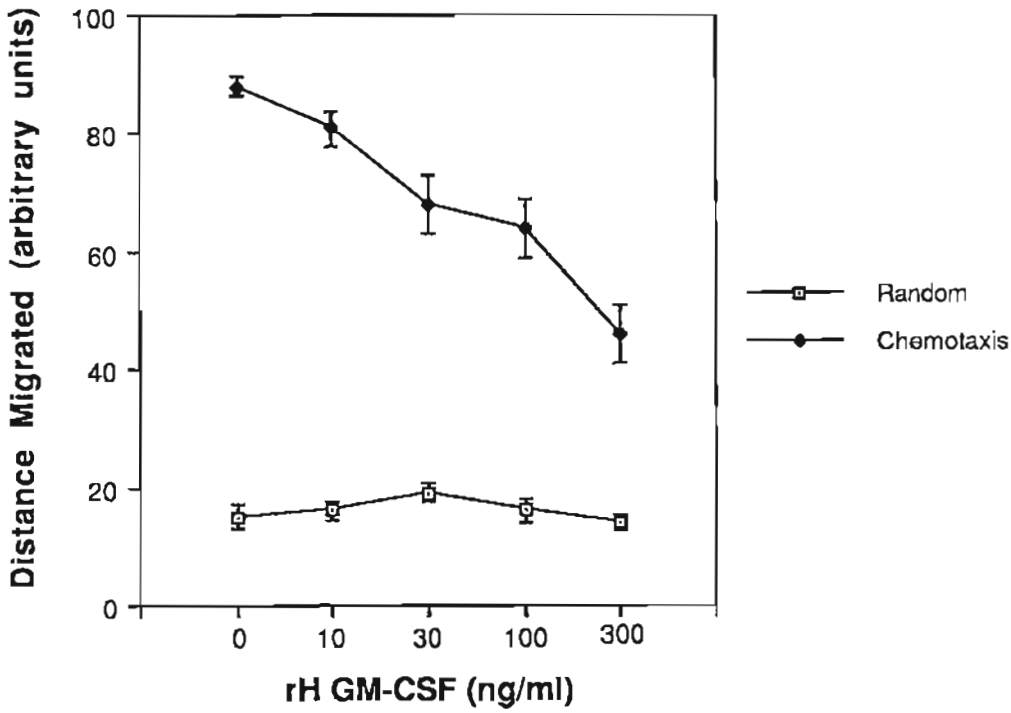
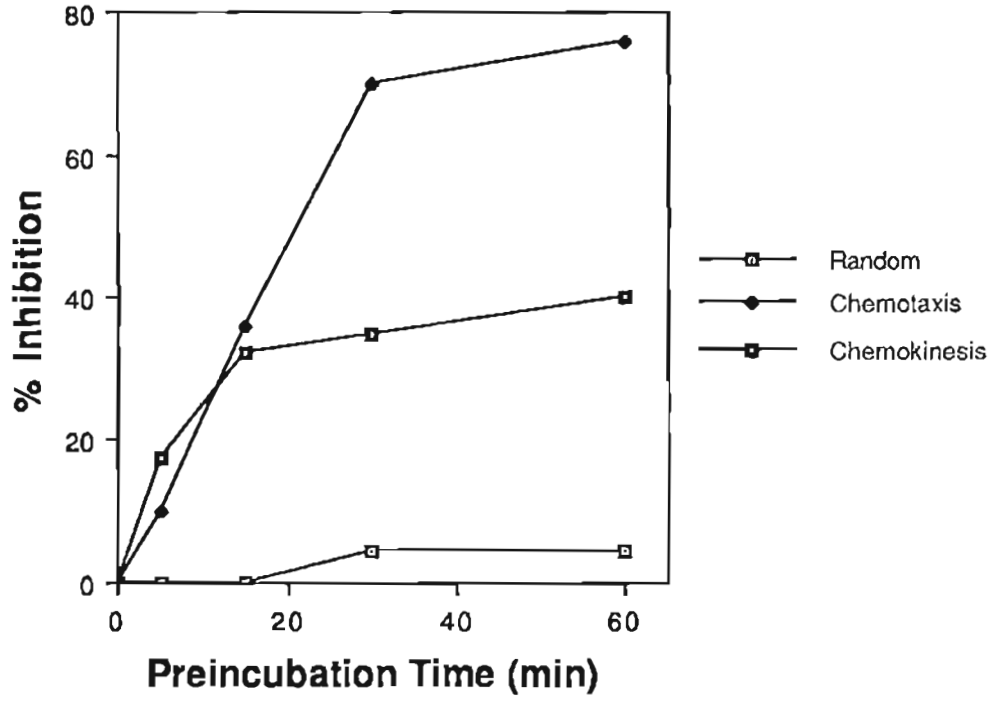

Distance Migrated (arbitrary units)
100
80
60
40
20
0
0
10
30
100
300
rH GM-CSF (ng/ml)
Random
Chemotaxis

FIGURE 5.3

Time-dependent inhibition by rH GM-CSF of neutrophil migration towards a gradient of f Met-Leu-Phe.

Following incubation with or without 100 ng/ml rH GM-CSF for various times at 37°C, the cells were washed and incubated in the presence or absence of a gradient of 10^{-7}M f Met-Leu-Phe for 2 hours at 37°C. Random (open squares), chemotactic (closed diamonds) and chemokinetic (closed squares) migration were determined in duplicate. Results are expressed as % inhibition of unstimulated neutrophil migration.


80
60
40
20
0
% Inhibition
0
20
40
60
Preincubation Time (min)
Random
Chemotaxis
Chemokinesis

After determining optimal rH GM-CSF concentrations and time of preincubation, all three kinds of neutrophil locomotion were studied. Figure 5.4 shows the summary data from nine different individuals. It was found that preincubating neutrophils with rH GM-CSF significantly inhibited both chemokinetic ($p<0.01$) and chemotactic ($p<0.001$) movement as determined by the student's two-tailed t test. In contrast, the random migration of unstimulated neutrophils was not significantly ($p>0.05$) altered by treatment with rH GM-CSF. As with superoxide anion release the response varied between different individuals. To ensure rH GM-CSF did not inhibit neutrophil migration due to oxidant toxicity, this assay was carried out in the presence of 100μg/ml superoxide dismutase and 70μg/ml catalase. No change in the pattern of inhibition caused by rH GM-CSF was observed.

5.2.3 Binding of f Met-Leu-[^{3}H]Phe to neutrophils:

To determine the characteristics of f Met-Leu-Phe receptor binding to neutrophils following stimulation with rH GM-CSF at 37^{o}C, cells were cooled to 4^{o}C and steady state receptor binding experiments performed. A typical specific binding saturation curve for unstimulated neutrophils and for neutrophils incubated with 200 ng/ml rH GM-CSF for 1 hour is shown in Figure 5.5A.

Statistical analysis of the binding data indicated that the two-saturable-sites model was preferable to the one-saturable-site model for unstimulated neutrophils, and Scatchard analysis of these same data was curvilinear (Figure 5.5B); neutrophils that had been incubated with rH GM-CSF for 60 minutes, however, fitted better to a one-saturable-site model and the Scatchard plot for these neutrophils was linear.

To determine what period of exposure to rH GM-CSF was necessary for a change in the characteristics of f Met-Leu-[^{3}H]Phe binding to occur, cells were incubated with 200 ng/ml rH GM-CSF at 37^{o}C for 5, 15, 30 and 60 minutes. The cells were then

FIGURE 5.4

rH GM-CSF inhibits the chemokinetic and chemotactic migration of neutrophils towards a gradient of f Met-Leu-Phe.

Following incubation with or without 100 ng/ml rH GM-CSF for 60 minutes at 37°C, the cells were washed and assayed in the presence or absence of a gradient caused by 10^{-7}M f Met-Leu-Phe for 2 hours at 37°C. Each point represents the mean of duplicate determinations from nine individuals ± S.E.M.

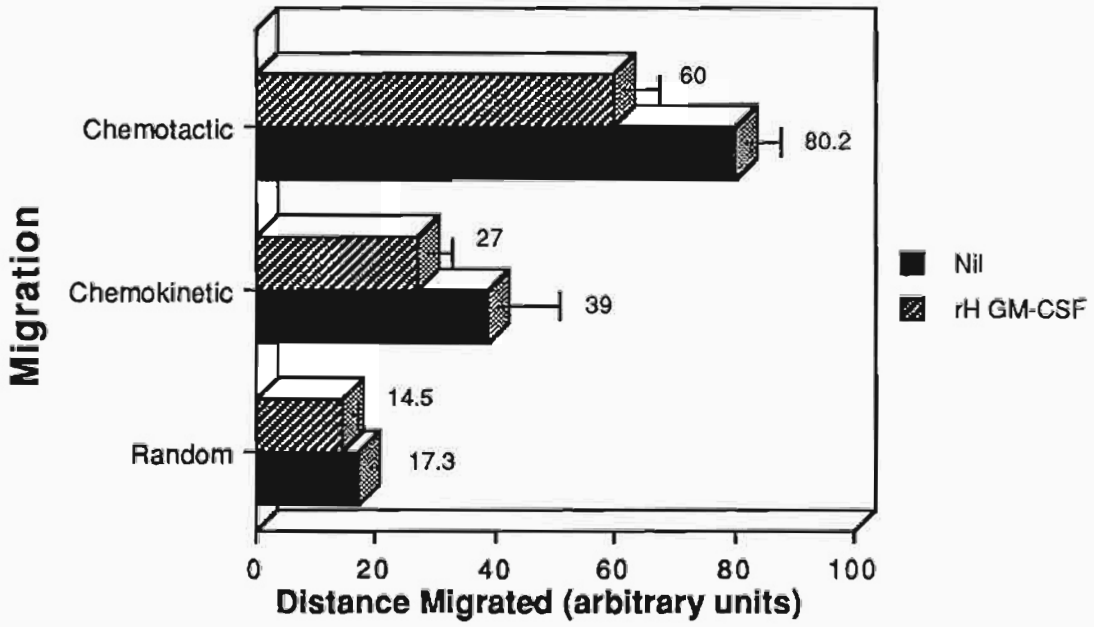
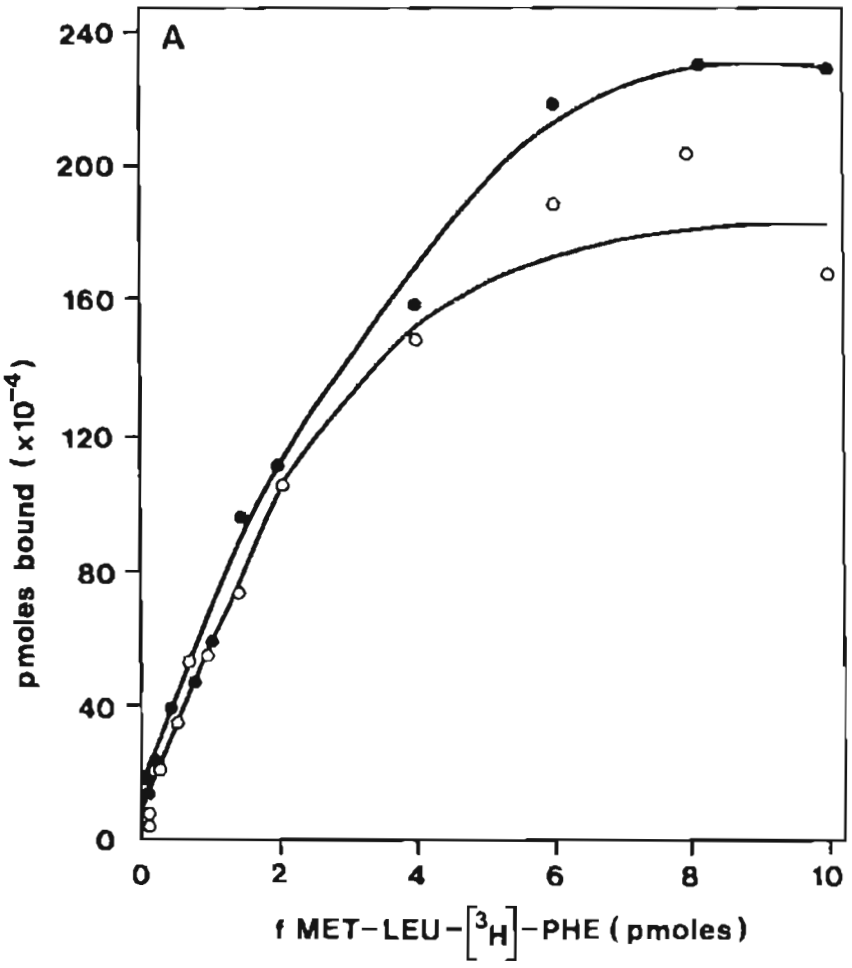

Migration
Chemotactic
Chemokinetic
Random
60
80.2
27
39
14.5
17.3
Nil
rH GM-CSF
0
20
40
60
80
100
Distance Migrated (arbitrary units)

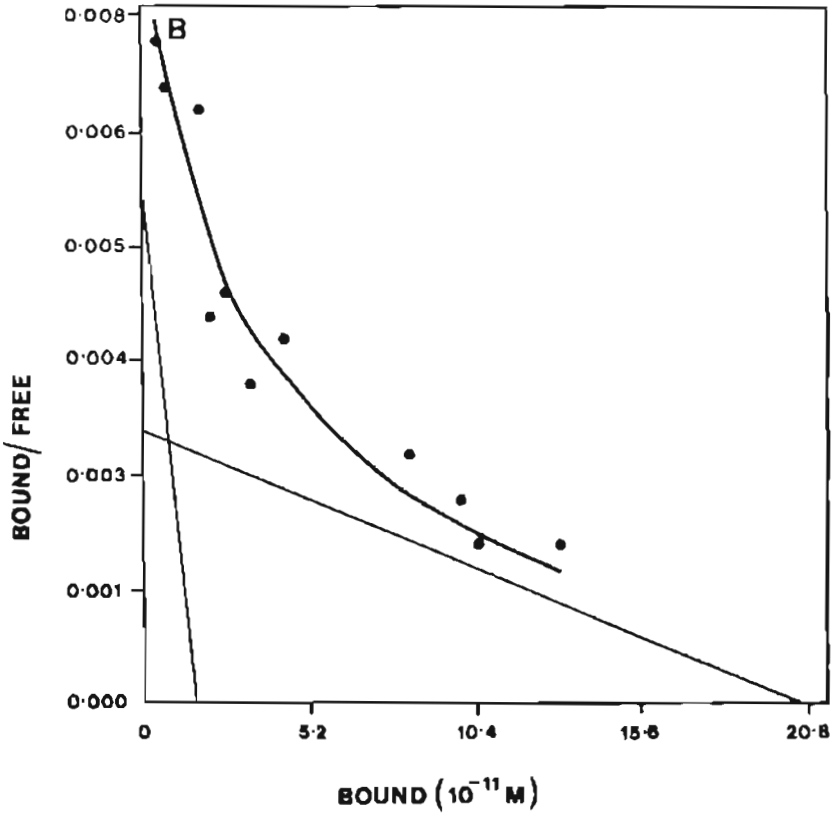
FIGURE 5.5

f Met-Leu-[^{3}H]Phe binding isotherms of unstimulated and rH GM-CSF-stimulated neutrophils.

(a) Following 60 minutes incubation at 37^{o}C in the presence (open circles) or absence (closed circles) of 200 ng/ml rH GM-CSF, neutrophils were washed, then incubated for 30 minutes at 4^{o}C with increasing concentrations of f Met-Leu-[^{3}H]Phe. Non-specific binding in the presence of 1000-fold or more excess of f Met-Leu-Phe was determined in duplicate, and subtracted from total binding, which was determined in triplicate.

(b) Computer generated Scatchard analysis of f Met-Leu-[^{3}H]Phe binding to unstimulated neutrophils.


A
240
200
160
120
80
40
0
pmoles bound (x10⁻⁴)
0
2
4
6
8
10
f MET-LEU-[³H]-PHE (pmoles)


B
0·008
0·006
0·005
0·004
0·003
0·001
0·000
BOUND/FREE
0
5·2
10·4
15·6
20·8
BOUND (10⁻¹¹ M)

cooled to 4°C, and the binding assay was performed.

The data in Figure 5.6 are representative of two experiments where each point was determined in triplicate. The appropriateness of each fit was determined using the F test as described in Chapter 2. The characteristic curvilinear Scatchard plot obtained without stimulation remained at 5 minutes (p=0,0.039) but had changed to a straight line Scatchard plot by 15 minutes (p=0.483,0.807), and remained straight for longer preincubation times (30 minutes, p=0.259,0.445; 60 minutes, p=0.362,0.384).

A summary of the analysis of the binding models from six separate experiments, both for unstimulated and rH GM-CSF-stimulated neutrophils is shown in Table 1. The characteristics of the two-saturable-sites model were a high affinity site with a Kd value of 4 ± 2nM, and a low affinity site with a Kd value of 230 ± 130nM. Two to seven percent of the total number of sites detected were of the higher affinity. In addition, these same data when applied to the Hill equation were characterised by a Hill coefficient of 1.0 ± 0.02, suggesting the curvilinearity of the Scatchard plot is not due to site-site interactions. Although the affinity was altered by rH GM-CSF stimulation, the total number of receptors remained significantly unchanged, as determined by the student's two-tailed t test.

SECTION 5.3 DISCUSSION

In this chapter it was shown that rH GM-CSF modulates the affinity but not the total number of f Met-Leu-Phe receptors on human neutrophils. These data may explain the biological findings that rH GM-CSF enhanced superoxide anion production by neutrophils in response to f Met-Leu-Phe, but inhibited their chemotaxis towards a gradient of f Met-Leu-Phe. This data is of particular interest considering it has been reported that these important functional roles of neutrophils are stimulated by f Met-Leu-Phe at greatly different concentrations.

FIGURE 5.6

Time-dependent change by rH GM-CSF in the characteristics of f Met-Leu-[^{3}H]Phe binding to neutrophils.

Computer generated Scatchard analysis of steady state binding of f Met-Leu-[^{3}H]Phe to neutrophils stimulated with rH GM-CSF for 5, 15, 30 and 60 minutes.

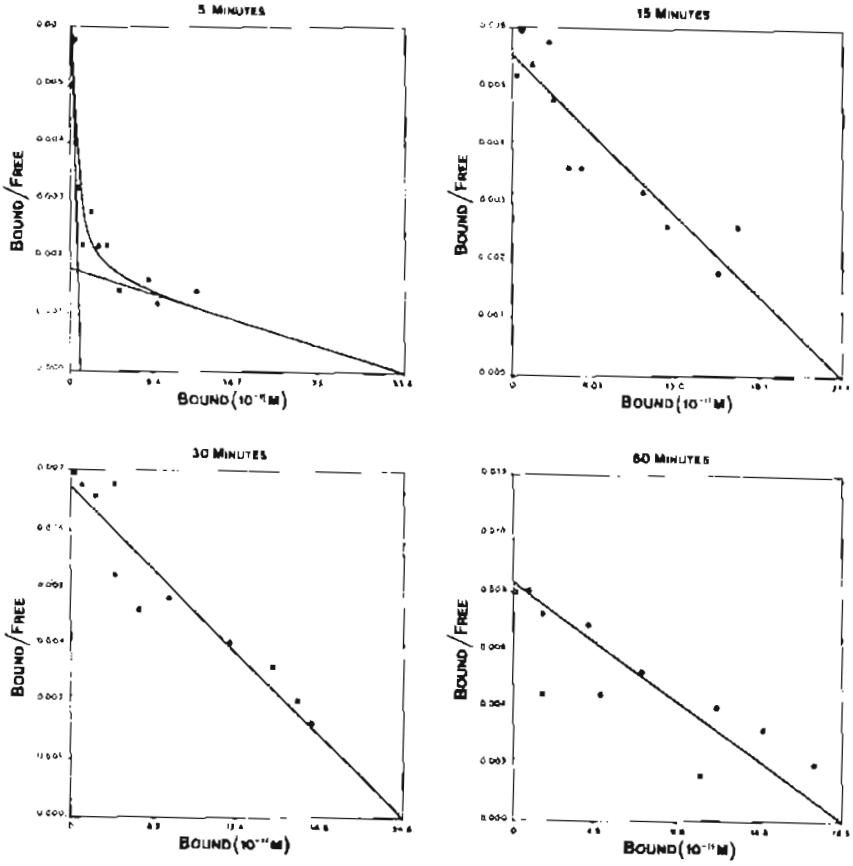

5 Minutes
15 Minutes
30 Minutes
60 Minutes
Bound/Free
Bound(10⁻¹¹M)

TABLE 1

rH GM-CSF alters the affinity of f Met-Leu-Phe receptors on human neutrophils.

Binding Parameters	Unstimulated Neutrophils	Neutrophils + rH GM-CSF
Kd_1 (nM)	$4 \pm 2^*$	$30 \pm 10^\dagger$
No. sites/cell	$2,000 \pm 830^\ddagger$	$26,000 \pm 5,000$
Kd_2 (nM)	220 ± 130	N/A
No. sites/cell	$40,000 \pm 13,000$	N/A
Significance	$p<0.01^\parallel$	$p>0.05$
	(1)	(2)

* Arithmetic means ± standard error of computer-estimated values from six separate experiments each with a curvilinear Scatchard plot for normal neutrophils and a linear Scatchard plot for rH GM-CSF-stimulated neutrophils.

† The Kd of the low affinity population was found to be significantly different from the Kd of the homogeneous receptor population of rH GM-CSF-stimulated neutrophils ($p = 0.004$).

‡ The number of sites per cell was compared to those on normal neutrophils from six separate experiments, and no significant change was found ($p>0.1$).

‖ Significance, indicated by results of F-ratio tests, pertains to the statistical difference of the data relative to those for the one saturable site model (1), and the two saturable sites model (2).

The study of the equilibrium binding of f Met-Leu-[^{3}H]Phe to unstimulated neutrophils revealed a curvilinear plot when analysed as described by Scatchard (Scatchard, 1949). As discussed in Chapter 4.3, curvilinear Scatchard plots have been widely interpreted as due to the presence of two binding sites, one of higher affinity and one of lower affinity (Koo, Lefkowitz, and Snyderman, 1982). There are several possible explanations for the curvilinearity of the Scatchard analysis of f Met-Leu-Phe receptors on normal neutrophils. The most favoured one is that there are two sites that are interconvertible states of the same receptor (Koo, Lefkowitz and Snyderman, 1983; Lohr and Snyderman, 1982; Yuli, Tomonaga and Snyderman, 1982), although other workers have suggested that the two sites may be independent (Mackin et al., 1983), or that the curvilinear Scatchard plot is due to negative cooperativity (Seligmann, Fletcher, and Gallin, 1982).

LIGAND analysis of the binding data reveals a high affinity and a low affinity component. When these data were fitted to the Hill equation, a Hill coefficient of 1 was observed, suggesting a two-site binding model was more appropriate than negative site-site interactions. Despite the various interpretations placed on a curvilinear Scatchard plot, the observations thus far suggest the existence of two components of the f Met-Leu-Phe receptor population on normal resting neutrophils.

The stimulation of cells with 200 ng/ml rH GM-CSF caused a time-dependent modulation of the f Met-Leu-Phe receptor population. Unstimulated neutrophils, and neutrophils incubated with rH GM-CSF for 5 minutes at 37^{o}C, exhibited curvilinear Scatchard plots indicative of two-affinity binding sites as described above. After 15 minutes incubation with rH GM-CSF however, the Scatchard plot was linear indicating a homogenous population of receptors. The Scatchard plot remained linear over 30 minutes and 60 minutes of stimulation with 200 ng/ml rH GM-CSF. In addition, the affinity of the resulting receptor population was invariably intermediate between the affinities of the high and low binding component of unstimulated neutrophils. Although the affinity of the receptors was altered, total receptor number did not change significantly following stimulation with rH GM-CSF. It appears that the relative numbers of lower affinity receptors are increased upon stimulation by rH GM-CSF. This suggests either that the high affinity receptors are internalised and lower affinity ones expressed from the intracellular pool (Fletcher and Gallin, 1983) or that the formyl peptide receptor exists in an interconvertible state as is the case for interleukin-2 receptors. It was also observed that the affinity of the homogenous receptor population increased slightly over the 45 minutes following the conversion: the reason for this is unknown but may be due to the progressive expression of more functionally relevant receptors.

The priming effect of rH GM-CSF on the ability of neutrophils to produce superoxide anion in response to f Met-Leu-Phe was time-dependent with activation being virtually

maximal at 30 minutes, and maintained over the time period studied. It is notable that the change in the characteristics of the f Met-Leu-Phe receptors after 15 minutes paralleled the enhancement of superoxide anion production by rH GM-CSF. Thus it appears that cells pre-exposed to rH GM-CSF are primed, and their subsequent response to a bacterial product such as f Met-Leu-Phe is both more efficient and of a greater magnitude; such a priming event caused by a natural cytokine might be expected to be physiologically significant.

One of the salient features of the activation of neutrophils by rH GM-CSF is that no detectable release of superoxide anion occurs in the presence of rH GM-CSF alone. This is not surprising as rH GM-CSF does not translocate protein kinase C in mature neutrophils (unpublished observations), which is a mediator in responses to f Met-Leu-Phe (Naccache et al., 1985). Previous reports have associated the formyl peptide receptor with a family of proteins that regulate andenylate cyclase known as the guanine nucleotide binding proteins or G proteins (Gilman, 1984). Evidence suggesting that the G proteins modulate the affinity of the formyl peptide receptor has been presented for human neutrophils (Koo, Lefkowitz, and Snyderman, 1983; Lad, Olson and Smiley, 1985; Goldman et al., 1985). Considering this, it is feasible that rH GM-CSF modulates the affinity of f Met-Leu-Phe receptor via the G proteins.

It was found that rH GM-CSF influences neutrophil function in a dose-dependent fashion as previously shown (Lopez et al., 1986). Preincubating neutrophils with rH GM-CSF caused an inhibition of both chemokinetic and chemotactic migration in response to f Met-Leu-Phe. Although rH GM-CSF inhibited random movement as suggested previously (Gasson et al., 1984), the effect was not significant. One possible reason for this discrepancy is that in these experiments the neutrophils were washed following preincubation with rH GM-CSF. This was done in order to eliminate the possibility of negative gradient effects as Wang et al. (1987) have shown that rH GM-CSF is

chemotactic by itself; indeed, without the washing step we found that random movement was reduced to a greater extent (Atkinson and Lucas, unpublished data).

In the course of this work a study of the regulation of f Met-Leu-Phe receptors by biosynthetic human GM-CSF was published (Weisbart, Golde and Gasson, 1986). These data differ from ours on several points. Firstly, they observe that preincubating neutrophils with rH GM-CSF enhances chemotaxis whereas the data presented here shows that rH GM-CSF inhibits both the chemokinesis and chemotaxis of neutrophils. This discrepancy may be due to the fact that Weisbart et al. (1986) have expressed migration as a 'chemotactic index'. This is defined as the ratio of migration towards a chemoattractant to migration away from the chemoattractant. Expressing chemotaxis in such a way may lead to misinterpretation of the data as illustrated by Nelson (Nelson, Quie, and Simmons, 1975). If one migration component in the chemotactic index is altered, whereas the other remains unaffected, the chemotactic index is altered and subsequently not representative of the observations.

Secondly, Weisbart et al. (1986) present binding data which represent f Met-Leu-Phe receptors as a homogenous population on unstimulated neutrophils. This is in disagreement with previous observations by others (Williams et al., 1977; Koo et al., 1982; Mehta and Spilberg, 1983). Furthermore, it is possible that the binding assays were performed under suboptimal conditions for a number of reasons: (a) the concentration of Ca^{++} and Mg^{++} were not specified, and these are important in f Met-Leu-Phe binding to neutrophils (Niedel, Wilkinson and Cuatrecasas, 1979), as it has been reported that millimolar concentrations of Ca^{++} and Mg^{++} enhanced binding from 4- to 6-fold, although the requirement for Mg^{++} was not as important as for Ca^{++}. (b) Metabolic inhibitors were not used, and these are essential to minimize internalisation which can occur even at $4^{o}C$ (Sullivan and Zigmond, 1980).

The changes in the affinity of the f Met-Leu-Phe receptor paralleled changes in the biological response studied. A decrease in the high affinity f Met-Leu-Phe receptor population was accompanied by an enhanced superoxide production but a decreased neutrophil chemotaxis. This is consistent with the previous findings (Sklar, 1986) showing that the chemotactic response is most sensitive to low formyl peptide concentrations, whereas the generation of superoxide anion requires higher concentrations of formyl peptide. The evidence above implies that the high affinity formyl peptide receptors may mediate neutrophil chemotaxis, whereas the lower affinity receptors mediate superoxide anion generation.

Such an interpretation of the data would make considerable physiological sense. Thus neutrophils far from the site of invading bacteria would encounter very low concentrations of f Met-Leu-Phe, detectable only by high affinity receptors. Upon migrating towards the source of infection (i.e. up the f Met-Leu-Phe gradient) the low affinity receptors would become responsive and superoxide production would be induced. GM-CSF, produced locally, would assist in maintaining the neutrophils at the site of inflammation and optimise superoxide production where it is required most. By contrast, the administration of GM-CSF by the intravenous route, as is being suggested for clinical trials, might lead, at least transiently, to deleterious effects.

CHAPTER 6

DIRECT AND INDIRECT STIMULATION OF NEUTROPHIL DEGRANULATION AND ACTIN POLYMERISATION BY RECOMBINANT HUMAN TUMOR NECROSIS FACTOR-ALPHA.

SECTION 6.1. INTRODUCTION

As mentioned in the Introduction to Chapter 3, neutrophil microbicidal mechanisms centre around two major events - degranulation and the respiratory burst. All phagocyte cells contain microbicidal and hydrolytic digestive enzymes which are packaged into lysosomes within the cytoplasm. There are two major forms of lysosomes within the neutrophil. These are the azurophilic granules and the specific granules (Bainton and Farquhar, 1966). Amongst other digestive enzymes, both types of granules contain lysosyme, approximately two-thirds of which is contained within the specific granules. (Spitznagel et al., 1974).

Neutrophils do not degranulate unless the granules come in contact with the cell membrane. This occurs during the process of phagocytosis (Bainton, 1973), or when a neutrophil spreads against a surface (Cramer and Gallin, 1979; Henson, 1973). In a resting neutrophil, granule contact with the plasma membrane is prevented by the presence of the cortical actin network that lies just below the plasma membrane (Southwick and Stossel, 1983). When this microfilament network is disrupted, granule fusion occurs (Bainton, 1973; Cramer and Gallin, 1979; Henson, 1973) and the cells degranulate. It appears that granule contact with the plasma membrane is not the sole requirement for degranulation, because cytochalasin B facilitates degranulation, but does not induce this response on its own (Zurier, Hoffstein and Weissmann, 1973). Similarly, surface stimulation by a soluble ligand such as f Met-Leu-Phe cannot induce degranulation unless cytochalasin B is present (Showell et al., 1976), or unless the stimulus is attached to a surface too large for the neutrophil to phagocytose (Becker et al., 1974; Becker and Showell, 1974: Henson and Oades, 1975). Therefore, exogenous surface stimulation by a chemotactic factor such as f Met-Leu-Phe (Showell et al., 1976) together with the microfilament disruption caused by cytochalasin B cause degranulation by the cells.

In Chapter 3, data were presented which indicate that rH TNF-α influences the respiratory burst and chemotactic responsiveness of neutrophils. As the generation of superoxide anion is a reflection of the activation of neutrophil microbicidal activity, and the other major microbicidal mechanism of neutrophils is the release of lysosomal enzymes, the effect of rH TNF-α on neutrophil degranulation was investigated. Because regulation of the microfilaments is an integral aspect of degranulation, the influence of rH TNF-α on actin polymerisation was also investigated.

SECTION 6.2. RESULTS

6.2.1 rH TNF-α stimulates neutrophil degranulation both directly and in response to f Met-Leu-Phe:

In order to determine whether rH TNF-α could stimulate degranulation by neutrophils, cells were incubated in the presence or absence of 100 u/ml rH TNF-α for 60 minutes at 37°C. Following this, neutrophils were either incubated in the presence or absence of 10^{-7}M f Met-Leu-Phe for 15 minutes at 37°C. Figure 6.1 represents the cumulative data from 18 separate experiments. rH TNF-α induces a three-fold increase in lysosyme release over unstimulated neutrophils ($p<0.001$). In addition, when cells were incubated with rH TNF-α, then stimulated with f Met-Leu-Phe, degranulation was increased in an additive manner ($p<0.001$). These experiments indicated that rH TNF-α could stimulate neutrophil degranulation directly, and could alter the cell's response to f Met-Leu-Phe.

Throughout this series of experiments, it became evident that although a consistent response occurred, the magnitude of the response to both f Met-Leu-Phe and rH TNF-α varied quite considerably. Figure 6.2 is a scatter diagram representing 18 separate experiments. Although the magnitude of response to rH TNF-α varied, only one person out of the eighteen tested did not elicit a significant response ($p >0.05$). When the cells

FIGURE 6.1

rH TNF-α stimulates neutophil degranulation.

Neutrophils were incubated with or without 100 u/ml rH TNF-α for 60 minutes at 37°C, then incubated with or without 10^{-7}M f Met-Leu-Phe for 15 minutes at 37°C. Numbers represent the mean ± S.E.M. from 18 separate experiments where each point was determined in duplicate or triplicate.

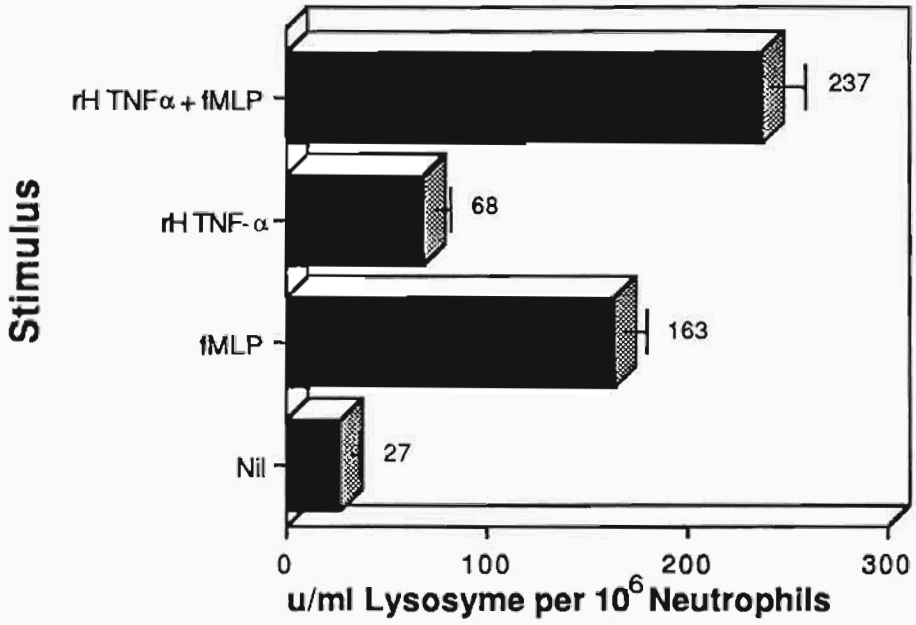
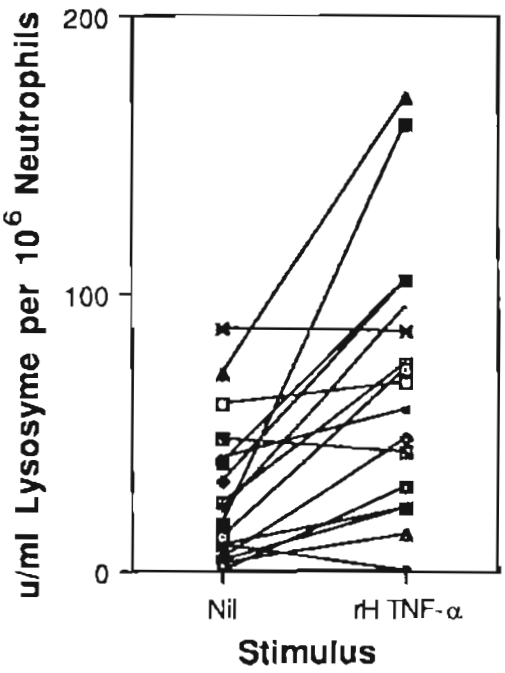
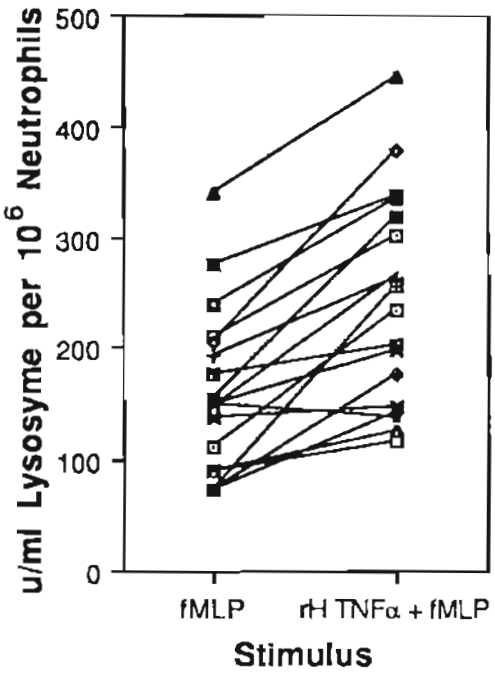

Stimulus
rH TNFα + fMLP
rH TNF-α
fMLP
Nil
237
68
163
27
0
100
200
300
u/ml Lysosyme per 10^6 Neutrophils

FIGURE 6.2

The response to rH TNF-α and f Met-Leu-Phe varies between donors.

Neutrophils were treated as described in Figure 6.1. Each symbol represents the mean of an individual experiment. The S.E.M. is calculated from the complete data set for 18 experiments.


u/ml Lysosyme per 10^6 Neutrophils
200
100
0
Nil
rH TNF-α
Stimulus


u/ml Lysosyme per 10^6 Neutrophils
500
400
300
200
100
0
fMLP
rH TNFα + fMLP
Stimulus

were incubated with rH TNF-α, then stimulated with f Met-Leu-Phe, all volunteers tested exhibited an enhanced response when compared to f Met-Leu-Phe alone.

To determine the optimal concentration of rH TNF-α required for this effect, neutrophils were preincubated with various concentrations of rH TNF-α for 60 minutes at 37°C, then stimulated with 10^{-7}M f Met-Leu-Phe for 15 minutes at 37°C. Figure 6.3 illustrates that rH TNF-α influences neutrophil degranulation to f Met-Leu-Phe in a dose-dependent manner. Although the dose of rH TNF-α required for maximal enhancement of degranulation varied among donors, 100 u/ml was the most consistent concentration of rH TNF-α required.

A titration of f Met-Leu-Phe in the presence or absence of rH TNF-α was performed to determine whether rH TNF-α enhanced degranulation at other f Met-Leu-Phe concentrations. Neutrophils were preincubated with 100 u/ml rH TNF-α for 60 minutes at 37°C. Following this, they were stimulated with various concentrations of f Met-Leu-Phe for 15 minutes at 37°C. Figure 6.4 illustrates that rH TNF-α increases degranulation to all concentrations of f Met-Leu-Phe in an additive manner. This effect was not due to cell leakage, as the supernatanats were simultaneously checked for lactate dehydrogenase and found to be virtually negative.

6.2.2 rH TNF-α upregulates cell surface CR3:

CR3 is the receptor for the complement fragment C3bi, and is present in the granule membrane of the neutrophil (Todd et al., 1984). When neutrophils degranulate, the upregulation of cell surface CR3 results from fusion of the granule membrane with the plasma membrane of the cell. This antigen can be detected by the binding of the monoclonal antibody anti Mo-1 (Todd et al., 1984). To determine whether rH TNF-α could upregulate CR3, cells were incubated with either 100u/ml rH TNF-α, or 10^{-7}M

FIGURE 6.3

rH TNF-α influences neutrophil degranulation to f Met-Leu-Phe in a dose-dependent manner.

Neutrophils were incubated with various concentrations of rH TNF-α for 60 minutes at 37°C, then stimulated with 10^{-7}M f Met-Leu-Phe. Numbers represent the mean ± S.E.M. from triplicate samples.

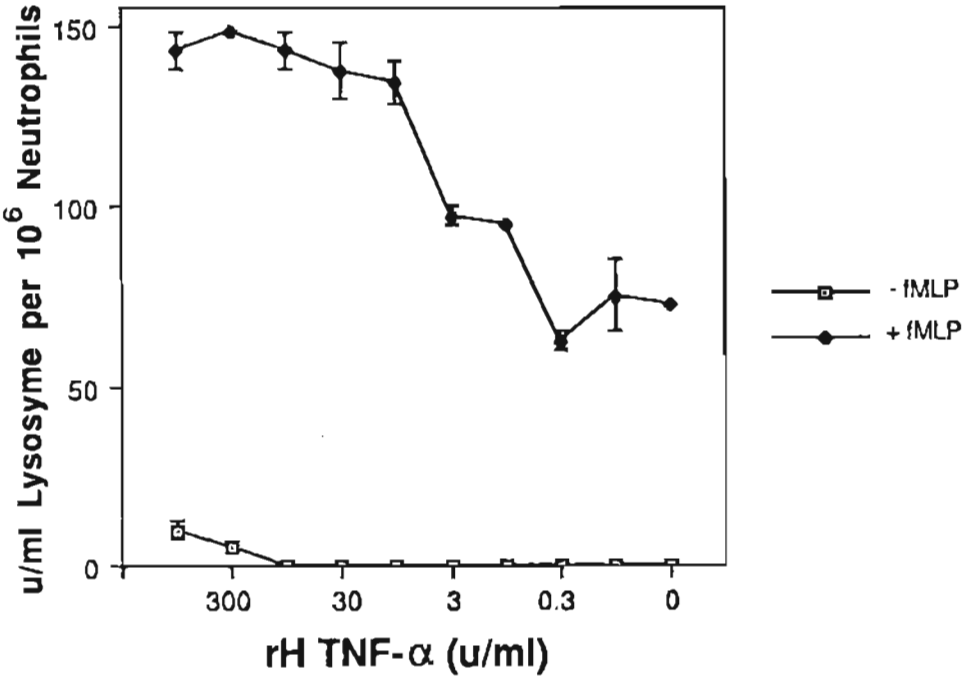
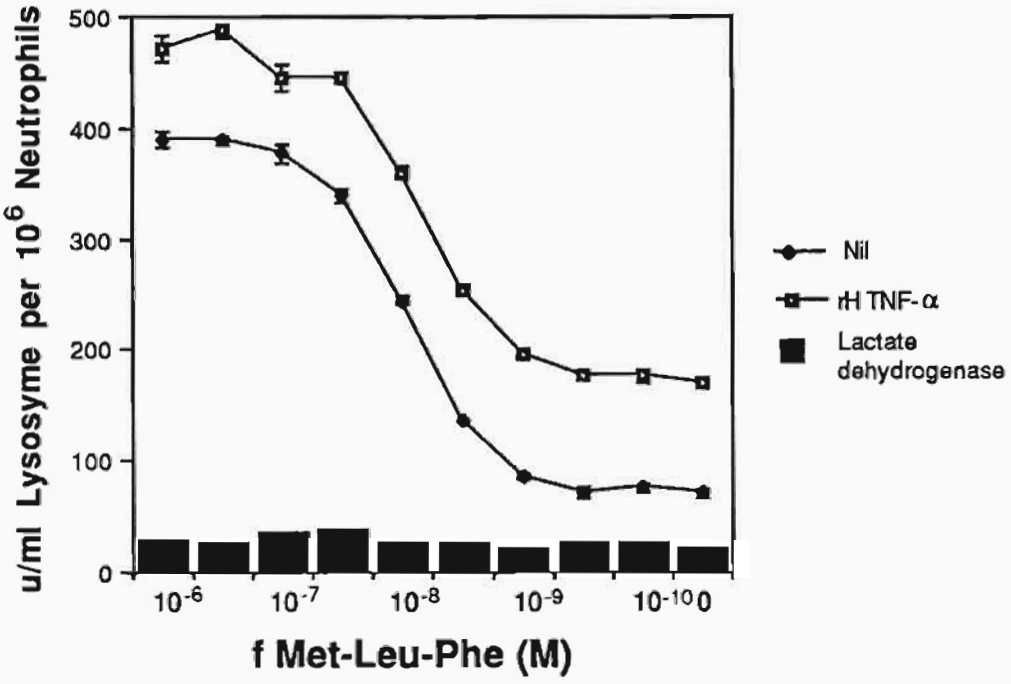

u/ml Lysosyme per 10^6 Neutrophils
150
100
50
0
300
30
3
0.3
0
rH TNF-α (u/ml)
- fMLP
+ fMLP

FIGURE 6.4

rH TNF-α increases the response to f Met-Leu-Phe in an additive manner.

Neutrophils were incubated 100 u/ml rH TNF-α for 60 minutes at 37°C, then stimulated with various concentrations of f Met-Leu-Phe for 15 minutes at 37°C. Numbers represent the mean ± S.E.M. of triplicates. Lactate dehydrogenase was assayed on aliquots from the same samples used for the lysosyme assay.


u/ml Lysosyme per 10^6 Neutrophils
500
400
300
200
100
0
10^{-6}
10^{-7}
10^{-8}
10^{-9}
10^{-10} 0
f Met-Leu-Phe (M)
Nil
rH TNF-α
Lactate dehydrogenase

f Met-Leu-Phe for 60 minutes at 37°C. Following this, they were stained with anti-Mo-1 and their fluorescence intensity determined by flow cytometry. Figure 6.5A represents the upregulation of CR3 by 10^{-7}M f Met-Leu-Phe. Over 6 experiments there was an average of 10 ± 2 mean log channel shifts over unstimulated neutrophils. When cells were incubated with rH TNF-α, there was an average of 6 ± 0.8 mean log channel shifts over six experiments (Figure 6.5B). These changes represent a 31% increase in CR3 expression by f Met-Leu-Phe and a 18% increase induced by rH TNF-α. As with lysosyme release, rH TNF-α can elicit a direct effect on CR3 expression, but is not as potent as f Met-Leu-Phe.

6.2.3 rH TNF-α directly stimulates actin polymerisation:

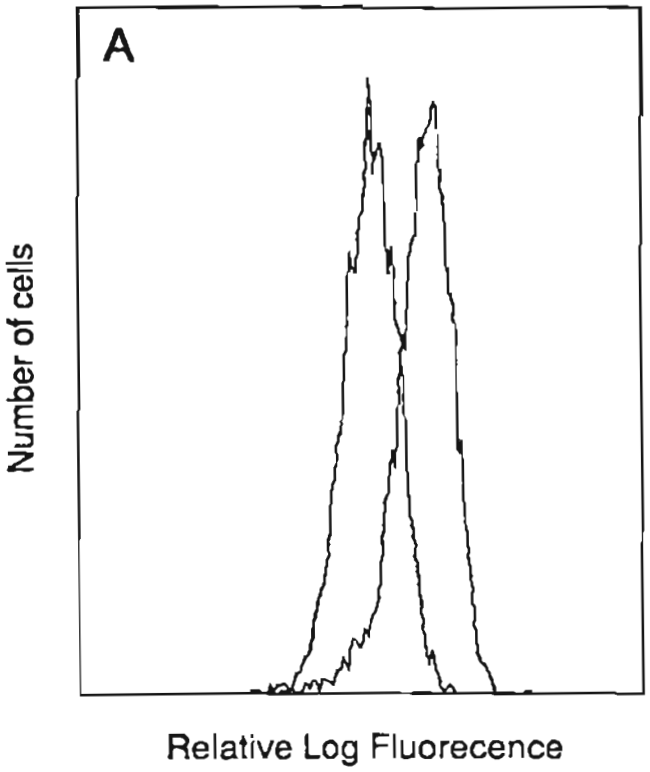
Neutrophil degranulation is dependent on extensive actin rearrangements within the neutrophil cytoskeleton. As rH TNF-α has been shown to influence neutrophil chemotaxis (see Chapter 3) and degranulation directly, and has previously been reported to induce the adherence of neutrophils (Gamble et al., 1985), it was important to determine whether rH TNF-α could influence the neutrophil cytoskeleton by studying actin polymerisation.

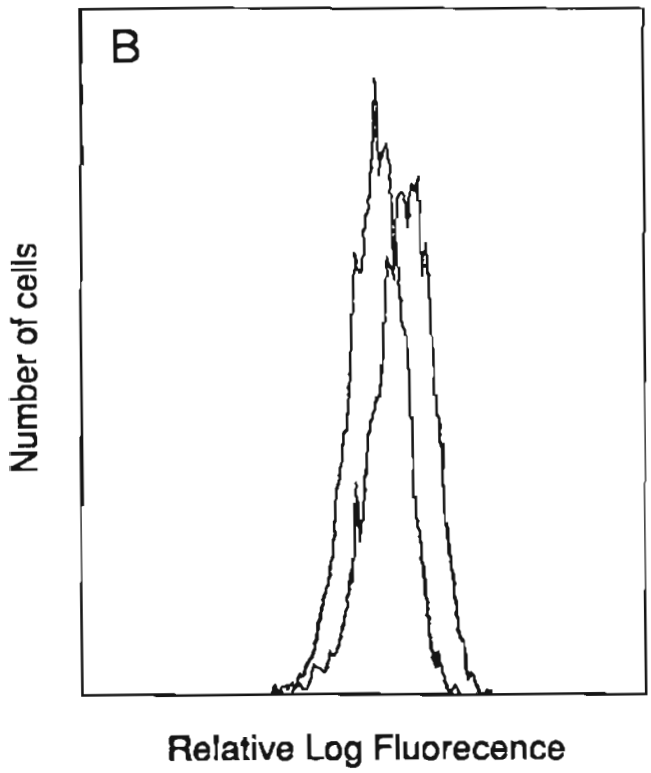
To determine whether rH TNF-α could directly stimulate actin polymerisation, it was first necessary to define the characteristics of the response with a known stimulus of actin polymerisation such as f Met-Leu-Phe. The cells were incubated with 10^{-7}M f Met-Leu-Phe for various times at 37°C, and the reaction stopped by fixing the cells in ice cold paraformaldehyde. As with other assays for neutrophil function, there was considerable variability in the response to f Met-Leu-Phe. Not only did the magnitude of the response to f Met-Leu-Phe vary between donors, but the basal actin content of the cells varied. For example, over ten experiments basal actin ranged from 40 to 65 mean log channels. The magnitude of the response varied from 30 to 90 mean log channel shifts over the baseline. This variability represents a two-fold change in basal actin, and more than a four-fold change in the magnitude of the response to f Met-Leu-Phe. In order to compare different

FIGURE 6.5

rH TNF-α upregulates surface expression of CR3 on neutrophils.

Neutrophils were incubated with either 100 u/ml rH TNF-α or 10^{-7}M f Met-Leu-Phe for 60 minutes at 37°C. They were then stained with fluorescently conjugated anti-Mo-1 and analysed by flow cytometry. The histrograms represent unstimulated *vs* f Met-Leu-Phe-stimulated neutrophils (A), and unstimulated *vs* rH TNF-α stimulated neutrophils (B).


A
Number of cells
Relative Log Fluorecence


B
Number of cells
Relative Log Fluorecence

donors, it was therefore necessary to express actin polymerisation as the shift in mean fluorescence intensity on a log scale over baseline.

Despite the variability between donors, the kinetics of f Met-Leu-Phe induced actin polymerisation were fairly consistent. Essentially, the response peaked between 10 and 30 seconds over ten experiments. In six out of ten experiments the response peaked at 10 seconds and returned to basal levels by 5 minutes. In the other four experiments, the response peaked at 20 (3 expts) or 30 (1 expt) seconds and returned to basal levels after 5 minutes. Figure 6.6 is representative of these ten experiments in which the kinetics of actin polymerisation to 10^{-7}M f Met-Leu-Phe was studied.

Considering that actin polymerisation in response to f Met-Leu-Phe varied in its return to basal levels, the kinetics of the response to rH TNF-α were studied over a 15 minute time span. Figures 6.7A and B are representative of three experiments where the kinetics of rH TNF-α-induced actin polymerisation was compared to f Met-Leu-Phe-induced actin polymerisation. Neutrophils stimulated with f Met-Leu-Phe exhibit a rapid polymerisation which peaks at approximately ten seconds after stimulation, followed by a prolonged depolymerisation. Filamentous actin returns to baseline approximately five to ten minutes after stimulation. Although the initial peak of the response was identical in rH TNF-α stimulated cells, the magnitude of the change in fluorescence intensity was always less than in f Met-Leu-Phe-stimulated cells. In addition, cells stimulated with rH TNF-α depolymerised much more rapidly, with filamentous actin returning to unstimulated levels by approximately two minutes. In all three experiments, the same difference in the kinetics of depolymerisation occurred for rH TNF-α-stimulated cells when compared to f Met-Leu-Phe-stimulated cells.

To determine the optimal concentration of rH TNF-α required for this effect, a titration was performed. Neutrophils were incubated with various concentrations of rH TNF-α or

FIGURE 6.6

f Met-Leu-Phe-induced actin polymerisation in neutrophils.

Neutrophils were stimulated with 10^{-7}M f Met-Leu-Phe for various times at 37°C. The reaction was terminated by fixation in ice-cold paraformaldehyde, then the cells were stained with NBD-phallacidin and analysed by flow cytometry. This figure is representative of ten experiments where each point was determined in duplicate.

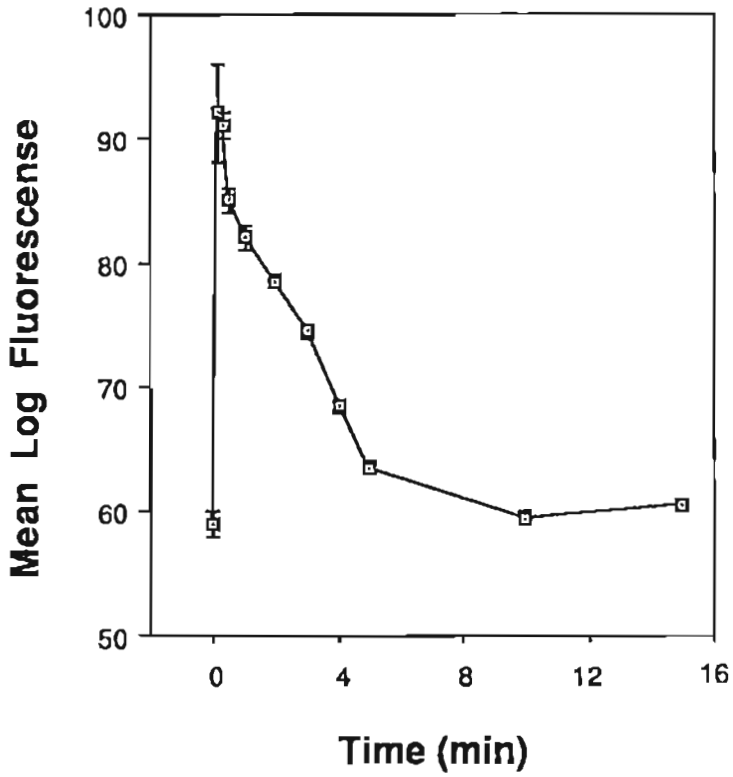
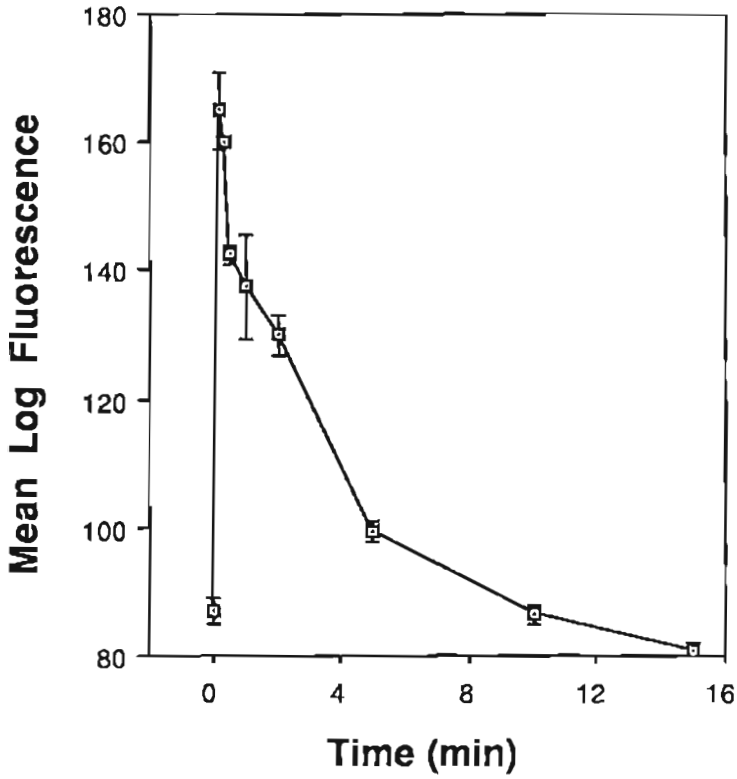
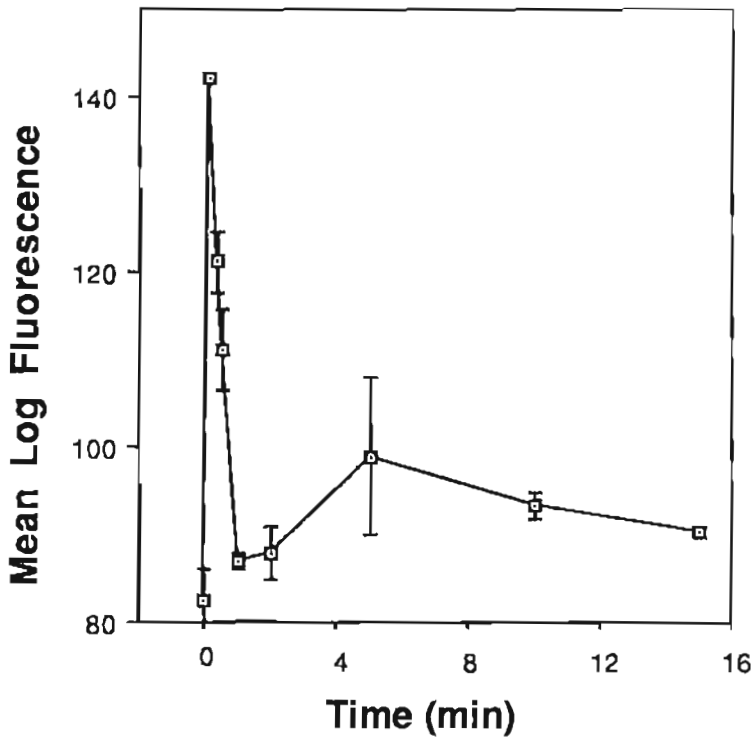

Mean Log Fluorescense
100
90
80
70
60
50
0
4
8
12
16
Time (min)

FIGURE 6.7

Kinetics of actin polymerisation in reponse to f Met-Leu-Phe and rH TNF-α.

Neutrophils were incubated with 10^{-7}M f Met-Leu-Phe (top) or 100 u/ml rH TNF-α (bottom) for various times at 37°C. The reaction was terminated by fixation, then the cells stained and analysed by flow cytometry. This experiment is representative of 3 experiments where each point was determined in duplicate.


Mean Log Fluorescence
180
160
140
120
100
80
0
4
8
12
16
Time (min)


Mean Log Fluorescence
140
120
100
80
0
4
8
12
16
Time (min)

10^{-7}M f Met-Leu-Phe for 10 seconds at 37°C. Figure 6.8 illustrates the direct effect of rH TNF-α on actin polymerisation. Over three separate experiments, 1000u/ml rH TNF-α elicited the maximal effect, and no plateau was reached at the higher rH TNF-α concentrations. Although 100u/ml to 1000u/ml rH TNF-α elicited a significant effect on actin polymerisation ($p<0.001$ and $p<0.01$, respectively) the magnitude of the response was never as high as the response to 10^{-7}M f Met-Leu-Phe.

To determine whether rH TNF-α changed the distribution of cellular F-actin, cells were stimulated in the presence or absence of 1000 u/ml rH TNF-α or 10^{-7}M f Met-Leu-Phe for 10 seconds at 37°C, then fixed and stained with NBD-phallacidin. Following this, they were placed on glass slides and photographed using a fluorescent microscope. Figure 6.9 illustrates the actin distribution in neutrophils. In unstimulated neutrophils, the actin is diffuse and distributed evenly across the cell. When neutrophils are stimulated with either rH TNF-α or f Met-Leu-Phe, a large amount of polymerisation occurs in the cortical actin network just below the plasma membrane. This pattern of actin polymerisation appears to be identical for both rH TNF-α and f Met-Leu-Phe.

6.2.4. rH TNF-α enhances actin polymerisation in response to f Met-Leu-Phe:

As rH TNF-α also alters neutrophil chemotaxis (see Chapter 3) and degranulation (this Chapter) in response to f Met-Leu-Phe, the possibility that it could also influence actin polymerisation in response to f Met-Leu-Phe was investigated.

To determine the effect of rH TNF-α on the response to f Met-Leu-Phe, the cells were preincubated with 100u/ml rH TNF-α for 30 minutes at 37°C, then stimulated with 10^{-7}M f Met-Leu-Phe for 10 seconds at 37°C. Figure 6.10 shows the cumulative data from 6 separate experiments where each point was determined in duplicate. As expected, cells

FIGURE 6.8

Dose-dependence of rH TNF-α-induced actin polymerisation.

Neutrophils were incubated with various concentrations of rH TNF-α or 10^{-7}M f Met-Leu-Phe for 10 seconds at 37°C. The reaction was terminated by fixation, then the cells were stained and analysed by flow cytometry. Numbers represent the mean ± S.E.M. from 3 separate experiments where each point was determined in duplicate. Values are expressed as % of the mean channel shift induced by 10^{-7}M f Met-Leu-Phe.

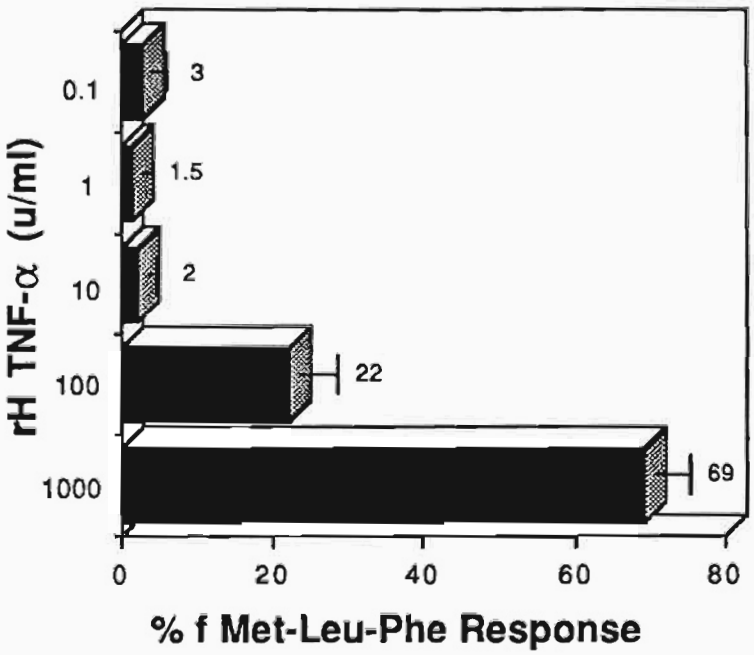
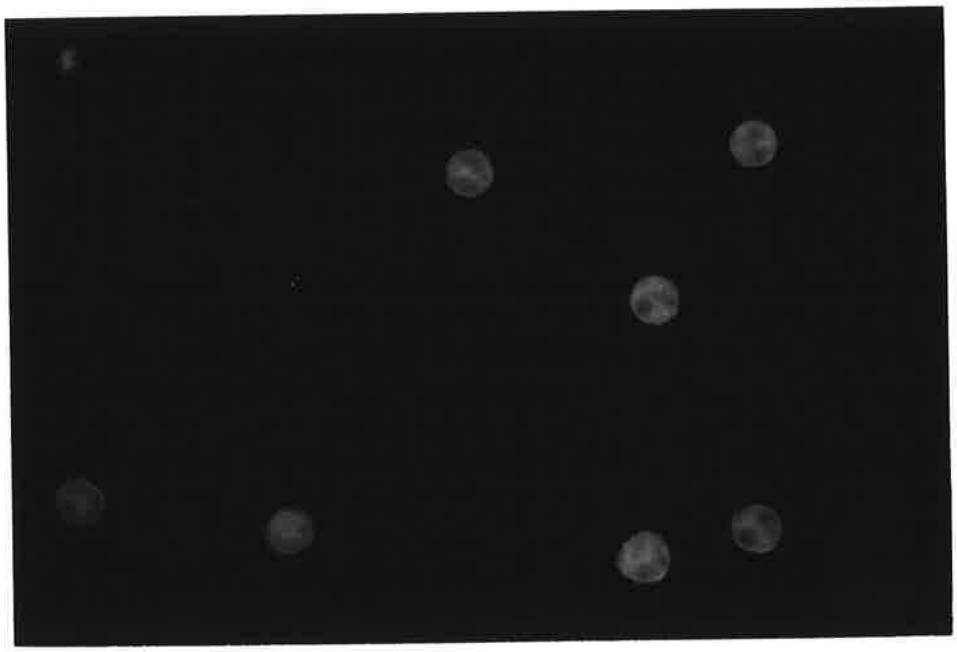
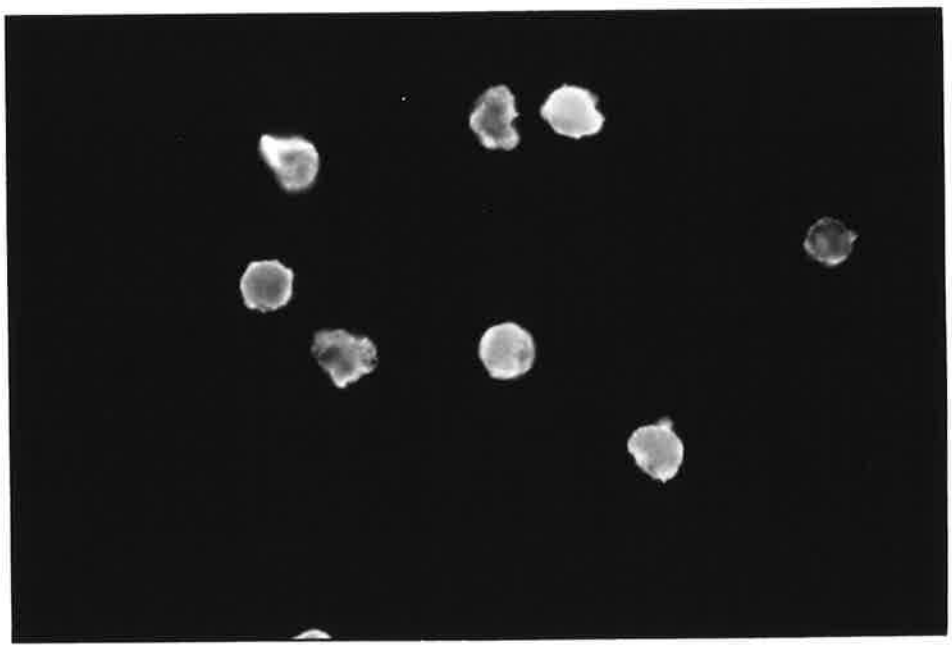

rH TNF-α (u/ml)
0.1
1
10
100
1000
3
1.5
2
22
69
0
20
40
60
80
% f Met-Leu-Phe Response

FIGURE 6.9

rH TNF-α induces the same pattern of actin polymerisation as f Met-Leu-Phe.

Neutrophils were incubated in medium (top) or with 1000u/ml rH TNF-α (centre) or 10^{-7}M f Met-Leu-Phe (bottom) for 10 seconds at 37°C. The cells were fixed, stained then placed on glass slides for visualisation with a fluorescent microscope. All photographic exposures are for the same amount of time, therefore fluorescence is comparable.





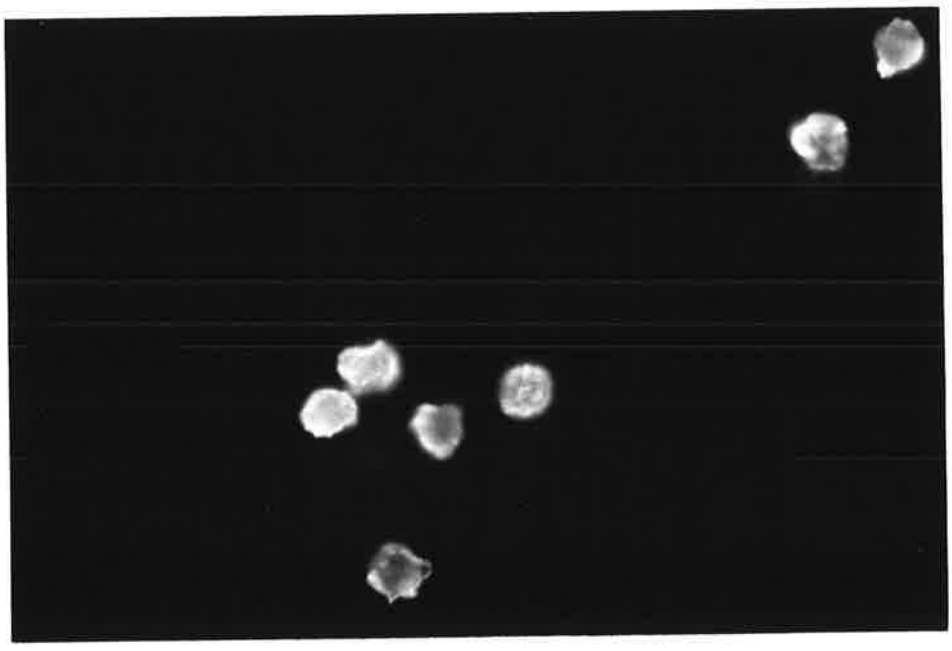
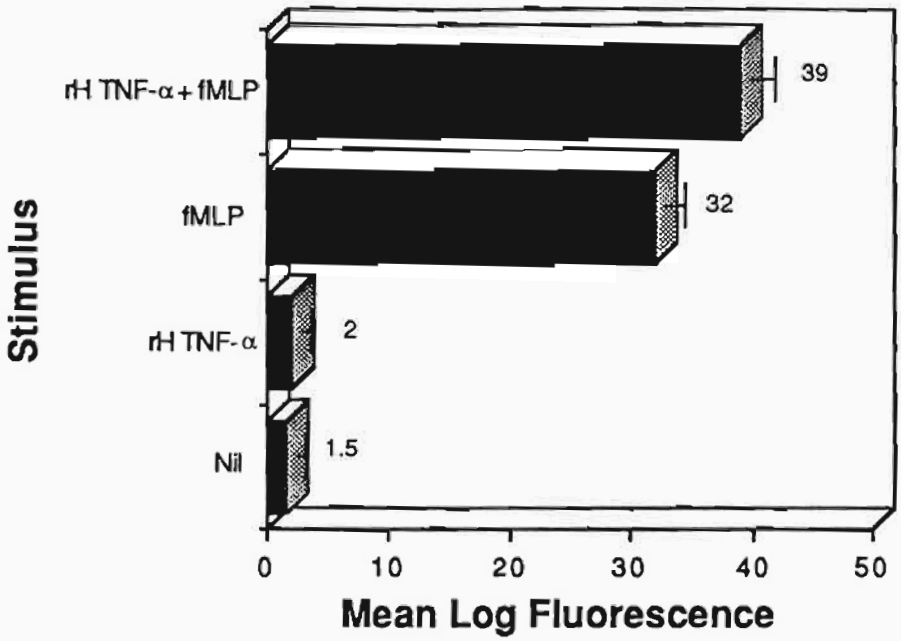


FIGURE 6.10

rH THF-α increases actin polymerisation in response to f Met-Leu-Phe.

Neutrophils were incubated with 100 u/ml rH TNF-α for 30 minutes at 37°C, then stimulated with 10^{-7}M f Met-Leu-Phe for 10 seconds at 37°C. Reactions were terminated by fixation, cells were stained then analysed by flow cytometry. Numbers represent the mean ± S.E.M. from 6 separate experiments where each point was determined in duplicate.


rH TNF-α + fMLP
fMLP
rH TNF-α
Nil
39
32
2
1.5
Stimulus
0
10
20
30
40
50
Mean Log Fluorescence

incubated with rH TNF-α for 30 minutes showed no increase in cellular actin ($p>0.5$), because complete depolymerisation would have already occurred (see Figure 6.7). When cells were incubated with rH TNF-α, then stimulated with f Met-Leu-Phe, actin polymerisation was enhanced slightly. This effect was significant over 6 experiments ($p<0.05$).

As it was observed that preincubation with rH TNF-α slightly enhanced the response to 10^{-7}M f Met-Leu-Phe, a titration was performed to determine whether rH TNF-α influences actin polymerisation in response to lower doses of f Met-Leu-Phe. It was first necessary to characterize the response to various concentration of f Met-Leu-Phe.

Figure 6.11A represents the response to various f Met-Leu-Phe concentrations from 8 separate experiments where each point was determined in duplicate. It appears that 10^{-7} and 10^{-8}M f Met-Leu-Phe are optimal concentrations for actin polymerization. To investigate the effect of rH TNF-α, the cells were preincubated with 100 u/ml rH TNF-α for 30 minutes at 37°C, then stimulated with various concentrations of f Met-Leu-Phe for 10 seconds at 37°C. Figure 6.11B illustrates cumulative data from seven separate experiments where each point was determined in duplicate. Thus, rH TNF-α significantly enhanced actin polymerisation in response to f Met-Leu-Phe by 10^{-8}M and 10^{-9}M ($p<0.05$), but this effect was not seen at lower concentrations.

Since rH TNF-α had a direct effect on actin polymerisation at 100u/ml and 1000u/ml, it was important to determine whether such high concentrations were also required for this enhancing effect. Neutrophils were preincubated with various concentrations of rH TNF-α for 30 minutes at 37°C, then stimulated with 10^{-9}M f Met-Leu-Phe for 10 seconds. Figure 6.12 represents a titration of rH TNF-α from three separate experiments where each point was determined in duplicate. One thousand units per ml rH TNF-α had an inhibitory effect on f Met-Leu-Phe induced actin polymerisation, whereas at lower doses the

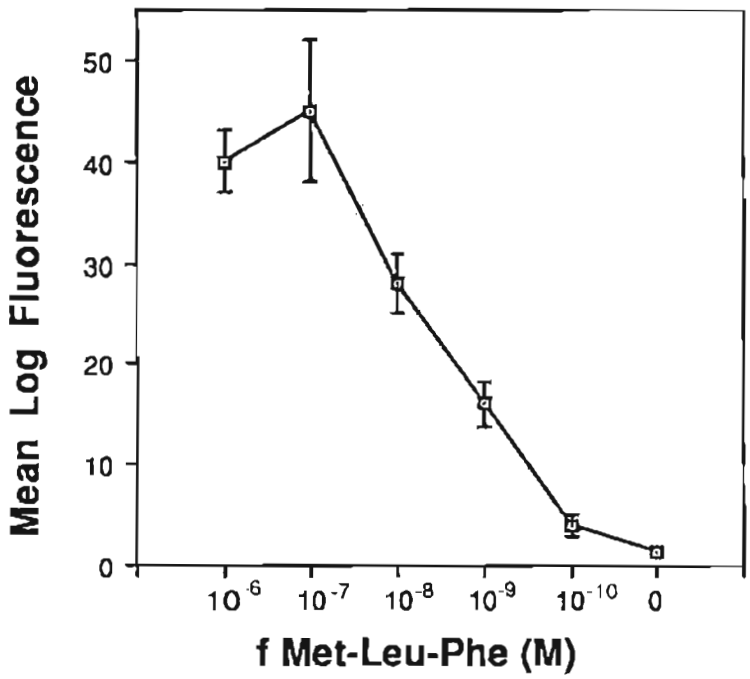
FIGURE 6.11

a) Dose-dependence of f Met-Leu-Phe-induced actin polymerisation.

Neutrophils were incubated with various concentrations of f Met-Leu-Phe for 10 seconds at 37°C. Reactions were terminated by fixation, cells were stained and analysed by flow cytometry. Numbers represent the mean ± S.E.M. from 8 separate experiments where each point was determined in duplicate

b) rH TNF-α enhances actin polymerisation in response to 10^{-8} and 10^{-9}M f Met-Leu-Phe.

Neutrophils were incubated 100 u/ml rH TNF-α for 30 minutes at 37°C, then stimulated with various concentrations of f Met-Leu-Phe for 10 seconds at 37°C. Reactions were terminated by fixation, cells stained and analysed by flow cytometry. Numbers represent the mean ± S.E.M. from seven separate experiments where each point was determined in duplicate.


Mean Log Fluorescence
50
40
30
20
10
0
10^{-6}
10^{-7}
10^{-8}
10^{-9}
10^{-10}
0
f Met-Leu-Phe (M)

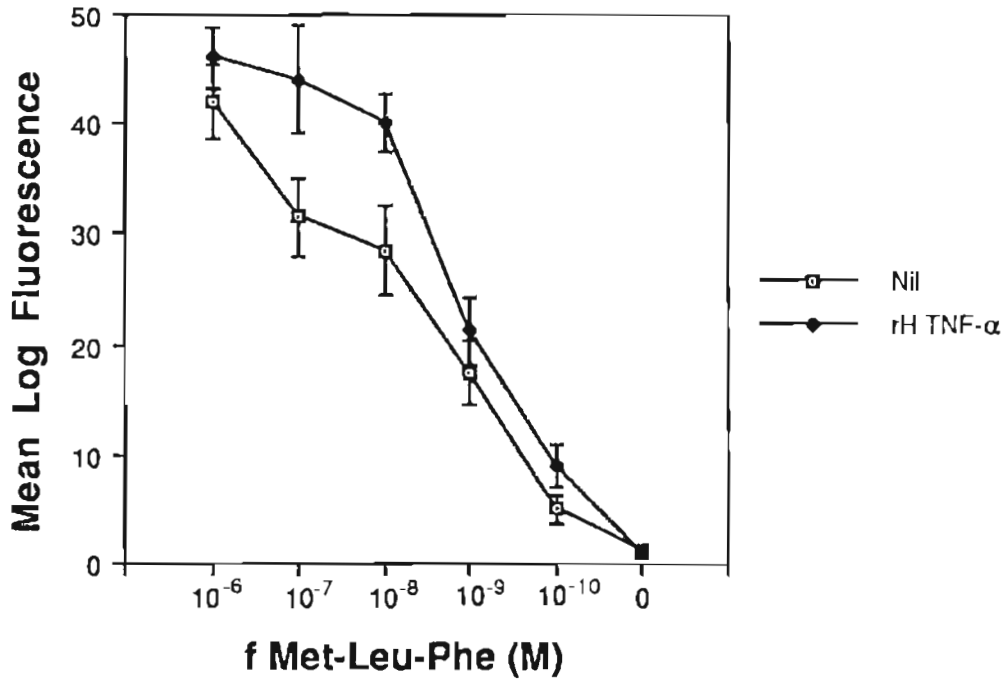
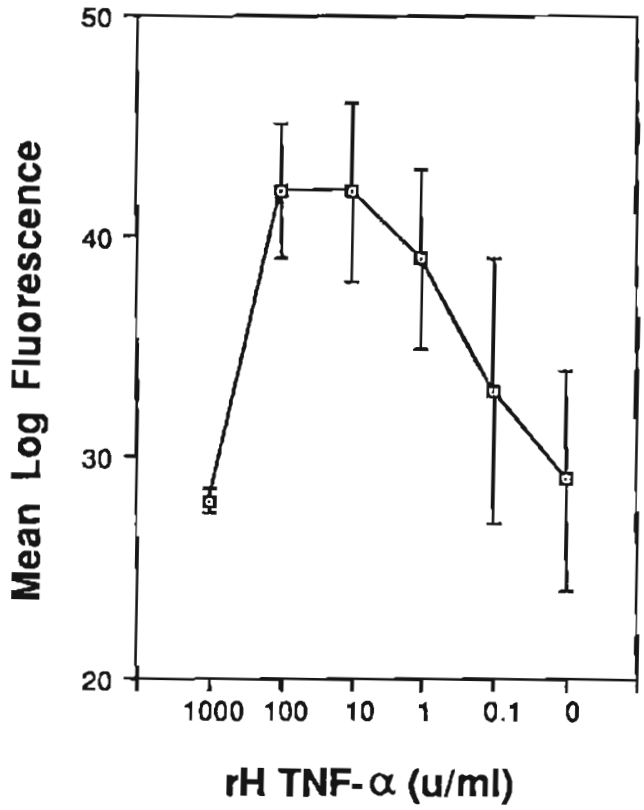

Mean Log Fluorescence
50
40
30
20
10
0
10^{-6}
10^{-7}
10^{-8}
10^{-9}
10^{-10}
0
f Met-Leu-Phe (M)
Nil
rH TNF-α

FIGURE 6.12

rH TNF-α enhances f Met-Leu-Phe-induced actin polymerisation in a dose-dependent manner.

Neutrophils were incubated with various concentrations of rH TNF-α for 30 minutes at 37°C then stimulated with 10^{-9}M f Met-Leu-Phe for 10 seconds at 37°C. Reactions were terminated by fixation, cells stained and analysed by flow cytometry. Numbers represent the mean ± S.E.M. from 3 separate experiments where each point was determined in duplicate.


50
40
30
20
Mean Log Fluorescence
1000
100
10
1
0.1
0
rH TNF-α (u/ml)

enhancing effect was evident. It appears that 10 to 100u/ml rH TNF-α is the optimal concentration required to enhance f Met-Leu-Phe induced actin polymerisation. This represents a ten-fold lower concentration of rH TNF-α than that which is required to directly induce actin polymerisation.

SECTION 6.3. DISCUSSION

In this chapter, data implicating rH TNF-α as a direct stimulant of neutrophil degranulation and actin polymerisation is presented. In addition, it is shown that rH TNF-α enhanced the response of neutrophils to the chemotactic factor, f Met-Leu-Phe.

The data presented here is in agreement with a previous report by Klebanoff et al. (1986) indicating that rH TNF-α directly stimulates neutrophil degranulation. Following a 60 minute incubation with rH TNF-α , neutrophils released significant amounts of lysosyme, but not myeloperoxidase and β-glucuronidase. One can therefore conclude that rH TNF-α induces the release of specific but not azurophilic granules from neutrophils. In contrast, Berkow et al. (1987) reported that rH TNF-α had no direct effect on neutrophil degranulation. This discrepancy may simply be due to the fact that they only incubated their cells with rH TNF-α for 25 minutes.

The observation that rH TNF-α can directly stimulate actin polymerisation is consistent with its ability to induce degranulation, considering that agents such as f Met-Leu-Phe also stimulate both functions. Although the exact relationship between actin polymerisation and degranulation has yet to be defined, Southwick and Stossel (1983) have reviewed the possible role of actin polymerisation in various neutrophil functions. Disruption of the cortical actin network, coupled to stimulation at the plasma membrane, are both required to induce degranulation. Their model of degranulation is as follows: a localized increase in calcium results from the binding of a ligand to its specific receptor; the local rise in

calcium activates gelsolin, causing disaggregation of the actin network, and movement of the network from the area of less structure (higher calcium concentration) to an area of higher structure (lower calcium concentration); these movements allow the granules to approach the plasma membrane in the areas of actin network disruption, resulting in degranulation. Therefore, the binding of rH TNF-α to its receptor, and the subsequent disruption of the cortical actin network appears to be sufficient to cause the neutrophil to degranulate. Evidence to support this theory can be seen in Figure 6.9 where the effect of rH TNF-α on actin polymerisation appears to be confined to the cortical actin network.

Another interesting aspect of these data involves the characteristics of the effect on various f Met-Leu-Phe concentrations. For degranulation, rH TNF-α has an additive effect on f Met-Leu-Phe responses at all concentrations tested, whereas for actin polymerisation, the effect is not additive, and is only evident at 10^{-8}M and 10^{-9}M f Met-Leu-Phe. This evidence implies that although rH TNF-α does alter actin polymerisation in response to f Met-Leu-Phe, this effect is not necessarily the sole cause for the observed change in degranulation. In fact, the additive effect of rH TNF-α on degranulation implies that its influence is independent of the f Met-Leu-Phe response. In contrast, the synergistic effect of rH TNF-α on actin polymerisation suggests some form of regulation of the f Met-Leu-Phe response by rH TNF-α.

The initial phase of actin polymerisation induced by rH TNF-α is identical to that induced by f Met-Leu-Phe. This raises an interesting point regarding signal transduction mechanisms, because the initiation of actin polymerisation appears to be independent of changes in the levels of intracellular calcium. A report by Sklar, Omann and Painter (1985), who studied actin polymerisation of quin 2-loaded neutrophils in calcium-depleted buffer, and in buffer with normal calcium, indicates that actin polymerisation occurred at similar rates in both buffers.

The depolymerisation phase of rH TNF-α-induced actin polymerisation was much more rapid than in f Met-Leu-Phe-stimulated cells. Depolymerisation was virtually complete by two minutes in rH TNF-α-stimulated cells, whereas in f Met-Leu-Phe-stimulated cells, depolymerisation was only complete after five to ten minutes. This sustained polymerisation is dependent on intracellular free calcium (Sklar, Omann and Painter, 1985). Therefore, it appears unlikely that rH TNF-α induces as large a change in the intracellular free calcium concentration as does f Met-Leu-Phe, because cells stimulated with rH TNF-α depolymerise much more rapidly.

It is likely that a GTP-binding protein is involved in coupling the TNF receptor to its signal transduction pathway, as GTP analogues stimulate the exocytosis of granules from neutrophils (Burgoyne, 1987). This occurs even in the absence of calcium and phospholipase C activation. Signal transduction mechanisms for the polymerisation of actin are still unidentified within neutrophils. Although rH TNF-α induces an increase in the oscillations of calcium fluxes across neutrophil membranes, it does not induce a major flux of calcium within neutrophils (J.M. Dayer, personal communication; Y.H. Atkinson, J.Crofts, G. Barritt, and A.F. Lopez, unpublished observations), or cause the translocation of protein kinase C (Lopez, et al., unpublished observations). It is also unlikely that an intracellular rise in pH is the initiating event, because a recent report by Sullivan et al. (1987) states that rH GM-CSF - a cytokine with similar activities to rH TNF-α - does not alter intracellular pH. Another possibility involves changes in the lipid composition of the membrane. Two recent reports discuss the influence of phospholipids on the cytoskeleton. Janmey and Stossel (1987) studied the modulation of gelsolin function by phosphatidylinositol 4,5-bisphosphate, and concluded that this phospholipid could promote the nucleated assembly of actin. Similarly, another study by Janmey et al. (1987) implicates phosphatidylinositol monophosphate in the regulation of gelsolin activity. Since rH TNF-α influences actin polymerisation, its effect on phospholipid metabolism will be investigated in the next chapter.

CHAPTER 7

HUMAN TUMOR NECROSIS FACTOR ALPHA DIRECTLY STIMULATES PHOSPHOLIPID METABOLISM ON HUMAN NEUTROPHILS

SECTION 7.1 INTRODUCTION

In the preceding chapters rH TNF-α has been shown to prime neutrophils by modulating their response to an activating agent. Thus rH TNF-α increases the phagocytosis and degranulation of neutrophils in response to both opsonized and unopsonized zymosan (Klebanoff et al., 1986), and enhances the respiratory burst and inhibits the chemotactic responsiveness of neutrophils to f Met-Leu-Phe (Atkinson et al., 1988b). Although these enhancing effects of rH TNF-α are well documented, little is known about direct effects of rH TNF-α that may help explain its mechanism of action.

Since the regulation of membrane receptors and adherence by rH TNF-α all require changes in membrane properties, it was hypothesized that rH TNF-α may directly alter the phospholipid turnover of neutrophils. In this chapter it is shown that rH TNF-α induces the release of significant amounts of arachidonic acid from neutrophils but does not stimulate the metabolism of this fatty acid. The stimulation of arachidonic acid release from neutrophils by rH TNF-α is shown to be equipotent to that induced by f Met-Leu-Phe. The stimulation of phospholipid turnover in neutrophils may play a role in the regulatory effects of rH TNF-α on neutrophil functions.

SECTION 7.2 RESULTS

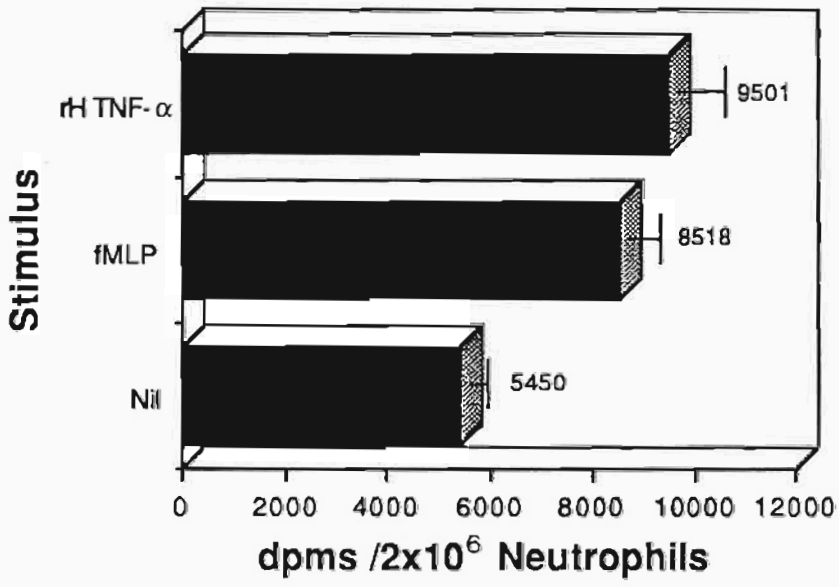
7.2.1 rH TNF-α stimulates [^{3}H]-arachidonic acid release from neutrophils:

Neutrophils were labelled with [^{3}H]-arachidonic acid, washed and then incubated for 45 minutes at 37^{o}C in the presence or absence of either 100u/ml rH TNF-α or 10^{-7}M f Met-Leu-Phe. Additional aliquots of cells were also incubated with 4μM A23187 for 15 minutes as a positive control. Figure 7.1 shows that rH TNF-α stimulated the release of

FIGURE 7.1

rH TNF-α induces arachidonic acid release from neutrophils.

[^{3}H]-arachidonic acid labelled-neutrophils ($2x10^6$) were incubated with medium, 10^{-7}M f Met-Leu-Phe or 100u/ml rH TNF-α for 45 minutes at 37°C. Results are shown as the mean ± S.E.M. of 16 experiments performed in triplicate. The values obtained in the presence of rH TNF-α were significantly different ($p<0.001$) from unstimulated controls.


rH TNF-α
fMLP
Nil
9501
8518
5450
Stimulus
0
2000
4000
6000
8000
10000
12000
dpms /2x10^6 Neutrophils

radioactive label from neutrophils prelabelled with [^{3}H]-arachidonic acid. In all experiments the release of radioactive label from the neutrophils was significantly increased over unstimulated cell turnover in the presence of rH TNF-α ($p<0.001$). This effect was as potent as that seen by 10^{-7}M f Met-Leu-Phe, a dose known to be optimal for the release of superoxide anion. The amount of radioactivity released by cells in response to rH TNF-α or f Met-Leu-Phe represents 13% and 11.5% respectively of the amount released in response to the calcium ionophore A23187.

7.2.2 The stimulation of [^{3}H]-arachidonic acid release by rh TNF-α is time and dose dependent:

Time course experiments were performed by incubating [^{3}H]-arachidonic acid-labelled neutrophils for various times in the presence or absence of 100 u/ml rH TNF-α or 10^{-7}M f Met-Leu-Phe. It was found that the time of maximum stimulated release of label varied between donors, with the majority of donors showing a maximal response at 45 minutes. Figure 7.2 shows a composite time course of the release of radioactive label from [^{3}H]-arachidonic acid labelled neutrophils from 20 separate experiments. In individual experiments the response either reached a plateau or dropped away following peak release of the label. This presumably reflected the reincorporation of the label into cellular lipids. The kinetics of rH TNF-α stimulated release were similar to the kinetics of f Met-Leu-Phe stimulated release of radioactive label.

A titration of rH TNF-α was performed to determine the optimal concentration for the release of radioactive label. Following labelling, the cells were washed and incubated with various concentrations of rH TNF-α for 30, 45 or 60 minutes. From 5 experiments it was evident that 100 u/ml rH TNF-α was consistently required for a maximum stimulatory effect on the release of label from [^{3}H]-arachidonic acid-labelled neutrophils (Figure 7.3)0

FIGURE 7.2

Time-dependent stimulation of arachidonic acid release.

[^{3}H]-arachidonic acid labelled neutrophils were incubated with medium, 10^{-7}M f Met-Leu-Phe or 100u/ml rH TNF-α for different times at 37°C. Results are shown as the mean ± S.E.M. of 20 experiments performed in triplicate. The values obtained with rH TNF-α were significantly different from unstimulated control values at 30 minutes ($p<0.005$) and 45 minutes ($p<0.001$).

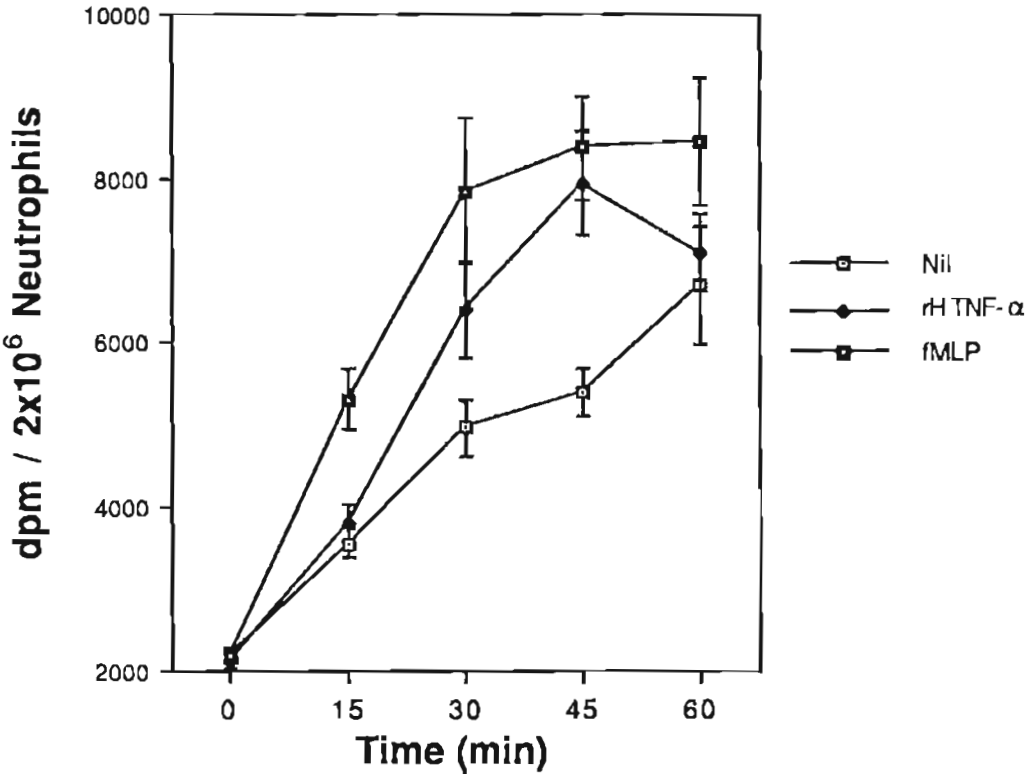
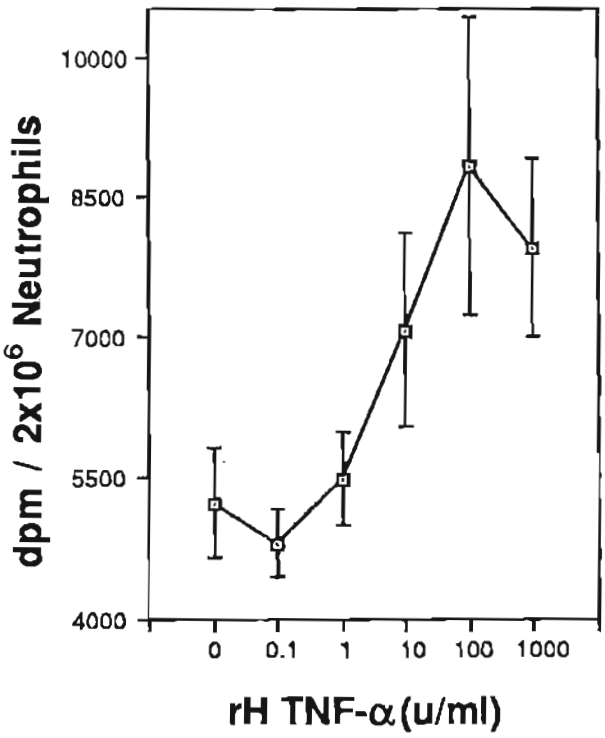

10000
8000
6000
4000
2000
dpm / 2x10^6 Neutrophils
0
15
30
45
60
Time (min)
Nil
rH TNF-α
fMLP

FIGURE 7.3

Dose-dependent stimulation of arachidonic acid release from neutrophils.

[^{3}H]-arachidonic acid labelled neutrophils were incubated with different concentrations of rH TNF-α for 45 minutes at 37^{o}C. Results are shown as the mean $\pm$ S.E.M. from 5 different experiments performed in triplicate.


10000
8500
7000
5500
4000
dpm / 2x10^6 Neutrophils
0
0.1
1
10
100
1000
rH TNF-α(u/ml)

Control experiments were performed to establish that rH TNF-α-stimulated turnover of [^{3}H]-arachidonic acid in neutrophils was not due to changes in membrane permeability with subsequent leakage of [^{3}H]-arachidonic acid not incorporated into cellular lipids. Neutrophils were labelled with arachidonic acid, the total lipids extracted and fractionated by thin layer chromatography, and radioactivity quantified by liquid scintillation counting. The levels of free [^{3}H]-arachidonic acid in the cells was monitored for up to 90 minutes following the labelling of the cells. This time span corresponds to the maximum incubation period covered in the[^{3}H]-arachidonic acid release assay. The intracellular free arachidonic acid never exceeded 2% of the total amount incorporated during the second incubation period (Table 1). Although rH TNF-α stimulates a range of responses, these were always greater than 2% release (2.25% - 7.7%), this indicates that the effect of rH TNF-α was to stimulate a small but significant turnover of arachidonic acid from cellular lipids, and was not due to cell leakage of free label.

TABLE I

Free [^{3}H]-arachidonic acid levels in neutrophils remain constant for up to 90 minutes incubation at 37°C.

TIME AFTER LOADING (MIN)*	% TOTAL LIPID LABELLED
0	1.75†
15	1.73
30	1.73
60	1.85
90	1.54

* Neutrophils were labelled with [^{3}H]-arachidonic acid at 37°C, then washed and incubated at 37°C for the times indicated above. Lipids were then extracted and analysed by thin-layer chromatography.

† Numbers represent % of the total amount of radioactivity incorporated into cellular lipids.

7.2.3 The stimulation of [^{3}H]-arachidonic acid release correlates with the ability to prime the neutrophil respiratory burst:

To determine whether the stimulation of [^{3}H]-arachidonic acid release from neutrophils by rH TNF-α could be correlated with the ability to prime neutrophil responses, two other cytokines were studied, recombinant human granulocyte-macrophage colony-stimulating factor (rH GM-CSF) and recombinant human interleukin-1β(rH IL-1β) both of which are known to bind to neutrophils (Rhyne et al., 1988; Gasson et al., 1986). Figure 7.4A shows that rH GM-CSF, like rH TNF-α stimulated a similar amount of [^{3}H]-arachidonic acid release from neutrophils whereas rH IL-1β did not. This effect was consistent at 30, 45 and 60 minutes after stimulation (data not shown). Similarly, Figure 7.4B shows that rH GM-CSF was able to prime neutrophil superoxide production in response to f Met-Leu-Phe, but that rH IL-1β had no effect.

7.2.4 rH TNF-α stimulates the release of [^{3}H]-arachidonic acid but not its metabolites:

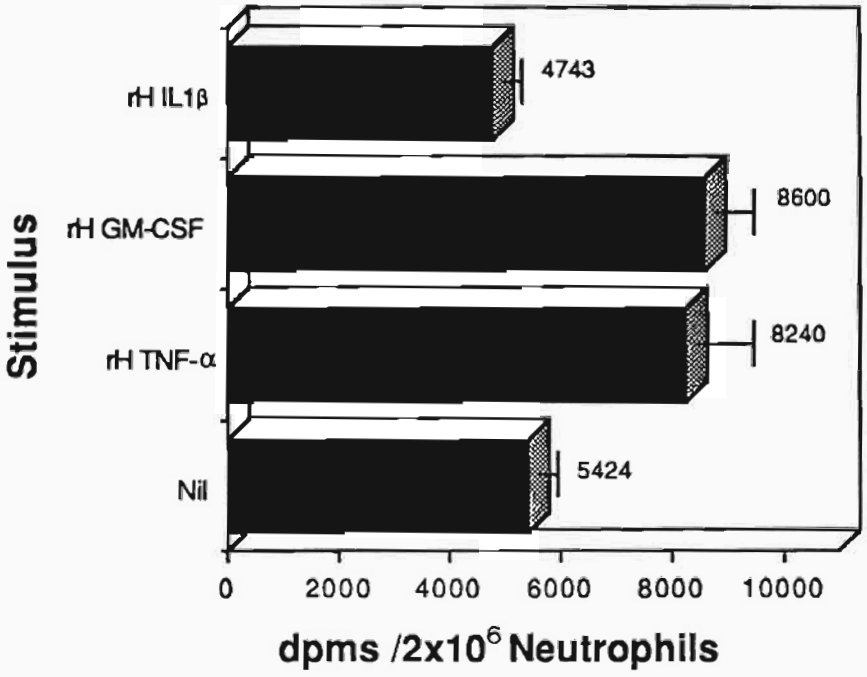
To determine whether the radioactivity released from [^{3}H]-arachidonic acid-labelled neutrophils was associated with free arachidonic acid or its metabolites, the supernatants from unstimulated or stimulated neutrophils were analysed by HPLC. Figure 7.5 shows the HPLC profiles from unstimulated neutrophils, A23187-stimulated neutrophils, and rH TNF-α-stimulated neutrophils. As expected, both arachidonic acid and its metabolites - the leukotrienes and the hydroxyeicosatetranoic acids (HETES) - were present in A23187-stimulated neutrophil supernatants. In contrast, although rH TNF-α induced the release of arachidonic acid from neutrophils, it did not stimulate the metabolism of this fatty acid. rH TNF-α did induce the release of small amounts of leukotriene C_4 (LTC_4), which was probably due to contamination with 5% eosinophils. These data indicate that although rH TNF-α can stimulate the release of arachidonic acid from neutrophils, it is unable to

FIGURE 7.4

The stimulation of [^{3}H]-arachidonic acid release correlates with the ability to prime superoxide anion production in response to f Met-Leu-Phe.

(Top) [^{3}H]-arachidonic acid labelled neutrophils were incubated with medium, 100u/ml rH TNF-α, 100ng/ml rH GM-CSF or 100u/ml rH IL-1β for 45 minutes at 37°C. Numbers represent the mean dpms from the supernatants of 2 x 10^6 neutrophils ± S.E.M.

(Bottom) Neutrophils were incubated with medium, 100u/ml rH TNF-α, 100ng/ml rH GM-CSF or 100u/ml rH IL-1β for 45 minutes at 37°C. Following this, they were stimulated with 10^{-7}M f Met-Leu-Phe for 15 minutes at 37°C. Numbers represent nmoles $O_2^-/10^6$ neutrophils as the mean of 6 determinations from 2 separate experiments ± S.E.M.


Stimulus
rH IL1β
rH GM-CSF
rH TNF-α
Nil
4743
8600
8240
5424
0
2000
4000
6000
8000
10000
dpms /2x10^6 Neutrophils

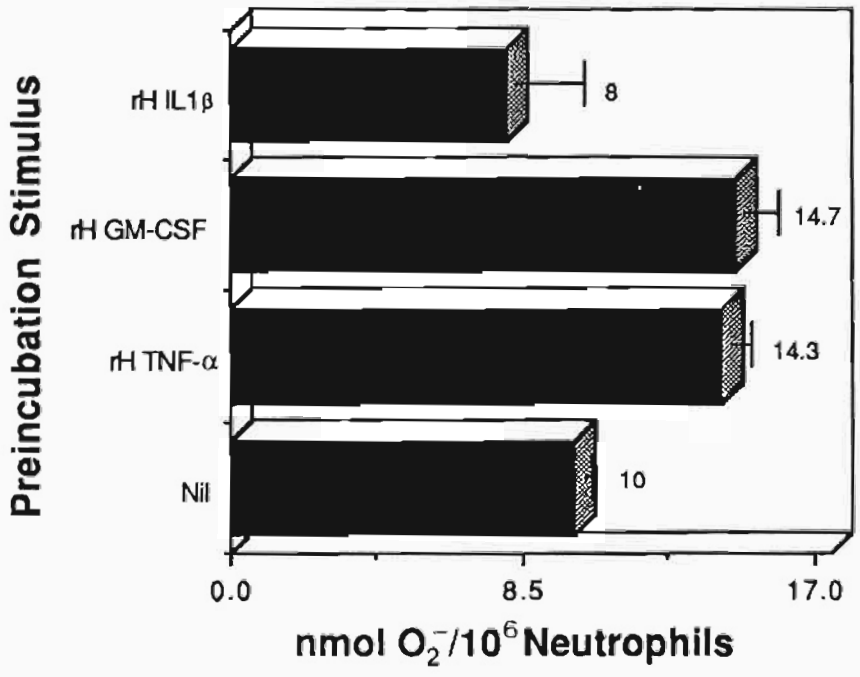
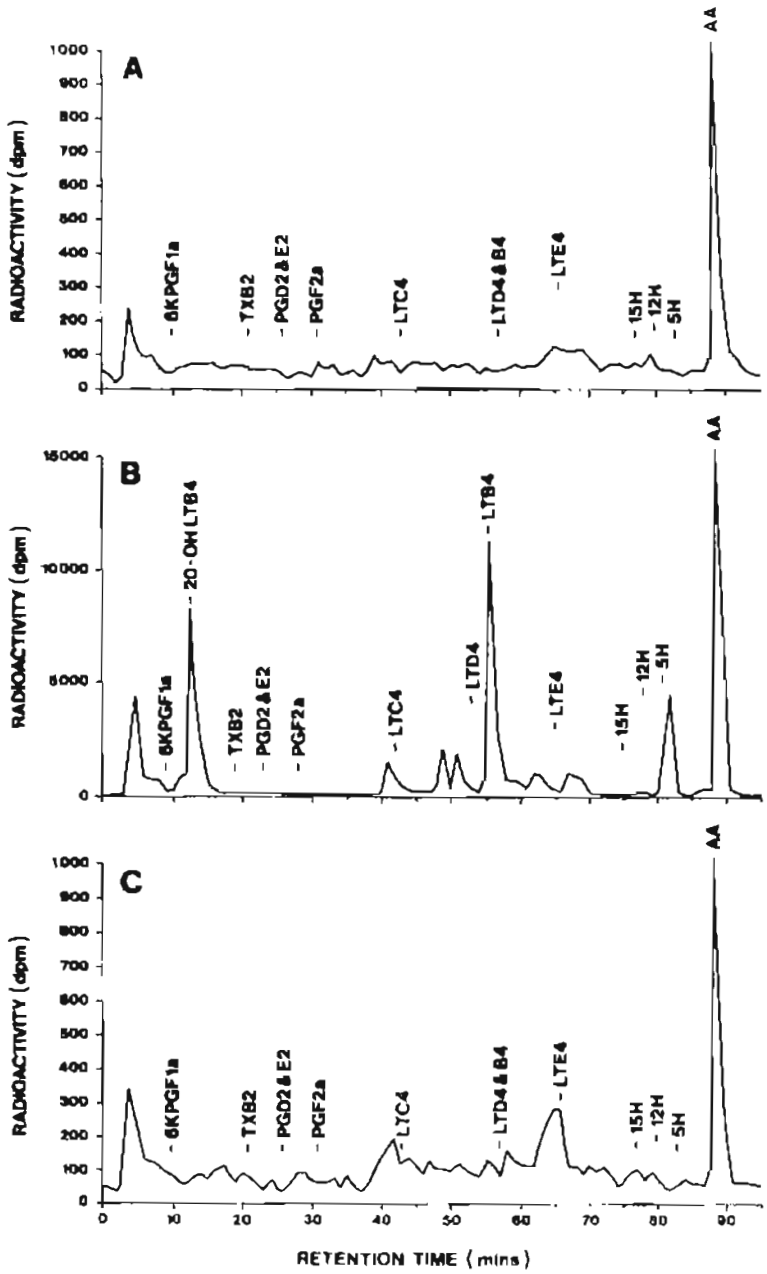

Preincubation Stimulus
rH IL1β
rH GM-CSF
rH TNF-α
Nil
8
14.7
14.3
10
0.0
8.5
17.0
nmol O2-/10^6 Neutrophils

FIGURE 7.5

rH TNF-α stimulates the release of [^{3}H]-arachidonic acid but not its metabolites.

[^{3}H]-arachidonic acid labelled neutrophils were incubated with medium (A), 4μM A23187 (B), or 100u/ml rH TNF-α (C) for 45 minutes at 37^{o}C. Supernatants were extracted and analysed by reverse phase-HPLC.


A
B
C
RADIOACTIVITY (dpm)
RETENTION TIME (mins)
6KPGF1a
TXB2
PGD2 & E2
PGF2a
LTC4
LTD4 & B4
LTE4
15H
12H
5H
20-OH LTB4
LTB4
LTD4
AA

directly activate either the lipoxygenase or cyclooxygenase pathways in these cells.

SECTION 7.3 DISCUSSION

The data presented here indicate that rH TNF-α directly stimulates the release of [^{3}H]-arachidonic acid from neutrophils prelabelled with this fatty acid. This effect is both time and dose dependent and as potent as that induced by f Met-Leu-Phe. The stimulation of arachidonic acid release is detectable in some individuals at 15 minutes and reaches a plateau at between 30 and 45 minutes. It was previously shown that rH TNF-α increases the production of superoxide anion in response to f Met-Leu-Phe in a manner which is detectable by 15 minutes and reaches a plateau at 30 minutes (see Chapter 4). It therefore appears that the effect of rH TNF-α on [^{3}H]-arachidonic acid release may be temporally related to its ability to prime the neutrophil respiratory burst.

Recombinant human TNF-α induces [^{3}H]-arachidonic acid release in a dose-dependent manner. Maximal response was obtained at a concentration of about 100u/ml over 6 experiments, which is consistent with the apparent dissociation constant of rH TNF-α for its receptor on neutrophils (Munker, Di Persio and Koeffler, 1987). It is also interesting to note that these doses of rH TNF-α are also optimal for the priming of the neutrophil respiratory burst in response to f Met-Leu-Phe (See Chapter 1). It was also shown here that rH GM-CSF induces the release of [^{3}H]-arachidonic acid from neutrophils, whereas rH IL-1β does not. This observation appears to correlate with the ability of these two molecules to influence neutrophil function. Thus both rH TNF-α and rH GM-CSF can prime the neutrophil respiratory burst to f Met-Leu-Phe, and regulate f Met-Leu-Phe receptors (see Chapter 4 & 5). In contrast rH IL-1β has been reported to have no effect on neutrophil function (Georgilis et al., 1987) despite the demonstration of IL-1β receptors on neutrophils (Rhyne et al., 1988). These observations are consistent with a role of [^{3}H]-arachidonic acid release in the modulation of various neutrophil responses by rH TNF-α.

Previous reports have indicated that TNF-α increases the permeability of phospholipid vesicles composed of acidic lipids (Oku et al., 1987). In addition, rH TNF-α has been reported to alter phospholipid metabolism in Friend leukemia cells and fibrosarcomas in mice (Podd et al., 1987), and liposome membranes composed of neutral lipids (Yoshimura and Sone, 1987). In both cases, changes in membrane permeability were correlated with the biological activities of rH TNF-α.

Roubin et al. (1987) have recently reported that rH TNF-α enhances the release of leukotriene B_4 in response to the calcium ionophore A23187. This is interesting considering that the availability of free arachidonic acid within the cell will influence leukotriene production. The results presented here are consistent with Roubin's observations in that rH TNF-α could increase the free arachidonic acid pool in the cell, thus enhancing leukotriene production in response to a second stimulus. Our data and that of Roubin and coworkers (1987) indicate that rH TNF-α is able to modulate cellular lipid metabolism both directly and in response to a second stimulus.

It is important to note that although rH TNF-α induces the release of [^{3}H]-arachidonic acid from neutrophils, it does not stimulate the metabolism of [^{3}H]-arachidonic acid by either the lipoxygenase or the cyclooxygenase pathways. The products of these pathways - leukotrienes, HETES, and prostaglandins and thromboxanes respectively - are potent activators of neutrophil function and would therefore directly activate the cells. These results are therefore consistent with the observation that rH TNF-α does not directly stimulate the production of superoxide anion from nonadherent neutrophils (Atkinson et al., 1988b).

The magnitude of the arachidonic acid release was found to be similar between rH TNF-α, rH GM-CSF and f Met-Leu-Phe. This raises the question of why f Met-Leu-Phe but not rH TNF-α induces the release of superoxide anion. It is well documented that f Met-Leu-

Phe stimulates both phospholipase C (Sha'afi, Volpi and Naccache, 1986) and phospholipase A_2 activity (Lackie and Lawrence, 1987). As arachidonic acid is esterified predominantly at position 2 of cell phospholipids, this observation implicates phospholipase A_2 activation by rH TNF-α and rH GM-CSF. It has previously been reported that rH GM-CSF did not induce the turnover of inositol phospholipids or translocate protein kinase C (Lopez et al., 1988), both of which are results of phospholipase C activation. In addition, rH TNF-α is unable to stimulate the turnover of inositol phospholipids (J. Eglinton and A.F Lopez, unpublished observation). It is therefore tempting to speculate that rH TNF-α and rH GM-CSF prime the cells by the activation of phospholipase A_2, but cannot stimulate full function due to their inability to activate phospholipase C as well. In contrast, f Met-Leu-Phe can activate neutrophil functions directly, as it stimulates both phospholipase C (Sha'afi et al., 1986) and phospholipase A_2.

The TNF-α induced stimulation of arachidonic acid release from neutrophils may be a relevant step in the production of platelet activating factor (PAF) in these cells. Thus, the release of arachidonic acid from position *sn*-2 (Mueller et al., 1984; Chilton and Murphy, 1986), with subsequent acetylation may lead to PAF formation in human neutrophils. Indeed, recent reports indicate that rH TNF-α stimulates the production of PAF from rat peritoneal macrophages, human vascular endothelial cells and neutrophils (Camussi et al., 1987; Bussolino, Camussi, and Baglioni, 1988). Extracellular PAF has been shown to prime neutrophil responses to f Met-Leu-Phe (Baggiolini and Dewald, 1986; Ingraham et al., 1987), and more recently Worthen and coworkers (1988) demonstrated that lipopolysaccharide - a bacterial product which also primes neutrophil responses - causes the intracellular accumulation of PAF. It remains to be established whether rH TNF-α stimulation of arachidonic acid release and PAF production are causally related, and the role of these intracellular mediators in neutrophil priming.

CHAPTER 8

GENERAL DISCUSSION

SECTION 8.1 rH TNF-α INFLUENCES NEUTROPHIL FUNCTION IN A DOSE AND TIME -DEPENDENT MANNER.

During inflammation, neutrophils migrate from the circulation into the tissues where they phagocytose microorganisms. This process activates the major microbicidal functions of neutrophils - degranulation and the respiratory burst. The data presented here support a role for TNF-α in the inflammatory response, because this cytokine is shown to either activate neutrophils directly, or influence the neutrophil response to another inflammatory stimulus. Table 1 summarises the influence of rH TNF-α on the neutrophil functions documented in this thesis. These observations extend other reports investigating the influence of rH TNF-α on neutrophil functions (see Chapters 3 to 7, Discussion).

Table 1
rH TNF-α both activates and modulates neutrophil functions

Function	Activate	Regulate
Superoxide anion release	-	+
Chemotaxis	-	+
f Met-Leu-Phe receptor expression	+	ND
Degranulation	+	+
CR3 expression	+	ND
Actin polymerisation	+	+
Arachidonic acid production	+	ND

rH TNF-α affects neutrophil functions in a dose-dependent manner. Generally, 100 to 1000 u/ml rH TNF-α are the maximal doses required to induce a change in neutrophil responsiveness (Table 2). These concentrations of rH TNF-α are consistent with the apparent dissociation constant reported for rH TNF-α (Munker, Di Persio and Koeffler, 1987), and the concentrations which influence peripheral blood neutrophils in other reports (Gamble et al., 1985; Klebanoff et al., 1986; Roubin et al., 1987; Berkow et al., 1987; and Wang, Bersani and Mantovani, 1987). In addition, the minimum doses required to elicit these effects are approximately the same for all functions observed, with the exception of actin polymerisation.

Table 2
rH TNF-α influences neutrophil function in a dose dependent manner

Function	rH TNF-α (u/ml) activate	regulate
Superoxide anion release	N/A	100
Chemotaxis	N/A	1000
Degranulation	100	100
Actin polymerisation	1000	100
Arachidonic acid release	100	ND

Another aspect of rH TNF-α induced modulation of neutrophil function is the dependence on the time of exposure to this cytokine.

Actin polymerisation is the earliest response to rH TNF-α, occurring by 10 seconds (Chapter 6). The rapidity of this response could reflect the internalisation of the TNF receptor, a cytoskeletal event that has been well characterised for the f Met-Leu-Phe receptor (Jesaitis, Tolley and Allen, 1986). The minimum concentration of rH TNF-α required to detect this functional change is 100 u/ml. This concentration is considerably higher than that required to induce other neutrophil functions. Thus, actin polymerisation, induced by rH TNF-α could well be attributed to ligand-receptor internalisation, the detection of which requires high receptor occupancy. This observation is interesting in that it suggests that the TNF receptor is associated with the cytoskeleton of the cell. This concept is consistent with *in vitro* studies demonstrating the influence of rH TNF-α on cytoskeletal associated functions such as spreading associated with adherence, degranulation and chemotaxis.

Other functions such as priming for superoxide anion production, and the inhibition of chemotaxis are just detectable at 5 minutes and take 15 to 30 minutes to manifest (Chapter 4). The induction of arachidonic acid release is detectable at 30 minutes and peaks at 45 minutes (Chapter 7). The release of arachidonic acid from the neutrophils is presumed to

be a reflection of metabolic events occurring within the cell (Hirata et al., 1979). There appears to be a temporal relationship between rH TNF-α induced priming of neutrophil responses such as superoxide anion production and chemotaxis, and the release of arachidonic acid (Chapters 4 and 7). To further define this phenomenon, it would be interesting to investigate the kinetics of the intracellular accumulation of arachidonic acid and its metabolites in response to rH TNF-α.

One of the most interesting effects of rH TNF-α-induced neutrophil activation, is that not only can this cytokine directly activate some neutrophil functions, but it can alter their response to other inflammatory stimuli. These include f Met-Leu-Phe and zymosan activated serum. These observations raise the question of the mechanism of action of rH TNF-α.

SECTION 8.2 rH TNF-α DIRECTLY ACTIVATES NEUTROPHILS AND PRIMES THEIR RESPONSE TO OTHER STIMULI.

rH TNF-α inhibits the random movement of neutrophils (Chapter 4), but this effect is not as complete as that observed when neutrophils were preincubated with cytochalasin B (data not shown). This observation, coupled to the data demonstrating both the activation and modulation of actin polymerisation (Chapter 6), preclude the disruption of cytoskeletal microfilaments as a possible cause. rH TNF-α induced aggregation of neutrophils is also unlikely to be the cause of reduced migration, because f Met-Leu-Phe, a potent chemokinetic stimulus of neutrophils, is also a potent neutrophil aggregator (Korchak et al., 1984). rH TNF-α increases neutrophil adherence, which may contribute to reduced motility. This possibility is unlikely considering that neutrophils from patients with CD-18 deficiencies exhibit decreased adherence motility (Anderson et al., 1985). Physiologically, increased neutrophil adherence is associated with migration into the tissues, therefore such a change in adhesion should not result in reduced motility. It is difficult to propose a

mechanism by which rH TNF-α influences neutrophil motility, because this function is an extremely complex process involving a number of neutrophil functions. Based on previously reported effects of rH TNF-α on neutrophil functions, factors contributing to this phenomenon could include the modulation of membrane receptors required for motility (Atkinson et al., 1988b), and changes in the membrane phospholipid content (Oku et al., 1987; Yoshimura and Sone, 1987; Podd et al., 1987), and therefore, adhesive properties of the plasma membrane. Whatever the mechanism, rH TNF-α induced reduction in neutrophil motility makes physiological sense, because immobilisation of neutrophils at inflammatory foci, a major site of TNF-α production by activated macrophages, would ensure the development of a successful localised reaction, thus limiting tissue damage.

The modulation of f Met-Leu-Phe receptor expression is another direct effect of rH TNF-α (Chapter 4). This phenomenon could well explain the modulation of neutrophil responses such as superoxide anion production and chemotaxis in response to f Met-Leu-Phe. The mechanism by which rH TNF-α modulates f Met-Leu-Phe receptor expression remains to be determined. Based on other studies of the modulation of f Met-Leu-Phe receptor affinity, two mechanisms can be proposed. Firstly, GTP binding proteins modulate the affinity of the receptor (Koo, Lefkowitz and Snyderman, 1983; Lad, Olson and Smiley, 1985; Goldman et al., 1985). rH TNF-α could achieve this by altering the coupling of the receptor from a "high affinity" GTP binding protein to a "low affinity" GTP binding protein. The possibility of the rH TNF-α receptor being coupled to a binding protein can be implied from the fact that rH TNF-α induces neutrophil degranulation, and this function is known to be controlled by GTP binding proteins. Alternatively, agents that alter membrane fluidity have been shown to modulate f Met-Leu-Phe receptor affinity (Snyderman, 1985). rH TNF-α alters the phospholipid composition of Friend leukemia cells, fibrosarcomas in mice, phospholipid vesicles and liposome membranes (Podd et al.,

1987; Oku et al., 1987; Yoshimura and Sone, 1987). Modulating f Met-Leu-Phe receptor affinity by altering membrane permeability is a feasible hypothesis.

Consistent with its effect on neutrophil motility, the modulation of f Met-Leu-Phe membrane receptors is physiologically relevant. Down regulation of the high affinity receptors responsible for chemotaxis and upregulating the low affinity receptors responsible for superoxide anion generation, at the site of inflammation would serve to immobilize neutrophils and localize the effects of their microbicidal activity.

Degranulation is another function directly activated by rH TNF-α (Chapter 6). This observation provides a clue as to the coupling mechanism of TNF-α, because GTP binding proteins are known to control the exocytotic process (Burgoyne, 1987). Of additional interest, is the additive rather than synergistic effect that rH TNF-α has on the response to f Met-Leu-Phe (Chapter 6). This observation implies that although the coupling mechanisms are similar i.e., GTP binding proteins, the signal transduction pathways are different. Because f Met-Leu-Phe responses are mediated by protein kinase C, this tends to preclude this pathway as the mechanism of activation by rH TNF-α.

Other signals reported to induce neutrophil degranulation include the arachidonic acid metabolites, leukotriene B_4 (LTB_4) and the hydroxyeicosatetranoic acids (HETEs, O'Flaherty, 1985). Platelet activating factor is also implicated in the induction of neutrophil degranulation (O'Flaherty, 1985). The effects of LTB_4 and PAF on degranulation are interesting when considered in the light of the data presented here. Similar to rH TNF-α and f Met-Leu-Phe, LTB_4 and PAF induce degranulation on their own and are additive in combination (O'Flaherty, 1985). These observations suggest a role for LTB_4 and PAF as effector signals of rH TNF-α induced neutrophil degranulation.

The effect of rH TNF-α on actin polymerisation has been previously discussed. The kinetics and dose required to detect this response indicate that it is probably a reflection of ligand-receptor internalisation. If this is not the case, then the effect of rH TNF-α provides an important clue as to its signal transduction pathways. Three signal transduction pathways are able to affect actin polymerisation. These include the activation of phospholipase C, phospholipase A_2, or altering the ionic fluxes within the cell (Omann et al., 1987). rH TNF-α appears to be incapable of activating phospholipase C for two reasons. Firstly, rH TNF-α is additive with f Met-Leu-Phe in degranulation, indicating separate signal transduction pathways. Secondly, rH TNF-α does not increase the production of IP3 in neutrophils (J. Eglinton and A.F. Lopez, unpublished observations). It remains to be determined whether rH TNF-α alters the intracellular pH of neutrophils, therefore this possibility cannot be discounted. Finally, the activation of phospholipase A_2, and subsequent generation of LTB_4 and PAF is a feasible mechanism by rH TNF-α could induce actin polymerisation, because previous reports have demonstrated that rH TNF-α has an effect on phospholipid metabolism, and enhances LTB_4 production in response to a second stimulus.

The priming effect of rH TNF-α on actin polymerisation in response to f Met-Leu-Phe is interesting when considering other primed functions. Because rH TNF-α is additive with f Met-Leu-Phe in its effect on degranulation, but synergistic for actin polymerisation, it is unlikely that these two phenomena are causally related. This change in actin polymerisation could reflect the altered internalisation of the f Met-Leu-Phe receptor-ligand complex. Interestingly, the effect only occurs at 1 to 10 nM, which corresponds to the apparent dissociation constant for the high affinity receptor (Chapters 4 and 5). Perhaps the selective internalisation of high affinity f Met-Leu-Phe receptors by rH TNF-α contributes to the modulating effects of this cytokine described in Chapter 4.

SECTION 8.3 rH TNF-α INDUCES ARACHIDONIC ACID RELEASE FROM NEUTROPHILS.

The influence of rH TNF-α on the various neutrophil functions presented here strongly suggests an effect on the phospholipid metabolism of the cell. Chapter 7 is concerned with the initial step in investigating this hypothesis. Arachidonic acid release is associated with the generation of the biologically active lipid PAF, and is metabolised to form leukotrienes and HETEs (Rouzer and Samuelsson, 1985). rH TNF-α was shown to induce the production of arachidonic acid from neutrophils in both a time- and dose- dependent manner. Two points of interest arise from this work. Firstly, the kinetics and concentration of rH TNF-α required for this effect parallel those observed for the priming of superoxide anion production and chemotaxis inhibition (Chapters 4 and 7). Notably, there appears to be a 10 to 15 minute delay in the release of arachidonic acid when compared to the other two functions. Because arachidonic acid is esterified to membrane phospholipids in resting neutrophils (Chilton et al., 1984), and metabolised by the 5-lipoxygenase pathway or re-esterified into membrane lipids (Rouzer and Samuelsson, 1985; Chilton et al., 1983), this work should be extended to define this effect of rH TNF-α. Initially, inhibitors specific for phospholipase A_2 should be employed to determine the source of arachidonic acid. The fact that arachidonic acid takes so long to be released by the neutrophil, implies that it is being metabolised or sequestered back into the membrane, and that the release reflects a spillover from these processes, rather than actively being released from the cell. To further define this possibility, lipoxygenase and cyclooxygenase inhibitors could be used.

Arachidonic acid release from neutrophils is tightly coupled to the formation of two other lipid-derived factors - LTB_4 and PAF. The synthesis of these two substances is dependent on the release of arachidonic acid from membrane phospholipids (see Introduction, Sections 1.6.2 and 1.6.3). The accumulation of intracellular LTB_4 and PAF have been

proposed as signal transduction mechanisms by which neutrophils are activated (Rola-Pleszcynski, Borgeat and Sirois, 1982; Worthen et al., 1988). In fact LTB_4 generation is tightly coupled to PAF synthesis, because the arachidonic acid released from phospholipids in the generation of the PAF precursor (lyso-PAF), is metabolised predominantly by the 5-lipoxygenase pathway (Billah, Bryant and Siegel, 1985). LTB_4 modulates intracellular PAF production (Sisson et al., 1987). Interestingly, the time course of intracellular LTB_4 and PAF accumulation in this report is identical to the time course of rH TNF-α induced priming of superoxide production and inhibition of chemotaxis (see Chapter 4). Thus, the intracellular signals generated by rH TNF-α could well be LTB_4, PAF, or both of these factors. This would explain the *in vitro* effects of rH TNF-α on neutrophil function.

The above hypothesis is feasible for four reasons. Firstly, exogenous LTB_4 and PAF have the capacity to activate the neutrophil functions described here (see Introduction, Sections 1.6.2 and 1.6.3.). Perhaps their intracellular accumulation can influence the subsequent response of the cell to a second stimulus. Secondly, the direct and modulatory effects of LTB_4 and PAF on degranulation parallel those observed for rH TNF-α and f Met-Leu-Phe (O'Flaherty, 1985). Thirdly, rH TNF-α cannot induce the release of LTB_4 from neutrophils, but enhances the release in response to calcium ionophore (Roubin et al., 1987). This situation could parallel the calcium flux induced by challenge with a second signal such as f Met-Leu-Phe. Additionally, Roubin's observations can be temporally correlated with the priming responses presented here, because the effect requires preincubation for 5 to 10 minutes with rH TNF-α. This time span is the same as that required to detect the beginning of an effect on f Met-Leu-Phe responses. Other data supporting this indicate that inhibition of f Met-Leu-Phe-induced arachidonic acid release causes inhibition of neutrophil degranulation (Bokoch and Gilman, 1984). Finally, recent reports investigating the mechanism of priming of neutrophil functions by

lipopolysaccharide correlate the intracellular accumulation of PAF with it's priming effect (Worthen et al., 1988).

SECTION 8.4 PRIMING SIGNALS INDUCED BY GM-CSF.

Another cytokine which exhibits similar effects on neutrophils is GM-CSF (Chapter 5). Whereas TNF-α is produced by macrophages, GM-CSF is a T lymphocyte product. The different sources of these cytokines highlights the importance of the interaction of lymphocytes, macrophages and neutrophils during the inflammatory process. rH GM-CSF has been used *in vivo*, and its effect on neutrophil activation studied (Baldwin et al., 1988). It was found that leukopenic AIDS patients demonstrated restoration of impaired neutrophil functions, suggesting that *in vivo* administration of rH GM-CSF leads to the production of functionally active neutrophils. These observations emphasize the *in vivo* relevance of data presented in this thesis, and indicate a need for the *in vitro* dissection of the regulation of neutrophil functions by cytokines.

It is interesting that two cytokines produced at the site of inflammation should have such similar effects on neutrophils. For example, both cytokines increase neutrophil adherence (Gamble et al., 1985; Gamble et al., 1989), and enhance degranulation (Klebanoff et al., 1986; Lopez et al., 1986) and superoxide anion production (Atkinson et al., 1988b; Lopez et al., 1986) in response to a second stimulus. Additionally, both cytokines inhibit chemotaxis in response to f Met-Leu-Phe, and regulate the affinity of f Met-Leu-Phe receptors (Atkinson et al., 1988a; Atkinson et al., 1988b). These data imply that the mechanism of activation by GM-CSF could be similar to TNF-α.

Studies on the nature of the signal induced by rH GM-CSF are more extensive than for rH TNF-α. Sullivan et al. (1987) reported that rH GM-CSF exerted no immediate detectable effect on the resting transmembrane electrical potential, the intracellular concentration of

free calcium ions, or the cytosolic pH of neutrophils. However, when the cells were primed by preincubation with rH GM-CSF, the rate of membrane depolarisation was enhanced, but no alteration in the pH or calcium reponses could be detected. In addition, although rH GM-CSF did not induce the translocation of protein kinase C, it induced the release of arachidonic acid from membrane phospholipids. In agreement with these observations, Naccache et al. (1988) also found no change in resting calcium levels, but augmentation of the response to f Met-Leu-Phe. Consistent with the findings of Sullivan et al. regarding arachidonic acid release, Di Persio et al. (1988) and Dahinden et al. (1988), reported that rH GM-CSF enhances the production of LTB_4 in response to f Met-Leu-Phe, although it had no effect on LTB_4 release on its own. Combined, these observations suggest that rH GM-CSF may enhance LTB_4 production in response to f Met-Leu-Phe by activating phospholipase A_2 and increasing the availability of free arachidonic acid. Interestingly, GM-CSF is unable to translocate protein kinase C or induce the formation of inositol trisphosphates (Lopez et al., 1988). This may explain why rH GM-CSF is unable to induce superoxide anion release on its own, but can prime the response to a second stimulus. Since rH TNF-α and rH GM-CSF exert similar effects on neutrophil function, these reports provide some insight into the possible signal transduction pathways activated by rH TNF-α.

SECTION 8.5 PROPOSED MECHANISM OF ACTION OF rH TNF-α.

The action of rH TNF-α is therefore proposed to be mediated by the intracellular accumulation of LTB_4 and PAF (see Figure 8.1). These agents have the capacity to mediate the direct effects such as degranulation, changes in CR3 and f Met-Leu-Phe receptor expression, increased adherence (perhaps through changes in membrane fluidity), actin polymerisation and reduced random motility (Figure 8.1). One or both of them could be responsible for the changes observed in these functions. Additionally, the intracellular accumulation of these factors could serve to augment superoxide production by increasing

FIGURE 8.1

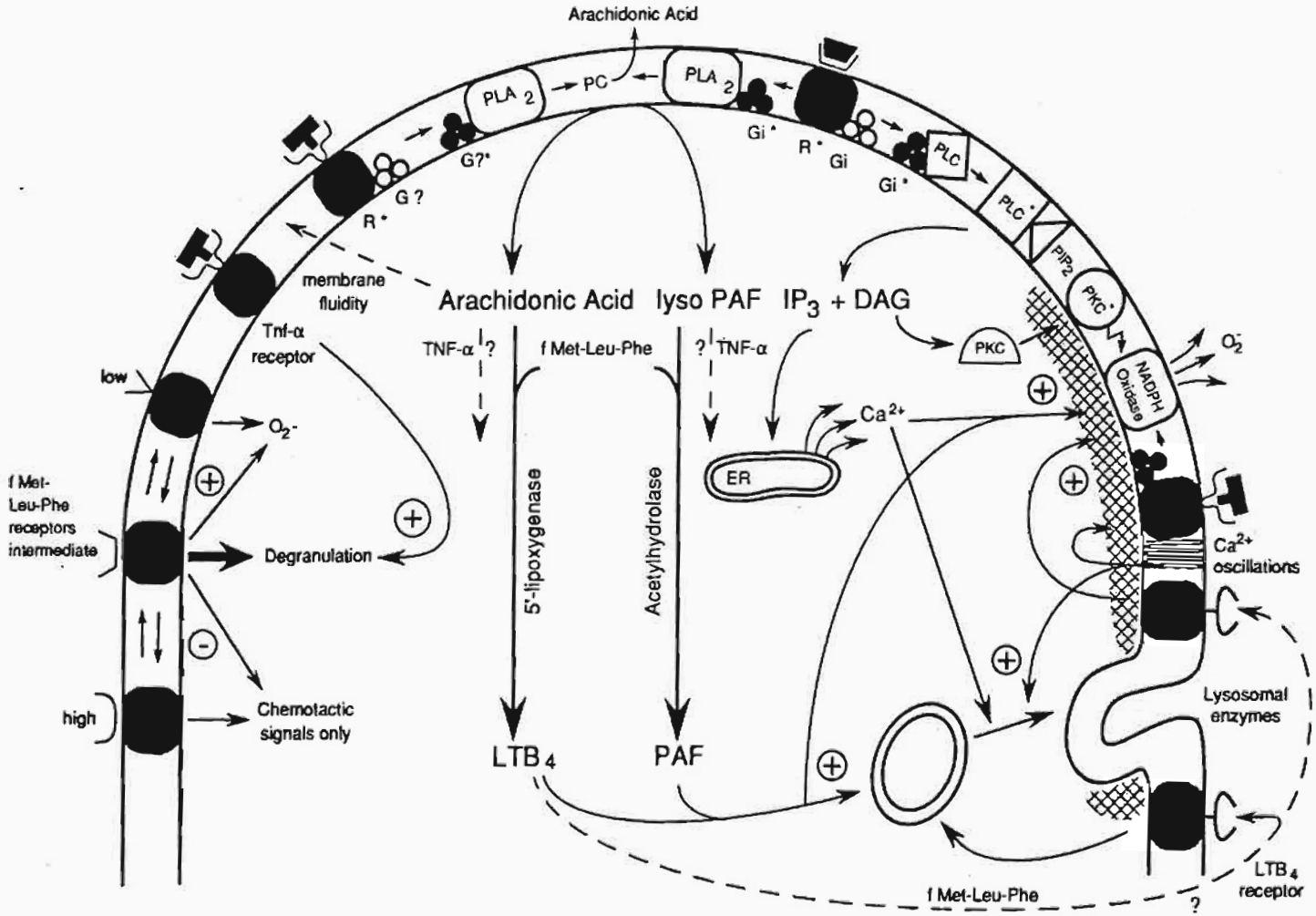
A model for the mechanism of action of rH TNF-α.

Left - Preincubation of neutrophils with rH TNF-α alters the affinity of f Met-Leu-Phe receptors. This could be a contributing factor in the regulation of various f Met-Leu-Phe-stimulated neutrophil functions such as O_2^- generation, degranulation, and chemotaxis.

Middle - The TNF receptor could be coupled to a GTP binding protein (G?), which transduces a signal to phospholipase A_2 (PLA_2) upon binding of rH TNFα. The arachidonic acid generated as a result of phospholipase A_2 activation could be directly released into the supernatant. It could also affect membrane fluidity and therefore f Met-Leu-Phe receptor affinity, or be metabolised to leukotriene B_4 (LTB_4). Lyso-PAF, generated by the action of phospholipase A_2 on phosphatidylcholine (PC), could be converted to PAF, and the levels of PAF and LTB_4 could influence degranulation and superoxide production. The subsequent binding of f Met-Leu-Phe would result in a larger response than in unprimed neutrophils, from the dual activation of PLA_2 (LTB_4 and PAF generated) and PLC (IP_3 and DAG generated), in addition to the signals generated by rH TNF-α.

Right - Binding of rH TNF-α to its receptor could induce calcium oscillations through the activation of a GTP binding protein. These calcium changes could influence actin polymerisation, and perhaps, the respiratory burst and degranulation.

(R^*=activated receptor; $G?^*$, Gi^*=activated alpha subunit of GTP binding proteins; ER=endoplasmic reticulum; cross hatched area=cortical actin network)


Arachidonic Acid
PLA 2
PC
PLA 2
G?*
G ?
R *
Gi *
R *
Gi
Gi *
PLC
PLC *
PIP2
PKC *
membrane fluidity
Arachidonic Acid
lyso PAF
IP3 + DAG
Tnf-α receptor
TNF-α ?
f Met-Leu-Phe
? TNF-α
PKC
NADPH Oxidase
O2-
low
O2-
Ca2+
ER
f Met-Leu-Phe receptors intermediate
Degranulation
5'-lipoxygenase
Acetylhydrolase
Ca2+ oscillations
high
Chemotactic signals only
Lysosomal enzymes
LTB4
PAF
LTB4 receptor
f Met-Leu-Phe
?

the existing pool of triggering stimuli for the NADPH oxidase. F Met-Leu-Phe activates both phospholipase A_2 (Lackie and Lawrence, 1987) and phospholipase C (Sha'afi, Volpi and Naccache, 1986). Leukotriene B_4 and PAF are "products" of phospholipase A_2 activation, and both stimulate phospholipase C-mediated formation of inositol trisphosphate in neutrophils (O'Flaherty and Nishihira, 1987). In effect then, subthreshold levels of LTB_4 and PAF could act in concert with the same lipids induced by f Met-Leu-Phe (Figure 8.1). Alternatively, stimulation with f Met-Leu-Phe could induce the release of LTB_4, which would subsequently bind to the cell and provide additional stimulation of the NADPH oxidase through receptor-mediated activation (O'Flaherty and Nishihira, 1987). These factors, combined with the direct stimulation of phospholipase C activity by f Met-Leu-Phe, could amplify the subsequent signal transduced to the NADPH oxidase (Figure 8.1).

Another potential second messenger in rH TNF-α-mediated neutrophil activation is intracellular free calcium. Studies on the mechanism of priming of superoxide anion production using calcium ionophore, indicate that changes in intracellular free calcium occurs with concentrations that prime the cells to subsequent stimulation with f Met-Leu-Phe, but this is insufficient accumulation of calcium to activate the NADPH oxidase (Finkel et al., 1987). This is supported by the fact that PMA induces superoxide production in the absence of changes in cytosolic calcium levels (Lehmeyer, Snyderman and Johnston, Jr., 1979). rH TNF-α and rH GM-CSF are unable to activate protein kinase C, but calcium may contribute to priming the respiratory burst (Figure 8.1).

What role then does calcium play in rH TNF-α-mediated neutrophil activation? The contribution of calcium to rH TNF-α-mediated neutrophil activation has yet to be defined. Since rH TNF-α induces the release of arachidonic acid from membrane lipids, presumably by activating phospholipase A_2, and this enzyme is calcium dependent (Simon, Chap and Douste-Blazy, 1986), although the role of calcium is uncertain. It

would therefore appear that rH TNF-α and rH GM-CSF should induce a change in intracellular free calcium levels. rH GM-CSF however does not change intracellular free calcium levels (Sullivan et al., 1987; Naccache et al., 1988). This could simply be due to the fact that rH GM-CSF is a less potent stimulus of neutrophils, i.e. it has no direct effect on degranulation (Lopez et al., 1986), and is less effective in inducing neutrophil adherence (Gamble et al., 1989). The inability of rH GM-CSF to change calcium levels is inconsistent with its ability to stimulate the release of arachidonic acid from phospholipids by a calcium dependent enzyme. This introduces the possibility that, although phospholipase A_2 is a calcium dependent enzyme, it does not neccessarily rely on a flux of calcium to express activity.

The effect of rH TNF-α on actin polymerisation provides another clue as to the contribution of calcium. Notably, although the initial calcium-independent phase of polymerisation is identical between rH TNF-α and f Met-Leu-Phe, the calcium-dependent depolymerisation phase is not sustained in rH TNF-α-stimulated neutrophils (see Chapter 6). These data suggest that rH TNF-α does not change absolute calcium levels, because it is unable to sustain prolonged actin polymerisation as does f Met-Leu-Phe.

Although rH GM-CSF has been reported negative for its effect on cytosolic calcium, Berridge and Galione (1988) propose some interesting models for the regulation of cells by calcium oscillators. They suggest that because cytosolic calcium oscillators act in a concentration dependent manner, their information might be encoded through changes in the frequency of calcium oscillations rather than through changes in the absolute level of calcium. In addition, an inositol trisphosphate-insensitive pool of calcium exists within the cell, suggesting that calcium release is not necessarily controlled by inositol trisphosphate alone. Interestingly, rH TNF-α and rH GM-CSF influence neutrophil functions in a concentration-dependent manner (Atkinson et al., 1988b; Lopez et al., 1988). The activation of GTP binding proteins can initiate calcium oscillatory activity. Since the

effect of rH TNF-α on degranulation suggests coupling to a GTP binding protein (Figure 8.1), perhaps this cytokine acts through the modulation of calcium oscillations (Figure 8.1). Recently, rH GM-CSF has been reported to prime neutrophils through the activation of a GTP binding protein (Corey and Rosoff, 1989). Additionally, the GTP binding protein that is coupled to the GM-CSF receptor does not activate phospholipase C activity, but does activate the liberation of arachidonic acid. These models would thus explain the physiological and biological data pertaining to rH TNF-α and perhaps to rH GM-CSF. A change in oscillatory activity through coupling to a GTP binding protein could prime neutrophils and activate phospholipase A_2, but no change in the absolute calcium levels could explain the kinetics of actin polymerisation (Figure 8.1).

rH TNF-α induces the cleavage of arachidonic acid from membrane phospholipids. Arachidonic acid exists predominantly in the alkyl species of phosphatidylcholine (PC) in neutrophils (Chilton and Murphy, 1986). Putative second messengers generated from this species of PC include the diacylglycerols (DAG), and the 1-*O*-alkyl-2-acyl-*sn*-glycerols (OAG). OAGs are generated by the activation of a phospholipase C specific for PC (Daniel, Waite and Wykle, 1986). Similar to rH TNF-α, they prime the neutrophil respiratory burst to stimulation with f Met-Leu-Phe, but do not directly induce superoxide anion production (Bass et al., 1989). Also similar to rH TNF-α, they enhance the conversion of arachidonic acid to LTB_4 upon subsequent stimulation with f Met-Leu-Phe (Bauldry, Wykle and Bass, 1988). Interestingly, this effect was induced by OAG, but not by DAG which is the lipid that binds to and activates protein kinase C. Since OAG exerts similar effects to rH TNF-α, it would be interesting to determine whether rH TNF-α activates the phosphatidylcholine-specific phospholipase C to generate OAG.

CHAPTER 9

BIBLIOGRAPHY

Aderka, D., Holtmann, H., Toker, L., Hahn, T., and Wallach, D. (1986). Tumor necrosis factor induction by Sendai virus. *J. Immunol.* **136**:2938-2942.

Aggarwal, B.B., Kohr, W.J., Hass, P., Moffat, B., Spencer, S.A., Henzel, W.J., Bringman, T.S., Nedwin, G.E., Goeddel, D.V., and Harkins, R.N. (1985). Human tumor necrosis factor. Production, purification, and characterization. *J. Biol. Chem.* **260**:2345-2354.

Agner, K. (1958). Crystalline myeloperoxidase. *Acta. Chem. Scand.* **12**:89-94.

Agner, K. (1963). Studies on myeloperoxidase activity. I. Spectrophotometry of the MPO-H_2O_2 compound. *Acta. Chem. Scand.* **17**:332-338.

Allan, R.B. and Wilkinson, P.C. (1978). A visual analysis of chemotactic and chemokinetic locomotion of human neutrophil leucocytes. Use of a new chemotaxis assay with *Candida albicans* as gradient source. *Exp. Cell. Res.* **111**:191-203.

Allen, R.A., Jesaitis, A.J., Sklar, L.A., Cochrane, C.G., and Painter, R.G. (1986). Physicochemical properties of the *N*-formyl peptide receptor on human neutrophils. *J. Biol. Chem.* **261**:1854-1857.

Allen, R.A., Parkos, C.A., Cochrane, C.G. and Jesaitis, A.J. (1985). A new method for labelling and purification of the *N*-formyl peptide receptor (abstr.) *Fed. Proc.* **44**:576a.

Allen, R.C., Stjernholm, R.L. and Steele, R.H. (1972). Evidence for the generation of an electronic excitation state(s) in human polymorphonuclear leukocytes and its participation in bactericidal activity. *Biochem. Biophys. Res. Commun.* **47**:679-684.

Alonso, F., Gil, M., Sanchez-Crespo, M., and Mato, J. (1982). Activation of 1-alkyl-2-lyso-glycero-3-phosphocholine: Acetyl-CoA transferase during phagocytosis in human polymorphonuclear leukocytes. *J. Biol. Chem.* **257**:3376-3378.

Andersen, B.R., Brendzel, A.M. and Lint, T.F. (1977). Chemiluminescence spectra of human myeloperoxidase and polymorphonuclear leukocytes. *Infect. Immun.* **17**:62-66.

Anderson, D.C., Schmalsteig, F.C., Finegold, M.J., Hughes, B.J., Rothlein, R., Miller, L.J., Kohl, S., Tosi, M.F., Jacobs, R.L., Waldrop, T.C., Goldman, A.S., Shearer, W.T., and Springer, T.A. (1985). The severe and moderate phenotypes of heritable Mac-1, LFA-1 deficiency: their quantitative definition and relation to leukocyte dysfunction and clinical features. *J. Infect. Dis.* **152**:668-689.

Anderson, D.C., Wible, L.J., Hughes, B.J., Smith, C.W. and Brinkley, B.R. (1982). Cytoplasmic microtubules in polymorphonuclear leukocytes: effects of chemotactic stimulation and colchicine. *Cell* **31**:719-729.

Arakawa, T., Wada, Y., Hayashi, T., Kakizaki, R., Chida, N., Chiba, R., and Konno, T. (1965). Uracil-uric refractory anemia with peroxidase negative neutrophils. *Tohuku Exp. Med.* **87**:52-76.

Arnaout, M.A., Wang, E.A., Clark, S.C. and Sieff, C.A. (1986). Human recombinant granulocyte-macrophage colony-stimulating factor increases cell-to-cell adhesions and surface expression of adhesion-promoting surface glycoproteins on mature granulocytes. *J. Clin. Invest.* **78**:597.

Atkinson, Y.H., Lopez, A.F., Marasco, W.A., Lucas, C.M., Wong, G.G., Burns, G.F. and Vadas, M.A. (1988a). Recombinant human granulocyte-macrophage colony-stimulating factor (rH GM-CSF). regulates f Met-Leu-Phe receptors on human neutrophils. *Immunology* **64**:519-525.

Atkinson, Y.H., Marasco, W.A., Lopez, A.F. and Vadas, M.A. (1988b). Recombinant human tumour necrosis factor-α. Regulation of N-formylmethionylleucylphenylalanine receptor affinity and function on human neutrophils. *J. Clin. Invest.* **81**:759-765.

Auron, P.E., Webb, A.C., Rosenwasser, L.J., Mucci, S.F., Rich, A., Wolff, S.M., and Dinarello, C.A. (1984). Nucleotide sequence of human monocyte interleukin-1 precursor cDNA. *Proc. Natl. Acad. Sci. USA* **81**:7907-7911.

Babior, B.M. and Kipnes, R.S. (1977). Superoxide-forming enzyme from human neutrophils: evidence for a flavin requirement. *Blood* **50**:517-524.

Babior, B.M., Curnutte, J.T. and Kipnes, R.S. (1975). Pyridine nucleotide-dependent superoxide production by a cell-free system from human granulocytes. *J. Clin. Invest.* **56**:1035-1042.

Babior, B.M., Kipnes, R.S. and Curnutte, J.T. (1973). Biological defense mechanisms: the production by leukocytes of superoxide, a potential bactericidal agent. *J. Clin. Invest.* **52**:741-744.

Babior, G.L., Rosin, R.E., McMurrich, B.J., Peters, W.A. and Babior, B.M. (1981). Arrangement of the respiratory burst oxidase in the plasma membrane of the neutrophil. *J. Clin. Invest.* **67**:1724-1728.

Bachner, R.L., and Nathan, D.G. (1967). Leukocyte oxidase: Defective activity in chronic granulomatous disease. *Science* **155**:835-836.

Baehner, R.L., and Nathan, D.G. (1968). Quantitative nitroblue tetrazolium test in chronic granulomatous disease. *New Eng. J. Med.* **278**:971-976.

Badwey, J.A. and Karnovsky, M.J. (1980). Active oxygen species and the functions of phagocytic leukocytes. *Annu. Rev. Biochem.* **49**:695-726.

Badwey, J.A., Curnutte, J.T. and Karnovsky, M.L. (1981). *cis*-Polyunsaturated fatty acids induce high levels of superoxide production by human neutrophils. *J. Biol. Chem.* **256**:12640-12643.

Baggiolini, M. (1972). The enzymes of the granules of polymorphonuclear leukocytes and their functions. *Enzyme* **13**:132-160.

Baggiolini, M. and Dewald, B. (1986). Stimulus amplification by PAF and LTB_4 in human neutrophils. *Pharmacol. Res. Comm.* **18**(suppl.):51-59 .

Bainton, D.F. (1973). Sequential degranulation of the two types of polymorphonuclear leukocyte granules during phagocytosis of microorganisms. *J. Cell. Biol.* **58**:249-264.

Bainton, D.F., and Farquhar, M.G. (1966). Origin of granules in polymorphonuclear leukocytes. Two types derived from opposite faces of the Golgi complexes in developing granulocytes. *J. Cell. Biol.* **28**:277-301.

Bainton, D.F., Nichols, B.A., and Farquhar, M.G. (1976). Primary lysosomes of blood leukocytes. *Front. Biol.* **45**:3-32.

Baldwin, G.C., Gasson, J.C., Quan, S.G., Fleischmann, J., Weisbart, R., Oette, D., Mitsuyasu, R.T., and Golde, D.W. (1988). Granulocyte-macrophage colony stimulating factor enhances neutrophil function in acquired immunodeficiency syndrome patients. *Proc. Natl. Acad. Sci. USA* **85**:2763-2766.

Baldwin, J.M., Bennet, J.P., and Gomperts, B.D. (1983). Detergent solubilisation of the rabbit neutrophil receptor for chemotactic formyl peptides. *Eur. J. Biochem.* **135**:515-518.

Barrett, A.J. (1974). Inhibitors of lysosomal proteinases. In: *Proteinase Inhibitors.* edited by H. Fritz, H. Tschesche, L.J. Greene, and E.E. Truskhe., pp.574-580, Springer-Verlag, New York.

Bass, D.A., and Szjeda, P. (1979). Eosinophils versus neutrophils in host defence: killing of newborn larvae of *Trichinella spiralis* by human granulocytes *in vitro*. *J. Clin. Invest.* **64**:1415-1422.

Bass, D.A., McPhail, L.C., Schmitt, J.D., Morris-Natschke, S., McCall, C.E., and Wykle, R.L. (1989). Selective priming of rate and duration of the respiratory burst of neutrophils by 1,2-diacyl and 1-*O*-alkyl-2-acyl diglycerides. Possible relation to effects on protein kinase C. *J. Biol. Chem.* **264**:19610-19617.

Bauldry, S.A., Wykle, R.L., and Bass, D.A. (1988). Phospholipase A_2 activation in human neutrophils. Differential actions of diacylglycerols and alkylacylglycerols in priming cells for stimulation by *N*-formyl-met-leu-phe. *J. Biol. Chem.* **263**:16787-16795.

Becker, E.L. (1979). A multifunctional receptor on the neutrophil for synthetic chemotactic oligopeptides. *J. Reticuloendothel. Soc.* (suppl.) **26**:701-709.

Becker, E.L. and Henson, P.M. (1973). *In vitro* studies of immunologically induced secretion of mediators from cells and related phenomena. *Adv. Immunol.* **17**:93-193.

Becker, E.L. and Showell, H.J. (1974). The ability of chemotactic factors to induce lysosomal enzyme release. II. The mechanisms of release. *J. Immunol.* **112**:2055-2062.

Becker, E.L., Henson, P.M., Showell, H.J. and Hsu, L.S. (1974). The ability of chemotactic factors to induce lysosomal enzyme release. I. The characteristics of the release, the importance of surfaces and relation of release to chemotactic responsiveness. *J. Immunol.* **112**:2047-2054.

Becker, E.L., Kermode, J.C., Naccache, P.H., Yassin, R., Marsh, M.L., Munoz, J.J., and Sha'afi, R.I. (1985). The inhibition of neutrophil granule enzyme secretion and chemotaxis by pertussis toxin. *J. Cell Biol.* **100**:1641-1646.

Bentwood, B.J. and Henson, P.M. (1980). The sequential release of granule constituents from human neutrophils. *J. Immunol.* **124**:855-862.

Benveniste, J., Henson, P.M., and Cochrane C.G. (1972). Leukocyte-dependent histamine release from rabbit platelets; The role of IgE, basophils, and a platelet-activating factor. *J. Exp. Med.* **136**:1356-1377.

Berkow, R.L., Wang, D., Labrick, W., Dodson, R.W. and Howard, T.H. (1987). Enhancement of neutrophil superoxide production by preincubation with recombinant human tumor necrosis factor. *J. Immunol.* **139**:3783-3791.

Berridge, M.J., and Galione, A. (1988). Cytosolic calcium oscillators. *FASEB J.* **2**:3072-3082.

Bertolini, D.R., Nedwin, G., Bringman, T., Smith, D., and Mundy, G.R. (1986). Stimulation of bone resorption and inhibition of bone formation *in vitro* by human tumour necrosis factors. *Nature (Lond.)* **319**:516-518.

Beutler, B. and Cerami, A. (1988). Tumour necrosis, cahexia, shock and inflammation: a common mediator. *Ann. Rev. Biochem.* **57**:505-518.

Beutler, B., Greenwald, D., Hulmes, J.D., Chang, M., Pan, Y.-C., Mathison, J., Ulevitch, R., and Cerami, A. (1985a). Identity of tumour necrosis factor and the macrophage-secreted factor, cachectin. *Nature (Lond.)* **316**:552-554.

Beutler, B., Krochin, N., Milsark, I.W., Goldberg, A., and Cerami, A. (1986a). Induction of cachectin (tumor necrosis factor) synthesis by Influenza virus-deficient production by endotoxin-resistant (C3H/HEJ) macrophages. *Clin. Res.* **34**:491A.

Beutler, B., Krochin, N., Milsark, I.W., Luedke, C., and Cerami, A. (1986b). Control of cachectin (tumor necrosis factor) synthesis: mechanisms of endotoxin resistance. *Science* **232**:977-980.

Beutler, B., Mahoney, J., LeTrang, N., Pekala, P., and Cerami, A. (1985b). Purification of cachectin, a lipoprotein lipase-suppressing hormone secreted by endotoxin-induced RAW 264.7 cells. *J. Exp. Med.* **161**:984-995.

Beutler, B., Milsark, I.W. and Cerami, A. (1985b). Cachectin/tumor necrosis factor: production, distribution and metabolic fate *in vivo*. *J. Immunol.* **135**:3972-3977.

Beutler, B., Tkacenko, V., Milsark, I.W., Krochin, N., and Cerami, A. (1986c). Effect of gamma interferon on cachectin expression by mononuclear phagocytes. Reversal by lpsd (endotoxin resistance) phenotype. *J. Exp. Med.* **164**:1791-1796.

Bevilacqua, M.P., Pober, J.S., Wheeler, M.E., Cotran, R.S., and Gimbrone, M.A., Jr. (1985). Interleukin-1 acts on cultured human vascular endothelium to increase the adhesion of polymorphonuclear leukocytes, monocytes, and related leukocyte cell lines. *J. Clin. Invest.* **76**:2003-2011.

Bielski, B.H.J., and Shiue, G.G. (1979). Reaction rates of superoxide radicals with the essential amino acids. In: *Oxygen Free Radicals and Tissue Damage, CIBA Foundation Symposium 65*, pp. 43-48. Excerpta Medica, Amsterdam.

Bielski, B.H.J., Arudi, R.L., and Sutherland, M.W. (1983). A study of the reactivity of $H0_2/0_2^-$ with unsaturated fatty acids. *J. Biol. Chem.* **258**:4759-4761.

Billah, M.M, Bryant, R.W., Siegel, M.I. (1985). Lipoxygenase products of arachidonic acid modulate biosynthesis of platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine). by human neutrophils via phospholipase A_2. *J. Biol. Chem.* **260**:6899-6906.

Blank, M.L., Lee, T.-C., Fitzgerald, V., and Snyder, F. (1981). A specific acetylhydrolase for 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (a hypotensive and platelet-activating lipid). *J. Biol. Chem.* **256**:175-178.

Bokisch, V.A., and Müller-Eberhard, H.J. (1970). Anaphylatoxin inactivator of human plasma: its isolation and characterization as a carboxypeptidase. *J. Clin. Invest.* **49**:2427-2436.

Bokoch, G.M., and Gilman, A.G. (1984). Inhibition of receptor-mediated release of arachidonic acid by pertussis toxin. *Cell* **39**:301-308.

Borregard, N. (1985). The respiratory burst of phagocytosis: biochemistry and subcellular localization. *Immunol. Lett.* **11**:165-171.

Borregard, N. and Tauber, A.I. (1984). Subcellular localization of the human neutrophil NADPH oxidase: b-cytochrome and associated flavoprotein. *J. Biol. Chem.* **259**:47-52.

Borregard, N., Heiple, J.M., Simons, E.R. and Clark, R.A. (1983). Subcellular localization of the b-cytochrome component of the human neutrophil microbicidal oxidase-translocation during activation. *J. Cell. Biol.* **97**:52-61.

Boyden, S. (1962). The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leukocytes. *J. Exp. Med.* **115**:453-466.

Boyles, J., and Bainton, D.F. (1979). The changing pattern of plasma-membrane-associated filaments during the initial phases of polymorphonuclear leukocyte adherence. *J. Cell. Biol.* **82**:347-368.

Bridges, R.A., Berendes, H., and Good, R.A. (1959). A fatal granulomatous disease of childhood. The clinical, pathological, and laboratory features of a new syndrome. *J. Dis. Child.* **97**:387-408.

Broudy, V.C., Kaushansky, K., Segal, G.M., Harlan, J.M., and Adamson, J.W. (1987). Tumor necrosis factor type stimulates human endothelial cells to produce granulocyte/macrophage colony-stimulating factor. *Proc. Natl. Acad. Sci. USA.* **83**:7467-7471.

Burgoyne, R.D. (1987). G proteins. Control of exocytosis. *Nature (Lond.)* **328**:112-113.

Bussolino, F., Camussi, G., and Baglioni, C. (1988). Synthesis and release of platelet-activating factor by human vascular endothelial cells treated with tumor necrosis factor or interleukin 1α. *J. Biol. Chem.* **263**:11856-11861.

Camussi, G., Bussolino, F., Salvidio, G., and Baglioni, C. (1987). Tumor necrosis factor/cachectin stimulates rat peritoneal macrophages and human endothelial cells to synthesize and release platelet-activating factor. *J. Exp. Med.* **166**:1390-1404.

Carlsson, L., Nystrom, L.E., Sundkvist, I., Markey, F. and Lindberg, U. (1976). Profilin, a low molecular weight protein controlling actin polymerisability. In: *Contractile Systems in Non-muscle Tissues*, edited by S.V. Perry, A. Margreth and R.S. Adelstein, pp. 39-48. Elsevier/North Holland, Amsterdam.

Carswell, E.A., Old, L.J., Kassel, R.L., Green, S., Fiore, N., and Williamson, B. (1975). An endotoxin-induced serum factor that causes necrosis of tumors. *Proc. Natl. Acad. Sci. USA* **72**:3666-3670.

Carter, S.B., (1965). Principles of cell motility: the direction of cell movement and cancer invasion. *Nature (Lond.)* **208**:1183-1187.

Catovsky, D., Galton, D.A.G., and Robinson, J. (1972). Myeloperoxidase-deficient neutrophils in acute myeloid leukemia. *Scand. J. Haemat.* **9**:142-148.

Changelian, P.S. and Fearon, D.T. (1986). Tissue-specific phosphorylation of complement receptors CR1 and CR2. *J. Exp. Med.* **163**:101-115.

Chenoweth, D.E., and Hugli, T.E. (1978). Demonstration of specific C5a receptor on intact human polymorphonuclear leukocytes. *Proc. Natl. Acad. Sci. USA.* **75**:3943-3947.

Cheson, B.D., Christensen, R.L., Sperling, R., Kohler, B.E. and Babior, B.M. (1976). The origin of the chemiluminescence of phagocytosing granulocytes. *J. Clin. Invest.* **58**:789-796.

Chilton, F.H. and Connell, T.R. (1988). 1-ether-linked phosphoglycerides. Major endogenous sources of arachidonate in the human neutrophil. *J. Biol. Chem.* **263**:5260-5265.

Chilton, F.H., and Murphy, R.C. (1986). Remodelling of arachidonate-containing phosphoglycerides within the human neutrophil. *J. Biol. Chem.* **261**: 7771-7777.

Chilton, F.H., Ellis, J.M., Olson, S.C., and Wykle, R.L. (1984). 1-*O*-alkyl-2-arachidonoyl-sn-glycero-3-phosphocholine. A common source of platelet-activatig factor and arachidonate in human polymorphonuclear leukocytes. *J. Biol. Chem.* **259**:12014-12019.

Chilton, F.H., Hadley, J.S. and Murphy, R.C. (1987). Incorporation of arachidonic acid into 1-acyl-2-lyso-sn-glycero-3-phosphocholine of the human neutrophil. *Biochim Biophys Acta.* **917**:48-56.

Chilton, F.H., O'Flaherty, J.T., Ellis, J.M., Swensden, C.L., and Wykle, R.L. (1983). Metabolic fate of platelet-activating factor in neutrophils. *J. Biol. Chem.* **258**:6357-6361.

Cline, M.J., Melmon, K.L., Davis, W.C., and Williams, H.E. (1968). Mechanisms of endotoxin interaction with human leukocytes. *Br. J. Haematol.* **15**:539-547.

Cochrane, C.G., Weigle, W.O. and Dixon, F.J. (1959). The role of polymorphonuclear leukocytes in the initiation and cessation of the Arthus vasculitis. *J. Exp. Med.* **110**:481-494.

Cockcroft, S., and Gomperts, B.D. (1985). Role of guanine nucleotide binding protein in the activation of polyphosphoinositide phosphodiesterase. *Nature (Lond.)* **314**:534-536.

Colditz, I.G., and Movat, H.Z. (1984). Kinetics of neutrophil accumulation in acute inflammatory lesions induced by chemotaxins and chemotaxinigens. *J. Immunol.* **133**:2169-2173.

Coley, W.B. (1906). Late results of the treatment of inoperable sarcoma by the mixed toxins of *Erysipelas* and *Bacillus prodigiosus*. *Am. J. Med. Sci.* **131**:375-430.

Colquhoun, D. (1979). The link between drug binding and response: Theories and observations. In: *The Receptors. A Comprehensive Treatise*. edited by R.D. O'Brien, pp. 96-98, 125. Plenum Press, New York.

Corey, S.J., and Rosoff, P.M. (1989). Granulocyte-macrophage colony-stimulating factor primes neutrophils by activating a pertussis toxin-sensitive G protein not associated with phosphatidylinositol turnover. *J. Biol. Chem.* **264**:14165-14171.

Cox, C.C., Dougherty, R.W., Ganong, B.K., Bell, R.M., Niedel, J.E. and Snyderman, R. (1986). Differential stimulation of the respiratory burst and lysosomal enzyme secretion in human polymorphonuclear leukocytes by synthetic diacylglycerols. *J. Immunol.* **136**:4611-4616.

Cramer, E., Pryzwansky, K.B., Villeral, J-L., Testa, U. and Breton-Gorius, J. (1985). Ultrastructural localisation of lactoferrin and myeloperoxidase in human neutrophils by immunogold. *Blood* **65**:423-432.

Cramer, E.B. and Gallin, J.I. (1979). Localisation of submembranous cations to the leading end of human neutrophils during chemotaxis. *J. Cell. Biol.* **82**:369-379.

Crawford, D.R. and Schneider, D.L. (1982). Identification of ubiquinone-50 in human neutrophils and its role in microbicidal events. *J. Biol. Chem.* **257**:6662-6668.

Cross, A.R., Jones, O.T.G., Harper, A.M. and Segal, A.W. (1981). Oxidation-reduction properties of the cytochrome b found in the plasma membrane fraction of human neutrophils: a possible oxidase in the respiratory burst. *Biochem. J.* **194**:599-606.

Cunningham, C.C., DeChatelet, L.R., Spach, R.I., Parce, J.W., Thomas, M.J., Lees, C.J. and Shirley, P.S. (1982). Identification and quantitation of electron-transport components in human polymorphonuclear neutrophils. *Biochim. Biophys. Acta* **682**:430-435.

Curnutte, J.T., Whitten, D.M., and Babior, B.M. (1974). Defective superoxide production by granulocytes from patients with chronic granulomatous disease. *N. Engl. J. Med.* **290**:593-597.

Cuturi, M.C., Murphy, M., Costa-Giomi, M.P., Weinmann, R., Perussia, B., and Trinchieri, G. (1987). Independent regulation of tumor necrosis factor and lymphotoxin production by human peripheral blood lymphocytes. *J. Exp. Med.* **165**:1581-1594.

Cybulsky, M.I., Colditz, I.G. and Movat, H.Z. (1986). The role of interleukin-1 in neutrophil leukocyte emigration induced by endotoxin. *Am. J. Pathol.* **124**:367-372.

Dahinden, C., and Fehr, J. (1983). Granulocyte activation by endotoxin. II. Role of granulocyte adherence, aggregation, and effect of cytochalasin B, and comparison with formylated chemotactic peptide-induced stimulation. *J. Immunol.* **130**:863-868.

Dahinden, C., Galanos, C., and Fehr, J. (1983). Granulocyte activation by endotoxin. I. Correlation between adherence and other granulocyte functions, and role of endotoxin structure in biologic activity. *J. Immunol.* **130**:857-862.

Dahinden, C.A., Zingg, J., Maly, F.E., and de Weck, A.L. (1988). Leukotriene production in human neutrophils primed by recombinant human granulocyte-macrophage colony-stimulating factor and stimulated with the complement component C5a and FMLP as second signals. *J. Exp. Med.* **167**:1281-1295.

Dale, M.M. and Penfield, A. (1984). Synergism between phorbol ester and A23187 in superoxide production by neutrophils. *FEBS Lett.* **175**:170-172.

Dancey, J.T., Deubelbeiss, K.A., Harker, L.A. and Finch, C.A. (1976). Neutrophil kinetics in man. *J. Clin. Invest.* **58**:705-715.

Daniel, L.W., Waite, M., and Wykle, R.L. (1986). A novel mechanism of diglyceride formation. 12-*O*-tetradecanoyl phorbol-13-acetate stimulates the cyclic breakdown and resynthesis of phosphatidylcholine. *J. Biol. Chem.* **261**:9128-9132.

Dayer, J.-M., Beutler, B., and Cerami, A. (1985). Cachectin/tumor necrosis factor stimulates collagenase and prostaglandin E_2 production by human synovial cells and dermal fibroblasts. *J. Exp. Med.* **162**:2163-2168.

De Lean, A. and Rodbard, A. (1979). Kinetics of co-operative binding. In: *The Receptors. A Comprehensive Treatise.* edited by R.D. O'Brien. p. 146, Plenum Press, New York.

Decker, T., Lohmann-Matthes, M.-L., Gifford, G.E. (1987). Cell-associated tumor necrosis factor (TNF) as a killing mechanism of activated cytotoxic macrophages. *J. Immunol.* **138**:957-962.

Dessein, A.J., Lee, T.H., Elsas, P., Ravalese, J.R., III, Silberstein, D, David, J.R., Austen, K.F., and Lewis, R.A. (1986). Enhancement by monokines of leukotriene generation by human eosinophils and neutrophils stimulated with calcium ionophore A23187. *J. Immunol.* **136**:3829-3838.

Dewald, B, Baggiolini, M., Curnutte, J.T., and Babior, B.M. (1979). Subcellular localization of the superoxide-forming enzyme in human neutrophils. *J. Clin. Invest.* **63**:21-29.

Dewald, B., Bretz, U. and Baggiolini, M. (1982). Release of gelatinase from a novel secretory compartment of human neutrophils. *J. Clin. Invest.* **70**:518-525.

Dewald, B., Payne, T.G. and Bagglioni, M. (1984). Activation of NADPH oxidase of human neutrophils: potentiation of chemotactic peptide by a diacylglycerol. *Biochem. Biophys. Res. Commun.* **125**:367-373.

Di Persio, J.F., Billings, P., Williams, R., and Gasson, J.C. (1988). Human granulocyte-macrophage colony-stimulating factor and other cytokines prime human neutrophils for enhanced arachidonic acid release leukotriene B_4 synthesis. *J. Immunol.* **140**:4315-4322.

Di Virgilio, F., Lew, D.P. and Pozzan, T. (1984). Protein kinase C activation of physiological processes in human neutrophils at vanishingly small cytosolic Ca^{2+} levels. *Nature (Lond.)* **310**:691-693.

Dinarello, C.A. (1984). Interleukin-1. *Rev. Infect. Dis.* **6**:51-95.

Dinarello, C.A. (1986a). Interleukin-1: Amino acid sequences, multiple biological activities and comparison with tumor necrosis factor (cachectin). *Year Immunol.* **2**:68-89.

Dinarello, C.A., Cannon, J.G., Mier, J.W., Bernheim, H.A., LoPreste,G., Lynn, D.L., Love, R.N., Webb, A.C., Auron, P.E., Reuben, R.C., Rich, A., Wolff, S.M.,

and Putney, S.D. (1986b). Multiple biological activities of human recombinant interleukin-1. *J. Clin. Invest.* **77**:1734-1739.

Dinarello, C.A., Cannon, J.G., Wolff, S.M., Bernheim, H.A., Beutler, B., Cerami, A., Figari, I.S., Palladino, M.A., Jr., and O'Connor, J.V. (1986c). Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. *J. Exp. Med.* **163**:1433-1450.

Dolmatch, B., and Niedel, J. (1983). Formyl peptide chemotactic receptor. Evidence for an active proteolytic fragment. *J. Biol. Chem.* **258**:7570-7577.

Donahue, R.E., Wang, E.A., Stone, D.K., Kamen, R., Wong, G.G., Sehgal, P.H., Nathan, D.G., and Clark, S.C. (1986). Stimulation of hematopoiesis in primates by continuous infusion of recombinant human GM-CSF. *Nature (Lond.)* **321**:872-875.

Dorfman, L.M. and Adams, G.E. (1973). Reactivity of the hydroxyl radical in aqueous solutions. In: *National Standard Reference Data System, National Bureau of Standards,* No. 46, pp. 1-59.

Drazin, R.E. and Lehrer, R.I. (1977). Fungicidal properties of a chymotrypsin-like cationic protein from human neutrophils: absorption to *Candida parapsilosis*. *Infect. Immun.* **17**:382-388.

Dubos, R.J. (1953). Effect of ketone bodies and other metabolites on the survival and multiplication of staphylococci and tubercle bacilli. *J. Exp. Med.* **98**:145-155.

Edwards, D.L., and Unger, B.W. (1980). Inhibition of the respiratory burst of human polymorphonuclear leukocytes by phenothiazine drugs. *Biochem. Int.* **1**:364-370.

Efrati, C., Sacks, T., Ne'eman, N., Lahor, M. and Ginsburg, I. (1976). The effect of leukocyte hydrolases on bacteria. VIII. The combined effect of leukocyte extracts, lysozyme, enzyme "cocktails", and penicillin on the lysis of *Staphylococcus aureus* and group A *Streptococci in vitro*. *Inflammation* **1**:371-407.

Elsbach, P. (1980). Degradation of microorganisms by phagocytic cells. *Rev. Infect. Dis.* **2**:106-128.

Evans, S.W., Beckner, S.K. and Farrar, W.L. (1987). Stimulation of specific GTP binding and hydrolysis activities in lymphocyte membrane by interleukin-2. *Nature (Lond.)* **325**:166-168.

Fearon, D.T. (1980). Identification of the membrane glycoprotein that is the C3b receptor of the human erythrocyte polymorphonuclear leukocyte, B lymphocyte, and monocyte. *J. Exp. Med.* **152**:20-30.

Figari, I.S., Mori, N.A. and Palladino, M.A., Jr. (1987). Regulation of neutrophil migration and superoxide production by recombinant tumor necrosis factors-α and -β: comparison to recombinant interferon-γ and interleukin-1α. *Blood* **70**:979-984.

Finkel, T.H., Pabst, M.J., Suzuki, H., Guthrie, L.A., Forehand, J.R., Phillips, W.A., and Johnston, Jr., R.B. (1987). Priming of neutrophils and macrophages for

enhanced release of superoxide anion by the calcium ionophore ionomycin. Implications for regulation of the respiratory burst. *J. Biol. Chem.* **262**:12589-12596.

Fittschen, C., Sandhaus, R.A., Worthen, G.S., and Henson, P.M. (1985). Human neutrophils express elastase in response to chemotactic concentrations of FMLP. *Fed. Proc.* **44**:1918a.

Fleit, A.B., Wright, S.D. and Unkeless, J.C. (1982). Human neutrophil Fcγ receptor distribution and structure. *Proc. Natl. Acad. Sci. USA* **79**:3275-3279.

Fletcher, M.P. and Gallin, J.I. (1980). Degranulating stimuli increase the availability of receptors on human neutrophils for the chemoattractant f-Met-Leu-Phe. *J. Immunol.* **124**:1585-1588.

Fletcher, M.P. and Gallin, J.I. (1983). Human neutrophils contain an intracellular pool of putative receptors for the chemoattractant N-formyl-methionyl-leucyl-phenylalanine. *Blood* **62**:792-799.

Foote, C.S. (1976). Photosensitized oxidation and singlet oxygen: consequences in biological systems. In: *Free Radicals in Biology, Vol. 2*, edited by W.A. Pryor, pp. 85-133. Academic Press, New York.

Forehand, J.R., Pabst, M.J., Phillips, W.A., and Johnston, R.B., Jr. (1989). Lipolpolysaccharide priming of human neutrophils for an enhenced respiratory burst. Role of intracellular free calcium. *J. Clin. Invest.* **83**:74-83.

Ford-Hutchinson, A.W., Bray, M.A., Doig, M.V., Shipley, N.E., and Smith, M.J. (1980). Leukotriene B, a potent chemokinetic and aggregating substance released from polymorphonuslear leukocytes. *Nature (Lond.)* **286**:264-265.

Franson, R., Patriarca, P. and Elsbach, P. (1974). Phospholipid metabolism by phagocytic cells. Phospholipase A_2 associated with rabbit polymorphonuclear leukocyte granules. *J. Lipid. Res.* **15**:380-388.

Freer, R.J., Day, A.R., Muthukumaraswamy, N., Pinon, D., Wu, A., Showell, H.J. and Becker, E.L. (1982). Formyl peptide chemoattractants: a model of the receptor on rabbit neutrophils. *Biochemistry* **21**:257-263.

Freer, R.J., Day, A.R., Radding, J.A., Schiffman, E., Aswanikumar, S., Showell, H.J. and Becker, E.L. (1980). Further studies on the structural requirements for synthetic peptide chemoattractants. *Biochemistry* **19**:2404-2410.

Fridovich, I. (1983). Superoxide radical: endogenous toxicant. *Annu. Rev. Pharmacol. Toxicol.* 23:239-257.

Frimer, A.A., Forman, A. and Borg, D.C. (1983). H_20_2-diffusion through lysosomes. *Isr. J. Chem.* **23**:442-445.

Fujita, I., Irita, K., Takeshige, K. and Minakami, S. (1984). Diacylglycerol, 1-oleoyl-2-acetyl-glycerol, stimulates superoxide-generation from human neutrophils. *Biochem. Biophys. Res. Commun.* **120**:318-324.

Gabig, T.G. and Lefker, B.A. (1985). Activation of the human neutrophil NADPH oxidase results in coupling of electron carrier function between ubiquinone and cytochrome b_{559}. *J. Biol. Chem.* **260**:3991-3995.

Gabig, T.G. and Babior, B.M. (1979). The O_2^- forming oxidase responsible for the respiratory burst in human neutrophils: properties of the solubilized enzyme. *J. Biol. Chem.* **254**:9070-9074.

Gallin, J.I. (1980). Degranulating stimuli decrease the surface charge and increase the adhesiveness of human neutrophils. *J. Clin. Invest.* **65**:298-306.

Gallin, J.I., Clark, R.A. and Frank, M.M. (1975). Kinetic analysis of the generation of the chemotactic factor in human serum via activation of the classical and alternate complement pathways. *Clin. Immunol. Immunopath.* **3**:334-346.

Gallin, J.I., Wright, D.G. and Schiffman, E. (1978). Role of secretory events in modulating human neutrophil chemotaxis. *J. Clin. Invest.* **62**:1364-1374.

Gamble, J.R., Harlan, J.M., Klebanoff, S.J. and Vadas, M.A. (1985). Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. *Proc. Natl. Acad. Sci. USA* **82**:8667-8671.

Gamble, J.R., Rand, T.H., Lopez, A.F., Clarke-Lewis, I., and Vadas, M.A. (1989). Heterogeneity of recombinant granulocyte-macrophage colony-stimulating factor-mediated enhancement of neutrophil adherence to endothelium (submitted).

Garcia, R.C. and Segal, A.W. (1984). Changes in the subcellular distribution of the cytochrome b_{-245} on stimulation of human neutrophils. *Biochem. J.* **219**:233-242.

Gasson, J.C., Weisbart, R.H., Kaufman, S.E., Clark, S.C., Hewick, R.M., Wong, G.G. and Golde, D.W. (1984). Purified human granulocyte-macrophage colony-stimulating factor: direct action on neutrophils. *Science* **226**:1339-1342.

Gebicki, J.M. and Bielski, B.H.J. (1981). Comparison of the capacities of the perhydroxyl and the superoxide radicals to initiate chain oxidation of linoleic acid. *J. Am. Chem. Soc.* **103**:7020-7022.

Geiger, B. (1987). Intermediate filaments: Looking for a function. *Nature (Lond.)* **329**:392-393.

Gennaro, R., Florio, C. and Romeo, D. (1985). Activation of protein kinase C in neutrophil cytoplasts: localization of protein substrates and possible relationship with stimulus-response coupling. *FEBS Lett.* **180**:185-190.

Georgilis, K., Schaeffer, C., Dinarello, C. and Klempner, N. (1987). Human recombinant interleukin 1β has no effect on intracellular calcium or on functional responses of human neutrophils. *J. Immunol.* **138**:3403-3407.

Gery, I., and Waksman, B.H. (1972). Potentiation of the T-lymphocyte response to mitogens. II. The cellular source of potentiating mediator(s). *J. Exp. Med.* **136**:143-155.

Gewurz, H., Shin, H.S., and Mergenhagen, S.E. (1968). Interactions of the complement system with endotoxin lipopolysaccharide: consumption of each of the six terminal complement components. *J. Exp. Med.* **128**:1049-1057.

Gilman, A.G. (1984). G Proteins and dual control of adenylate cyclase. *Cell* **36**:577-579.

Glick, A.D., Ranhard, J.M. and Cole, R.M. (1972). Degradation of group A streptococcal cell walls by egg-white lysozymes and human lysosomal enzymes. *Infect. Immun.* **6**:403-413.

Goetzl, E.J. (1981). Selective feedback inhibition of the 5-lipoxygenation of arachidonic acid in human T lymphocytes. *Biochem. Biophys. Res. Commun.* **101**:344-350.

Goetzl, E.J. and Austen, K.F. (1974). Stimulation of neutrophil leukocyte aerobic glucose metabolism by purified chemotactic factors. *J. Clin. Invest.* **53**:591-599.

Goetzl, E.J., and Pickett, W.C. (1981). Novel structural determinants of the human neutrophil chemotactic activity of leukotriene B. *J. Exp. Med.* **153**:482-487.

Goetzl, E.J., Boeynaems, J.M., Oates, J.A., and Hubbard, W.G. (1981). Stimulus-specificity of the chemotactic deactivation of human neutrophils by lipoxygenase products of arachidonic acid. *Prostaglandins* **22**:279-288.

Goldman, D.W., and Goetzl, E.J. (1984). Heterogeneity of human polymorphonuclear leukocyte receptors for leukotriene B_4: Identification of a

subset of high affinity receptors that transduce the chemotactic response. *J. Exp. Med.* **159**:1027-1041.

Goldman, D.W., Chang, F.-H., Gifford, L.A., Goetzl, E.J. and Bourne, H.R. (1985). Pertussis toxin inhibition of chemotactic factor-induced calcium mobilization and function in human polymorphonuclear leukocytes. *J. Exp. Med.* **162**:145-156.

Goldstein I.M., Hoffstein, S., Gallin, J.I., and Weissmann, G. (1973). Mechanisms of lysosomal enzyme release from human leukocytes: Microtubule assembly and membrane fusion induced by a component of complement. *Proc. Natl. Acad. Sci. USA* **70**:2916-2920.

Goldstein, I.M., Feit, F., and Weissmann, G. (1975). Enhancement of nitroblue tetrazolium dye reduction by leukocytes exposed to a component of complement in the absence of phagocytosis. *J. Immunol.* **114**:516-518.

Goldstein, I.M., Roos, D., Weissmann, G., and Kaplan, H. (1975). Complement and immunoglobulins stimulate superoxide production by human leukocytes independently of phagocytosis. *J. Clin. Invest.* **56**:1155-1165.

Goodman, M.L., Way, B.A., and Irwin, J.W. (1979). The inflammatory response to endotoxin. *J. Pathol.* **128**:7-14.

Gomperts, B.D., Barrowman, M.M., and Cockcroft, S. (1986). Dual role for guanine nucleotides in stimulus-secretion coupling. *Fed. Proc.* **45**:2156-2161.

Grabstein, K.H., Urdal, D.L., Tushinski, R.J., Mochizuki, D.Y., Price, V.L., Cantrell, M.A., Gillis, S., and Conlon, P.J. (1986). Induction of macrophage tumoricidal activity by granulocyte-macrophage colony-stimulating factor. *Science* **232**:506-508.

Graham, G.S. (1920). The neutrophilic granules of the circulating blood in health and in disease - a preliminary report. *N. Y. St. J. Med.* **20**:46-55.

Green, T.R. and Wu, D.E. (1986). The NADPH:O_2 oxidoreductase of human neutrophils: stoichiometry of univalent and divalent reduction of O_2. *J. Biol. Chem.* **261**:6010-6015.

Green, T.R., Shaefer, R.E. and Makler, M.T. (1980). Orientation of the NADPH dependent superoxide generating oxidoreductase of the outer membrane of human PMNs. *Biochem. Biophys. Res. Commun.* **94**:262-269.

Griffin, F.M., Griffin, J.A. and Silverstein, S.C. (1976). Studies on the mechanism of phagocytosis. II. The interaction of macrophages with anti-immunoglobulin IgG-coated bone marrow derived lymphocytes. *J. Exp. Med.* **144**:788-809.

Griffin, F.M., Griffin, J.A., Leider, J. and Silverstein, S.C. (1975). Studies on the mechanism of phagocytosis. I. Requirements for circumferential attachment of particle-bound ligands to specific receptors in the macrophage plasma membrane. *J. Exp. Med.* **142**:1263-1282.

Griffith, L.M., and Pollard, T.D. (1978). Evidence for actin filament-microtubule interaction mediated by microtubule-associated proteins. *J. Cell. Biol.* **78**:958-965.

Guthrie, L.A., and Johnston, R.B., Jr. (1982). Neutrophil "priming" by bacterial endotoxin (LPS). (abstr.) *Fed. Proc.* **41**:933.

Guthrie, L.A., McPhail, L.C., Henson, P.M., and Johnston, R.B., Jr. (1984). Priming of neutrophils for enhanced release of oxygen metabolites by bacterial lipopolysaccharide. Evidence for increased activity of the superoxide producing enzyme. *J. Exp. Med.* **160**:1656-1671.

Gutteridge, J.M.C., Richmond, R. and Halliwell, B. (1976). Inhibition of the iron-catalyzed formation of hydroxyl radicals from superoxide and of lipid peroxidation by desferrioxamine. *Biochem. J.* **184**:469-472.

Haber, F. and Weiss, J. (1934). The catalytic decomposition of hydrogen peroxide by iron salts. *Proc. R. Soc. Lond. [A]* **147**:332-351.

Halliwell, B. and Gutteridge, J.M.C. (1986). Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. *Arch. Biochem. Biophys.* **246**:501-513.

Harbour, J.R., Chow, V. and Bolton, J.R. (1974). An electron spin resonance study of the spin adducts of OH and HO_2 radicals with nitrones in the ultraviolet photolysis of aqueous hydrogen peroxide solutions. *Can. J. Chem.* **52**:3549-3553.

Harris, E.D., Jr., and Crane, S.M. (1974). Collagenases (in 3 parts). *N. Engl. J. Med.* **291**:557-563, 605-609, 652-661.

Harrison, J.E. (1976). The functional mechanism of myeloperoxidase. In: *Cancer Enzymology*, edited by J. Schultz and F. Ahmad, pp. 305-317. Academic Press, New York.

Haslett, C., Guthrie, L.A., Kopaniak, M.M., Johnson, R.B., Jr., and Henson, P.M. (1985). Modulation of multiple neutrophil functions by preparative methods or trace concentrations of bacterial lipopolysaccharide. *Am. J. Pathol.* **119**:101-110.

Hawkins, D. (1972). Neutrophilic leukocytes in immunologic reactions: evidence for the selective release of lysosomal constituents. *J. Immunol.* **108**:310-317.

Henke, D.C., Kouzan, S. and Eling, T.E. (1984). Analysis of leukotrienes, prostaglandins and other oxygenated metabolites of arachidonic acid by high-performance liquid chromatography. *Anal. Biochem.* **140**:87-94.

Henry, R.J., Canon, D.C., and Winkleman, J.W. (1974). *Clinical Chemistry: Principles and Technics., (2nd edition).* p 819. Harper and Rowe, Hagerston, M.D.

Henson, P.M. (1971). The immunologic release of constituents from neutrophil leukocytes. II. Mechanisms of release during phagocytosis and adherence to nonphagocytosable surfaces. *J. Immunol.* **107**:1547-1557.

Henson, P.M. (1973). Mechanisms of release of granule enzymes from human neutrophils phagocytosing aggregated immunoglobulin. An electron microscopic study. *Arthritis Rheum.* **16**:208-216.

Henson, P.M. and Oades, Z.G. (1975). Stimulation of human neutrophils by soluble and insoluble immunoglobulin aggregates. Secretion of granule constituents and increased oxidation of glucose. *J. Clin. Invest.* **56**:1053-1061.

Henson, P.M., Henson, J.E., Fittschen, C., Kimani, G., Bratton, D.L. and Riches, D.W.H. (1988). Phagocytic cells: degranulation and secretion. In: *Inflammation: Basic Principles and Clinical Correlates*, edited by J.I. Gallin, I.M. Goldstein, and R. Snyderman, pp. 363-90. Raven Press, Ltd., New York.

Higashi, O., Katsuyama, N., and Satodate, R. (1965). A case with haematological abnormality characterized by the absence of peroxidase activity in blood polymorphonuclear leukocytes. *Tohoku J. Exp. Med.* **87**:77-93.

Higson, F.K., Durbin, L., Pavlotsky, N. and Tauber, A.I. (1985). Studies of cytochrome B_{-245} translocation in the PMA stimulation of the human neutrophil NADPH-oxidase. *J. Immunol.* **135**:519-524.

Hirata, F., Corcoran, B.A., Venkatasubramanian, K., Schiffman, E., and Axelrod, J. (1979). Chemoattractants stimulate degradation of methylated phospholipids and release of arachidonic acid in rabbit leukocytes. *Proc. Natl. Acad. Sci. USA* **76**:2640-2643.

Hirsch, J.G. (1956). Phagocytin: a bactericidal substance from polymorphonuclear leukocytes. *J. Exp. Med.* **103**:589-611.

Hohn, D.C., and Lehrer, R.I. (1975). NADPH oxidase deficiency in X-linked chronic granulomatous disease. *J. Clin. Invest.* **55**:707-713.

Holmes, B., Page, A.R. and Good, R.A. (1967). Studies of the metabolic activity of leukocytes from patients with a genetic abnormality of phagocytic function. *J. Clin. Invest.* **46**:1422-1432.

Huey, R., and Hugli, T.E. (1985). Characterization of a C5a receptor on human polymorphonuclear leukocytes (PMN). *J. Immunol.* **135**:2063-2068.

Hyslop, P.A., Oades, Z.G., Jesaitis, A.J., Painter, R.G., Cochrane, C.G., and Sklar, L.A. (1984). Evidence for N-formyl chemotactic peptide-stimulated GTPase activity in human neutrophil homogenates. *FEBS Lett.* **166**:165-169.

Ilan, Y.A., Czapski, G. and Meisel, D. (1976). The one-electron transfer redox potentials of free radicals. I. The oxygen/superoxide system. *Biochim. Biophys. Acta.* **430**:209-224.

Ingraham, L.M., Coates, T.D., Allen, J.M., Higgins, C.P., Baehner, R.L., and Boxer, L.A. (1982). Metabolic, membrane, and functional responses of human polymorphonuclear leukocytes to platelet-activating factor. *Blood* **59**:1259-1266.

Ingraham, L.M., Lafuze, J.E., Boxer, L.A. and Baehner, R.L. (1987). *In vitro* and *in vivo* effects of treatment by platelet-activating factor on *N*-formyl-met-leu-phe-mediated responses of polymorphonuclear leucocytes. *Br. J. Haematol.* **66**:219-225.

Irvine, R.F. (1982). How is the level of free arachidonic acid controlled in mammalian cells? *Biochem. J.* **204**:3-16.

Jamuar, M.P. and Cronkite, E.P. (1980). The fate of granulocytes. *Exp. Hematol.* **8**:884-894.

Janatova, J., Lorenz, P.E., Schechter, A.N., Prahl, J.W., and Tack, B.F. (1980). Third component of human complement: appearance of a sulfhydryl group following chemical or enzymatic inactivation. *Biochemistry* **19**:4471-4478.

Janmey, P.A. and Stossel, T.P. (1987). Modulation of gelsolin function by phosphatidylinositol-4,5-bisphosphate. *Nature (Lond.)* **325**:362-364.

Janmey, P.A., Chaponnier, C., Lind, S.E., Zaner, K.S., Stossel, T.P. and Yin, H.L. (1985). Interactions of gelsolin and gelsolin actin complexes with actin: effects of calcium on actin nucleation, filament severing and end blocking. *Biochemistry* **24**:3714-3723.

Janmey, P.A., Iida, K., Yin, H.L. and Stossel, T.P. (1987). Polyphosphoinositide micelles and polyphosphoinositide containing vesicles dissociate endogenous gelsolin-actin complexes and promote actin assembly from the fast-growing end of actin filaments blocked by gelsolin. *J. Biol. Chem.* **262**(25):12228-12236.

Janmey, P.A., Peetermans, J., Zaner, K.S., Stossel, T.P. and Tanaka, T. (1986). Structure and mobility of actin as measured by quasielastic light scattering, viscometry and electron microscopy. *J. Biol. Chem.* **261**:8357-8362.

Janoff, A. (1972). Human granulocyte elastase: further delineation of its role to connective tissue damage. *Am. J. Pathol.* **68**:579-591.

Janoff, A. and Blondin, J. (1973). The effect of human granulocyte elastase on bacterial suspensions. *Lab. Invest.* **29**:454-457.

Janzen, E.G., Nutter, D.E. Jr., Davis, E.R., Blackburn, B.J., Poyer, J.L. and McCay, D.B. (1978). On spin trapping hydroxyl and hydroperoxyl radicals. *Can. J. Chem.* **56**:2237-2242.

Jensen, M.S. and Bainton, D.F. (1973). Temporal changes in pH within the phagocytic vacuoli of the polymorphonuclear neutrophilic leukocyte. *J. Cell. Biol.* **56**:379-388.

Jesaitis, A.J., Nacmura, J.R., Painter, R.G., Sklar, L.A., and Cochrane, C.G. (1982). Intracellular localisation of N-formyl chemotactic receptor and Mg++ dependent ATPase in human granulocytes. *Biochim. Biophys. Acta.* **719**:556-568.

Jesaitis, A.J., Naemura, J.R., Sklar, L.A., Cochrane, C.G., and Painter, R.G. (1984). Rapid modulation of *N*-formyl peptide receptors on the surface of human granulocytes: formation of high affinity ligand-receptor complexes in transient association with the cytoskeleton. *J. Cell. Biol.* **98**:1378-1387.

Jesaitis, A.J., Tolley, J.O., and Allen, R.A. (1986). Receptor-cytoskeleton interactions and membrane traffic may regulate chemoattractant-induced superoxide production in human granulocytes. *J. Biol. Chem.* **261**:13662-13669.

Jesaitis, A.J., Tolley, J.O., Painter, R.G., Sklar, L.A., and Cochrane, C.G. (1985). Membrane-cytoskeleton interactions and the regulation of chemotactic

peptide-induced activation of human granulocytes; the effects of dihydrocytochalasin B. *J. Cell. Biochem.* **27**:241-253.

Johnston, R.B. Jr., Keele, B.B. Jr., Misra, H.P., Lehmeyer, J.E., Webb, L.S., Baehner, R.L. and Rajagopalan, K.V. (1975). The role of superoxide anion generation in phagocytic bactericidal activity: Studies with normal and chronic granulomatous disease leukocytes. *J. Clin. Invest.* **55**:1357-1372.

Jubiz, W., Radmark, O., Malmsten, C., Hansson, G., Lindgren, J.A., Palmblad, J., Uden, A.m., and Samuelsson, B. (1982). A novel leukotriene produced by stimulation of leukocytes with formylmethionylleucylphenylalanine. *J.Biol. Chem.* **257**:6106-6110.

Kajdacsy-Balla, A.A., and Mendes, N.F. (1976). Rosétte formation between human B lymphocytes and zymosan-C3 complexes using different complement sources. *J. Immunol. Methods* **9**:205-209.

Kakinuma, K. (1970). Metabolic and intracellular pH during phagocytosis by polymorphonuclear leucocytes. *J. Biochem.* **68**:177-185.

Kakinuma, K., Kaneda, M., Chiba, T. and Ohnishi, T. (1986). Electron spin resonance studies on a flavoprotein in neutrophil plasma membranes: redox potentials of the flavin and its participation in NADPH oxidase. *J. Biol. Chem.* **261**:9426-9432.

Kane, S.P. and Peters, T.J. (1975). Analytical subcellular fractionation of human granulocytes with special reference to the localization of vitamin B_{12} binding proteins. *Clin. Sci. Mol. Med.* **49(suppl.)**:171-182.

Kasha, M. and Khan, A.U. (1970). The physics, chemistry and biology of singlet molecular oxygen. *Ann. N.Y. Acad. Sci.* **171**:5-23.

Kawakami, M. and Cerami, A. (1981). Studies of endotoxin-induced decrease in lipoprotein lipase activity. *J. Exp. Med.* **154**:631-639.

Kawakami, M., Murase, T., Ogawa, H., Ishibashi, S., Mori, N., Takaku, F., and Shibata, S. (1987). Human recombinant TNF suppresses lipoprotein lipase activity and stimulates lipolysis in 3T3-L1 cells. *J. Biochem. (Tokyo).* **101**:331-338.

Kawakami, M., Pekala, P.H., Lane M.D., and Cerami, A. (1982). Lipoprotein lipase suppression in 3T3-L1 cells by an endotoxin-induced mediator from exudate cells. *Proc. Natl. Acad. Sci. USA* **79**:912-916.

Kearns, D.R. (1979). Solvent and solvent isotope effects on the lifetime of singlet oxygen. In: *Singlet Oxygen*, edited by H.H. Wasserman and R.W. Murray, pp. 115-137. Academic Press, New York.

Keller, H.U., Naef, A. and Zimmerman, A. (1984). Effects of colchicine, vinblastine, and nocodazole on polarity, motility, chemotaxis, and cAMP levels of human polymorphonuclear leukocytes. *Exp. Cell. Res.* **153**:173-185.

Keller, H.U., Wilkinson, P.C., Abercombie, M., Becker,E.L., Hirsch, J.G., Miller, M.E., Ramsey, W.S. and Zigmond, S.H. (1977). A proposal for the definition of terms related to locomotion of leucocytes and other cells. *Clin. Exp. Immunol.* **27**:377-380.

Khan, A.U. (1978). Activated oxygen: singlet molecular oxygen and superoxide anion. *Photochem. Photobiol.* **28**:615-627.

Klass, H.J., Hopkins, J., Neale, G., and Peters, T.J. (1977). The estimation of serum lysosyme: a comparison of four assay methods. *Biochem. Med.* **18**:52-57.

Klebanoff, S.J. (1967). Iodination of bacteria: a bactericidal mechanism. *J. Exp. Med.* **126**:1063-1078.

Klebanoff, S.J. (1968). Myeloperoxidase-halide-hydrogen peroxide antimicrobial system. *J. Bacteriol.* **95**:2131-2138.

Klebanoff, S.J. (1977). Estrogen binding by leukocytes during phagocytosis. *J. Exp. Med.* **145**:983-998.

Klebanoff, S.J. (1982). The iron-H_2O_2-iodide cytotoxic system. *J. Exp. Med.* **156**:1262-1267.

Klebanoff, S.J. (1988). Phagocytic cells: products of oxygen metabolism. In: *Inflammation: Basic Principles and Clinical Correlates.* edited by J.I. Gallin, I.M. Goldstein and R. Snyderman. pp. 391-444, Raven Press, New York.

Klebanoff, S.J., and Clark,R.A. (1978). Chediak-Higashi syndrome. In: *The Neutrophil: Function and Clinical Disorders*. pp 735-783, North-Holland Publishing Company.

Klebanoff, S.J., and Green, W.L. (1973). Degradation of thyroid hormones by phagocytosing human leukocytes. *J. Clin. Invest.* **52**:60-72.

Klebanoff, S.J., and Hamon, C.B. (1972). Role of myeloperoxidase-mediated antimicrobial systems in intact leukocytes. *J. Reticuloendothel. Soc.* **12**:170-196.

Klebanoff, S.J., and White, L.R. (1969). Iodination defects in the leukocytes of a patient with chronic granulomatous disease of childhood. *N. Engl. J. Med.* **280**:460-466.

Klebanoff, S.J., Clem, W.H. and Leubke, R.G. (1966). The peroxidase-thiocyanate-hydrogen peroxide antimicrobial system. *Biochim. Biophys. Acta.* **117**:63-72.

Klebanoff, S.J., Vadas, M.A., Harlan, J.M., Sparks, L.H., Gamble, J.R., Agosti, J.M. and Waltersdorph, A.M. (1986). Stimulation of neutrophils by tumor necrosis factor. *J. Immunol.* **136**:4220-4225.

Klempner, M.S., Dinarello, C.A. and Gallin, J.I. (1978). Human leukocyte pyrogen induces release of specific granule contents from human neutrophils. *J. Clin. Invest.* **61**:1330-1336.

Koo, C., Lefkowitz, R.J. and Snyderman, R. (1983). Guanine nucleotides modulate the binding affinity of the oligopeptide chemoattractant receptor on human polymorphonuclear leukocytes. *J. Clin. Invest.* **72**:748-753.

Koo, C., Lefkowtiz, R.J. and Snyderman, R. (1982). The oligopeptide chemotactic factor receptor on human polymorphonuclear leukocyte membranes exists in two affinity states. *Biochem. Biophys. Res. Comm.* **106**:442-449.

Kopaniak, M.M. and Movat, H.Z. (1983). Kinetics of acute inflammation induced by *Escherichia coli* in rabbits. II. The effect of hyperimmunization, complement depletion, and depletion of leukocytes. *Am. J. Pathol.* **110**:13-29.

Korchak, H.M., Wilkenfeld, C., Rich, A.M., Radin, A.R., Vienne, K., and Rutherford,L.E. (1984). Stimulus response coupling in the human neutrophil. Differential requirements for receptor occupancy in neutrophil responses to a chemoattractant. *J. Biol. Chem.* **259**:7439-7445.

Krane, S.M., Dayer, J.-M., Simon, L.S., and Byrne, S. (1985). Mononuclear cell conditioned medium containing mononuclear cell factor (MCF), homologous with interleukin-1, stimulates collagen and fibronectin synthesis by adherent rheumatoid synovial cells: Effects of prostaglandin E_2 and indomethacin. *Collagen Related Res.* **5**:99-117.

Kreutzer, D.L., O'Flaherty, J.T., Orr, W., Showell, H.J., Ward, P.A. and Becker, E.L. (1978). Quantitative comparisons of various biological responses of neutrophils to different active and inactive chemotactic factors. *Immunopharmacol.* 1:39-47.

Kronheim, S.R., March, C.J., Erb, S.K., Conlon, P.J., Mochizuki, D.Y., and Hopp, T.P. (1985). Human interleukin 1. Purification to homogeneity. *J. Exp. Med.* **161**:490-502.

Lachman, L.B. (1983). Interleukin-1; release from LPS-stimulated mononuclear phagocytes. In: *Beneficial Effects of Endotoxin.* edited by A. Nowotny, pp.283-305, Plenum Press, New York.

Lackie, J.M. and Lawrence, A.J. (1987). Signal response transduction in rabbit neutrophil leucocytes. The effects of exogenous phospholipase A_2 suggests two pathways exist. *Biochem. Pharmacol.* **36**(12):1941-5.

Lad, P.M., Olson, C.V. and Smiley, P.A. (1985). Association of the N-formyl-Met-Leu-Phe receptor in human neutrophils with a GTP-binding protein sensitive to pertussis toxin. *Proc. Natl. Acad. Sci. USA* **82**:869-873.

Lad, P.M., Olson, C.V., Grewal, I.S., Scott, S.J., Learn, D.B., Smiley, P.A., and Lafrance-Flolich, M. (1987). Molecular mechanisms in the action of platelet-activating factor and other mediators of inflammation. In: *New Horizons in Platelet Activating Factor Research.* edited by C.M. Winslow and J.L.Lee, pp. 103-122. John Wiley and Sons, New York.

Lane, B., and Snyderman, R. (1984). High and low affinity binding sites for oligopeptide chemoattractants in detergent extracts of human neutrophil membranes (abstr.) *Fed. Proc.* **43**:1417A

Lassing, I. and Lindberg, U. (1985). Specific interaction between phosphatidyl inositol-4,5-biphosphate and profilactin. *Nature (Lond.)* **314**:472-473.

Law, S.K., Lichtenberg, N.A., Levine, R.P. (1979). Evidence for an ester linkage between the labile binding site of C3b and receptive surfaces. *J. Immunol.* **123**:1388-1394.

Lee, C.W., Lewis, R.A., Corey, E.J., and Austen, K.F. (1983). Conversion of leukotriene D_4 to leukotriene E_4 by a dipeptidase released from the specific granule of human polymorphonuclear leukocytes. *Immunology* **48**:27-35.

Lee, M.D., Zentella, A., Pekala, P.H., and Cerami, A. (1987). Effect of endotoxin-induced monokines on glucose metabolism in the muscle cell line L6. *Proc. Natl. Acad. Sci. USA* **84**:2590-2594.

Leffell, M.S. and Spitznagel, J.K. (1975). Fate of human lactoferrin and mycloperoxidase in phagocytizing human neutrophils: effects of immunoglobulin G subclasses and immune complexes on coated latex beads. *Infect. Immun.* **12**:813-820.

Lehmeyer, J.E., Snyderman, R., and Johnston, R.B., Jr. (1979). Stimulation of neutrophil oxidative metabolism by chemotactic peptides: influence of calcium ion concentration and cytochalasin B and comparison with stimulation by phorbol myristate acetate. *Blood* **54**:35-45.

Lehrer, R.I., Goldberg, L.S., Apple, M.A., and Rosenthal, N.P. (1972). Refractory megaloblastic anemia with myeloperoxidase-deficient neutrophils. *Ann. Intern. Med.* **76**:447-453.

Lehrer, R.I., and Cline, M.J. (1969). Leukocyte myeloperoxidase deficiency and disseminated candidiasis: The role of myeloperoxidase in resistance to *Candida* infection. *J. Clin. Invest.* **48**:1478-1488.

Levin, J. and Bang, F.B. (1968). Clottable proteins in Limulus: its localisation and kinetics of its coagulation by endotoxin. *Thromb. Diath. Haemorrh.* **19**:186-187.

Levitz, S.M. and Diamond, R.D. (1984). Killing of *Aspergillus fumigatus* spores and *Candida albicans* yeast phase by the iron-hydrogen peroxide-iodide cytotoxic system: comparison with the myeloperoxidase-hydrogen peroxide-halide system. *Infect. Immun.* **43**:1100-1102.

Lew, P.D., Monod, A., Waldvogel, F.A., Dewald, B., Baggiolini, M., and Pozzan, T. (1986). Quantitative analysis of the cytosolic free calcium dependency of exocytosis from three subcellular compartments in intact human neutrophils. *J. Cell. Biol.* **102**:2197-2204.

Lewis, R.A., and Austen, K.F. (1984). The biologically acive leukotrienes.: Biosynthesis, metabolism, receptors, function and pharmacology. *J. Clin. Invest.* **73**:889-897.

Libby, P., Warner, S.J.C., and Galin, C.B. (1987). Human vascular smooth muscle can transcribe the tumor necrosis factor/cachectin gene and respond to this inflammatory mediator. *Clin. Res.* **35**:297A.

Lohr, R.M. and Snyderman, R. (1982). Amphotericin B alters the affinity and functional activity of the oligopeptide chemotactic factor receptor on human polymorphonuclear leukocytes. *J. Immunol.* **129**:1594-1599.

Lomedico, P.T., Gubler, U., Hellman, C.P., Dukovich, M., Gery, J.G., Pan, Y.E., Collier, K., Semionow, R., Chua, A.O., and Mizel, S.B. (1984). Cloning and

expression of murine interleukin-1 in *Escherichia coli*. *Nature (Lond.)* **312**:458-462.

Lopez, A.F. and Vadas, M.A. (1984). Stimulation of human granulocyte function by monoclonal antibody WEM-G1. *Proc. Natl. Acad. Sci. USA.* **81**:1818-1821.

Lopez, A.F., Begley, G., Andrews, P., Butterworth, A.E. and Vadas, M.A. (1985). Identification of a human granulocyte functional antigen (GFA-2). involved in antibody-dependent cell-mediated cytotoxicity and phagocytosis. *J. Immunol.* **134**:3969-3977.

Lopez, A.F., Dyson, P.,G., Bik To, L., Elliott, M.J., Milton, S.E., Russell, J.A., Juttner, C.A., Yang, Y-C., Clark, S.C. and Vadas, M.A. (1988). Recombinant human interleukin-3 stimulation of hematopoiesis in humans: loss of responsiveness with differentiation in the neutrophilic myeloid series. *Blood* **72**(5):1797-1804.

Lopez, A.F., Nicola, N.A., Burgess, A.W., Metcalf, D., Battye, F.L., Sewell, W. and Vadas, M.A. (1983). Activation of granulocyte cytotoxic function by purified mouse colony-stimulating factors. *J. Immunol.* **131**:2983-2988.

Lopez, A.F., Williamson, D.J., Gamble, J.R., Begley, C.G., Harlan, J.M., Klebanoff, S.J., Waltersdorph, A., Wong, G., Clark, S.C. and Vadas, M.A. (1986). A recombinant human granulocyte-macrophage colony-stimulating factor (rH GM-CSF) stimulates .. mature human neutrophil and eosinophil function, surface receptor expression and survival. *J. Clin. Invest.* **78**:1220-1228.

Ludwig, J.C., and Pinckard, R.N. (1987). Diversity in the chemical structures of neutrophil-derived platelet-activating factors. In: *New Horizons in Platelet Activating Factor Research.* edited by C.M. Winslow and J.L.Lee, pp. 59-71. John Wiley and Sons, New York.

Ludwig, J.C., Hoppens, C., McManus, L.M., Mott, G.E., and Pinckard, R.N. (1985). Modulation of platelet-activating factor (PAF). synthesis and release from human polymorphonuclear leukocytes (PMN): Role of extracellular albumin. *Arch. Biochem. Biophys.* **241**:337-347.

Ludwig, J.C., McManus, L.M. and Pinckard, R.N. (1986). Synthesis-release coupling of platelet-activating factors (PAF). from stimulated human neutrophils. *Adv. Inflam. Res.* **11**:111-125.

Ludwig, J.C., McManus, L.M., Clark, P.O., Hanahan, D.J., and Pinckard, R.N. (1984). Modulation of platelet-activating factor (PAF) synthesis and release from human polymorphonuclear leukocytes (PMN): Role of extracellular calcium. *Arch. Biochem. Biophys.* **232**:102-110.

Luger, T.A., Charon, J.A., Calot, M., Mieksche, M. and Oppenheim, J.J. (1983). Chemotactic properties of partially purified human epidermal cell derived thymocyte activating factor (ETAF). for polymorphonuclear leukocytes and mononuclear cells. *J. Immunol.* **131**:816-820.

Lusis, A.J., Quon, D.H., and Golde, D.W. (1981). Purification and characterization of a human T-lymphocyte-derived granulocyte-macrophage colony-stimulating factor. *Blood* **57**:13-21.

Lutter, R., van Schaik, M.J.L., van Zwieten, R., Weever, R., Roos, D. and Halmers, M.N.J. (1985). Purification and partial characterization of the b-type cytochrome from human polymorphonuclear leukocytes. *J. Biol. Chem.* **260**:2237-2244.

Lutter, R., van Zwicten, R., Weening, R.S., Halmer, M.N., and Roos, D. (1985). Cytochrome b, flavins and ubiquinone-50 in enucleated human neutrophils (polymorphonuclear leukocyte cytoplasts). *J. Biol. Chem.* **259**:9603-9606.

MacGlashin, D.W., Jr., Peters, S.P., Warner, J., and Lichtenstein, L.M. (1986). Characteristics of human basophil sulfidopeptide leukotriene release; Relcasability defined as the ability of the basophil to respond to dimeric cross-links. *J. Immunol.* **136**:2231-2239.

Mackin, W.M., Huang, C-K. and Becker, E.L. (1982). The formylpeptide chemotactic receptor on rabbit peritoneal neutrophils. 1. Evidence for two binding sites with different affinities. *J. Immunol.* **129**:1608-1611.

Mackin, W.M., Michaels, M., Naccache, P., Huang, C-K., Bormann, B.J. and Becker, E.L. (1983). Effects of dithiothreitol (DTT). on rabbit neutrophil formylpeptide binding sites and biological responsiveness. *Fed. Proc.* **42**:1036a.

Malech, H.L., Root, R.K., and Gallin, J.I. (1977). Structural analysis of human neutrophil migration. Centriole, microtubule and microfilament orientation and function during chemotaxis. *J. Cell. Biol.* **75**:666-693.

Malawista, S.E., and Bensch, K.G. (1967). Human polymorphonuclear leukocytes: Demonstration of microtubules and effect of colchicine. *Science* **156**:521-522.

Mandell, G.L. (1970). Intraphagosomal pH of human polymorphonuclear neutrophils. *Proc. Soc. Exp. Biol. Med.* **134**:447-449.

Mandell, G.L. (1974). Bactericidal activity of aerobic and anaerobic polymorphonuclear neutrophils. *Infect. Immun.* **9**:337-341.

Marasco, W.A., Becker, E.L., Feltner, D.E., Brown, C.S., Ward, P.A., and Nairn, R. (1985). Covalent affinity labeling, detergent solubilization, and fluid-phase characterization of the rabbit neutrophil formyl peptide chemotaxis receptor. *Biochemistry* **24**:2227-2236.

Marasco, W.A., Feltner, D.E. and Ward, P.A. (1985). Formyl peptide chemotaxis receptors on the rat neutrophil: experimental evidence for negative cooperativity. *J. Cell. Biochem.* **27**:359-375.

Marasco, W.A., Phan, S.H., Krutzsch, H., Showell, H.J., Feltner, D.E., Nairn, R., Becker, E.L. and Ward, P.A. (1984). Purification and identification of formyl-methionyl-leucyl-phenylalanine as the major peptide neutrophil chemotactic factor produced by *Escherichia coli*. *J. Biol. Chem.* **259**:5430-5439.

Markert, M., Glass, G.A. and Babior, B.M. (1985). Respiratory burst oxidase from human neutrophils: purification and some properties. *Proc. Natl. Acad. Sci. USA* **82**:3144-3148.

Masson, P.L., Heremans, J.F., and Schonne, E. (1969). Lactoferrin, an iron-binding protein in neutrophilic leukocytes. *J. Exp. Med.* **130**:643-656.

Mathison, J.C., and Ulevitch, R.J. (1979). The clearance, tissue distribution, and cellular localization of intravenously injected lipopolysaccharide in rabbits. *J. Immunol.* **123**:2133-2143.

McAdam, K.P.W.J., and Dinarello, C.A. (1980). Induction of serum amyloid A synthesis by human leukocyte pyrogen. In: *Bacterial Endotoxins and Host Response.* edited by M.K. Agawal, pp. 167-178. Elsevier/North Holland Press, Amsterdam.

McCord, J.M. and Day, E.D. Jr. (1978). Superoxide-dependent production of hydroxyl radical catalyzed by iron-EDTA complex. *FEBS Lett.* **86**:139-142.

McCord, J.M., Crapo, J.D. and Fridovich, I. (1977). Superoxide dismutase assays: a review of methodology. In: *Superoxide and Superoxide Dismutase, 1st edn.* edited by A.M. Michelson, J.M. McCord, and I. Fridovich. p.1. Academic Press, New York.

McGee, J.E., and Fitzpatrick, F.A. (1986). Erythrocyte-neutrophil interactions: Formation of leukotriene B_4 by transcellular biosynthesis. *Proc. Natl. Acad. Sci. USA* **83**:1349-1353.

McIntyre, T.M., Zimmerman, G.A., and Prescott, S.A. (1986). Leukotrienes C_4 and D_4 stimulate human endothelial cells to synthesize platelet-activating factor and bind neutrophils. *Proc. Natl. Acad. Sci. USA* **83**:2204-2208.

McManus, L.M., Hanahan, D.J., Demopoulos, C.A., and Pinckard, R.N. (1980). Pathobiology of the intravenous infusion of acetyl glyceryl ether phosphorylcholine (AGPEC), a synthetic platelet-activating factor (PAF), in the rabbit. *J. Immunol.* **124**:2919-2924.

McPhail, L.C., Clayton, C.C., and Snyderman, R. (1984). The NADPH oxidase of human polymorphonuclear leukocytes. Evidence for regulation by multiple signals. *J. Biol. Chem.* **259**:5768-5775.

McPherson, G.A. (1985). Analysis of radioligand binding experiments: A collection of computer programmes for the IBM PC. *J. Pharmacol. Methods* **14**:213-228.

Mehta, J. and Spilberg, I. (1983). Heterologous receptor population for a chemotactic factor f-met-leu-phe on the human neutrophil. Effect of pH and temperature. *Inflammation* **7**:301-309.

Melamed, J., Arnaout, M.A., and Colten, H.R. (1982). Complement (C3b). interaction with the human granulocyte receptor: Correlation of binding of fluid-phase radiolabelled ligand with histamine release. *J. Immunol.* **128**:2313-2318.

Metcalf, D. (1984). *The Hemopoietic Colony-stimulating Factors*. pp. 1-486, Elsevier, Amsterdam.

Metcalf, D., Begley, C.G., Johnson, G.R., Nicola, N.A., Vadas, M.A., Lopez, A.F., Williamson, S.J., Wong, G.G., Clark, S.C. and Wang, E.A. (1986). Biological

properties *in vitro* of a recombinant human granulocyte-macrophage colony stimulating factor. *Blood* **67**:37-45.

Miller, T.E. (1968). Killing and lysis of gram-negative bacteria through the synergistic effect of hydrogen peroxide, ascorbic acid, and lysozyme. *J. Bacteriol.* **98**:949-955.

Min, H.Y., and Spiegelman, B.M. (1986). Adipsin, the adipocyte serine protease: gene structure and control of expression by tumor necrosis factor. *Nucleic Acids Res.* **14**:8879-8892.

Mollinedo, F. and Schneider, D.L. (1984). Subcellular localization of cytochrome b and ubiquinone in a tertiary granule of resting human neutrophils and evidence for a proton pump ATPase. *J. Biol. Chem.* **259**:7143-7150.

Molski, T.F.P., Naccache, P.H., Marsh, M.L., Kermode, J., Becker, E.L. and Sha'afi, R.I. (1984). Pertussis toxin inhibits the rise in the intracellular concentration of free calcium that is induced by chemotactic factors in rabbit neutrophils: Possible role of the "G proteins" in calcium mobilization. *Biochem. Biophys. Res. Comm.* **124**:644-650.

Morrison, D.C., and Kline, L.F. (1977). Activation of the classical and properdin pathways of complement by bacterial lipopolysaccharide (LPS). *J. Immunol.* **118**:362-368.

Movat, H.Z., Burrowes, C.E., Cybulsky, M.I., and Dinarello, C.A. (1987a). Role of complement, Interleukin-1 and tumor necrosis factor in a local Shwartzman-

like reaction. In: *Leukocyte Emigration and Its Sequelae*. edited by H.Z. Movat, pp 69-78. Karger, Basel, Switzerland.

Movat, H.Z., Cybulksy, M.I., Colditz, I.G., Chan, M.K.W. and Dinarello, C.A. (1987b). Acute inflammation in gram-negative infection: endotoxin, interleukin 1, tumor necrosis factor, and neutrophils. *Fed. Proc.* **46**(1):97-104.

Mrowietz, U., Schroder, J-M. and Christophers, E. (1988). Recombinant human tumor necrosis factor alpha lacks chemotactic activity for human peripheral blood neutrophils and monocytes. *Biochem. Biophys. Res. Comm.* **153**(3):1223-1228.

Mueller, H.W., O'Flaherty, J.T., Greene, D.G., Samuel, M.P. and Wykle, R.L. (1984). 1-*O*-alkyl-linked glycerophospholipids of human neutrophils: Distribution of arachidonate and other acyl residues in the ether-linked and diacyl species. *J. Lipid Res.* **25**:383-388.

Müller-Eberhard, H.J. (1986). The membrane attack complex of complement. *Annu. Rev. Immunol.* **4**:503-528.

Munker, R., Di Persio, J. and Koeffler, H.P. (1987). Tumor necrosis factor: receptors on hemopoietic cells. *Blood* **70**(6):1730-1734.

Munker, R., Gasson, J., Ogawa, M., and Koeffler, H.P. (1986). Recombinant human tumor necrosis factor induces production of granulocyte-monocyte colony

stimulating factor mRNA and protein from lung fibroblasts and vascular endothelial cells *in vitro*. *Nature (Lond.)* **323**:79-82.

Munson, P.J. and Rodbard, D. (1980). LIGAND: A versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* **107**:220-239.

Murphy, M., Loudon, R., Kobayashi, M., and Trinchieri, G. (1986). Gamma interferon and lymphotoxin, released by activated T cells, synergise to inhibit granulocyte/monocyte colony formation. *J. Exp. Med.* **164**:263-279.

Naccache, P.H., Faucher, N., Borgeat, P., and Gasson, J.C., Di Persio, J.F. (1988). Granulocyte-macrophage colony-stimulating factor modulates the excitation-response coupling sequence in human neutrophils. *J. Immunol.* **140**:3541-3546.

Naccache, P.H., Molski, T.F.P., Borgeat, P., White, J.R. and Sha'afi, R.I. (1985). Phorbol esters inhibit the f Met-Leu-Phe- and leukotriene B_4-stimulated calcium mobilization and enzyme secretion in rabbit neutrophils. *J. Biol. Chem.* **260**:2125-2131.

Nagano, T. and Fridovich, I. (1985). Does the aerobic xanthine oxidase reaction generate singlet oxygen? *Photochem. Photobiol.* **41**:33-37.

Nagata, S., Tsuchiya, M., Asano, S., Kaziro, Y., Yamazaki, T., Yamamoto, O., Hirata, Y., Kubota, N., Oheda, M., Nomura, H., and Ono, M. (1986). Molecular cloning and expression of cDNA for human granulocyte colony-stimulating factor. *Nature (Lond.)* **319**:415-418.

Nakamura, M., Baxter, C.R. and Masters, B.S.S. (1981). Simultaneous demonstration of phagocytosis-connected oxygen consumption and corresponding NAD(P)H oxidase activity: direct evidence for NADPH as the predominant electron donor to oxygen in phagocytozing human neutrophils. *Biochem. Biophys. Res. Commun.* **98**:743-751.

Nathan, C.F. (1987). Neutrophil activation on biological surfaces. Massive secretion of hydrogen peroxide in response to products of macrophages and lymphocytes. *J. Clin. Invest.* **80**:1550-1560.

Nawroth, P.P., and Stern, D.M. (1986). Modulation of endothelial cell hemostatic properties by tumor necrosis factor. *J. Exp. Med.* **163**:740-745.

Nawroth, P.P., Bank, I., Handley, D., Cassimeris, J., Chess, L., and Stern, D. (1986). Tumor necrosis factor/cachectin interacts with endothelial cell receptors to induce release of interleukin 1. *J. Exp. Med.* **163**:1363-1375.

Nelson, R.D., Quie, P.G. and Simmons, R.L. (1975). Chemotaxis under agarose: A new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes. *J. Immunol.* **115**:1650-1656.

Newburger, P.E., Speier, C., Borregard, N., Walsh, C.E., Whitin, J.C. and Simons, E.R. (1984). Development of the superoxide-generating system during differentiation of the HL-60 human promyelocytic leukemia cell line. *J. Biol. Chem.* **259**:3771-3776.

Newton, N., Morell, D.B., Clarke, L. and Clezy, P.S. (1965). The haem prosthetic groups of some animal peroxidases. II. Myeloperoxidase. *Biochim. Biophys. Acta.* **96**:476-486.

Ng, D.S., and Wong, K. (1986). GTP regulation of platelet-activating factor binding to human neutrophil membranes. *Biochem. Biophys. Res. Commun.* **141**:353-359.

Nicola, N., and Metcalf, D. (1984). Binding of the differentiation-inducer, granulocyte colony-stimulating factor, to responsive but not unresponsive leukemic cell lines. *Proc. Natl. Acad. Sci. USA* **81**:3765-3769.

Niedel, J., Davis, J., and Cuatrecasas, P. (1980). Covalent affinity labelling of formyl peptide chemotactic receptors. *J. Biol. Chem.* **255**:7063-7066.

Niedel, J., Wilkinson, S. and Cuatrecasas, P. (1979). Receptor-mediated uptake and degradation of ^{125}I-chemotactic peptide by human neutrophils. *J. Biol. Chem.* **254**:10700-10706.

Nilsson, R. and Kearns, D.R. (1974). Role of singlet oxygen in some chemiluminescence and enzyme oxidation reactions. *J. Phys. Chem.* **78**:1681-1683.

Nishizuka, Y. (1986). Studies and perspectives of protein kinase C. *Science* **233**:305-312.

Nomura, H., Imazeki, I., Oheda, M., Kubota, N., Tamura, M., Ono, M., Ueyama, Y., and Asano,S. (1986). Purification and characterization of human granulocyte colony-stimulating factor (G-CSF). *EMBO J.* **5**:871-876.

Nüße, O., and Lindau, M. (1988). The dynamics of exocytosis in human neutrophils. *J. Cell Biol.* **107**:2117-2123.

O'Flaherty, J.T. (1985). Neutrophil degranulation: evidence pertaining to its mediation by the combined effects of leukotriene B_4, platelet-activating factor, and 5-HETE. *J. Cell. Physiol.* **122**:229-239.

O'Flaherty, J.T., Surles, J.R., Redman, J., Jacobson, D., Piantadosi, C., and Wykle, R. (1986). Binding and metabolism of platelet-activating factor by human neutrophils. *J. Clin. Invest.* **78**:381-388.

O'Flaherty, J.T., and Nishihira, J. (1987). Arachidonite metabolites, platelet-activating factor, and the mobilization of protein kinase C in human polymorphonuclear neutrophils. *J. Immunol.* **138**:1889-1895.

O'Flaherty, J.T., Schmitt, J.D. and Wykle, R.L. (1985). Interactions of arachidonate metabolism and protein kinase C in mediating neutrophil function. *Biochem. Biophys. Res. Commun.* **127**:916-923.

O'Shea, J.J., Brown, E.J., Seligmann, E., Metcalf, J.A., Frank, M.M. and Gallin, J.I. (1985). Evidence for distinct intracellular pools of receptors for C3b and C3bi in human neutrophils. *J. Immunol.* **134**:2580-2587.

Odajima, T. and Yamazaki, I. (1970). Myeloperoxidase of the leukocyte of normal blood. I. Reaction of myeloperoxidase with hydrogen peroxide. *Biochim. Biophys. Acta.* **206**:71-77.

Odeberg, H. and Olsson, I. (1975). Antibacterial activity of cationic proteins from human granulocytes. *J. Clin. Invest.* **56**:1118-1124.

Ohno, Y., Seligmann, B.E. and Gallin, J.I. (1985). Cytochrome b_2 translocation to human neutrophil plasma membranes and superoxide release: differential effects of N-formylmethionylleucylphenylalanine, phorbol myristate acetate and A23187. *J. Biol. Chem.* **260**:2409-2415.

Ohno, Y., Buescher, E.S., Roberts, R., Metcalf, J.A. and Gallin, J. (1986). Reevaluation of cytochrome b and flavin adenine dinucleotide in neutrophils from patients with chronic granulomatous disease and description of a family with probably autosomal recessive inheritance of cytochrome b deficiency. *Blood* **67**:1132-1138.

Okajima, F., Katada, T. and Ui, M. (1985). Coupling of guanine nucleotide regulatory protein to chemotactic peptide receptors in neutrophil membranes and its uncoupling by islet-activating protein, pertussis toxin. *J. Biol. Chem.* **260**:6761-6768.

Oku, N., Araki, R., Araki, H., Shibamoto, S., Ito, F., Nishitiara, T. and Tsujimoto, M. (1987). Tumor necrosis factor-induced permability increase of negatively charged phospholipid vesicles. *J. Biochem. (Tokyo).* **102**(5):1303-1310.

Oliver, J.M., Lalchandani, R., and Becker, E.L. (1977). Actin redistribution during concanavilin A cap formation in rabbit neutrophils. *J. Reticuloendothelial. Soc.* **21**:359-364.

Omann, G.M., Rodger, A.A., Bokoch, G.M., Painter, R.G., Traynor, A.E. and Sklar, L.A. (1987). Signal transduction and cytoskeletal activation in the neutrophil. *Physiol. Rev.* **67**(1):285-322.

Painter, R.G., Schmitt, M., Jesaitis, A.J., Sklar, L.A., Preissner, K., and Cochrane, C.G. (1982). Photoaffinity labelling of the *N*-formyl peptide receptor of human polymorphonuclear leukocytes. *J. Cell. Biochem.* **20**:203-214.

Parente, L., and Flower, R.J. (1985). Hydrocortisone and 'macrocortin' inhibit the zymosan-induced release of lyso-PAF from rat peritoneal leucocytes. *Life Sci.* **36**:1225-1231.

Parmley, R.T., Takagi, M., Barton, J.C., Boxer, L.A. and Austin, R.L. (1982). Ultrastructural localization of lactoferrin and iron-binding protein in human neutrophils and rabbit heterophils. *Am. J. Path.* **109**:343-358.

Payan, D.G., and Goetzl, E.J. (1984). Recognition of leukotriene B_4 by a unique subset of human T-lymphocytes. *J. Allergy Clin. Immunol.* **74**:403-406.

Pekala, P.H., Kawakami, M., Angus, C.W., Lane, M.D., and Cerami, A. (1983). Selctive inhibition of synthesis of enzymes for *de novo* fatty acid biosynthesis by an endotoxin-induced mediator from exudate cells. *Proc. Natl. Acad. Sci. USA* **80**:2743-2747.

Penfield, A. and Dale, M.M. (1984). Synergism between A23187 and 1-oleoyl-2-acetyl-glycerol in superoxide production by human neutrophils. *Biochem. Biophys. Res. Commun.* **125**:332-336.

Pennica, D., Hayflick, J.S., Bringman, T.S., Palladino, M.A. and Goeddel, D.V. (1985). Cloning and expression in *Escherichia coli* of the cDNA for murine tumor necrosis factor. *Proc. Natl. Acad. Sci. USA* **82**:6060-6064.

Pennica, D., Nedwin, G.E., Hayflick, J.S., Seeburg, P.H., Derynck, R., Palladino, M.A., Kohr, W.J., Aggarwal, B.B. and Goeddel, D.V. (1984). Human tumor necrosis factor: precursor structure, expression and homology to lymphotoxin. *Nature (Lond.)* **312**:724-729.

Perez, H.D., Goldstein, I.M., Webster, R.O., and Henson, P.M. (1981). Enhancement of chemotactic activity of human C5a des Arg by an anionic polypeptide ("cochemotaxin") in normal serum and plasma. *J. Immunol.* **126**:800-804.

Perlmutter, D.H., Dinarello, C.A., Punsal, P.I., Colten, H.R. (1986). Cachectin/tumor necrosis factor regulates hepatic acute-phase gene expression. *J. Clin. Invest.* **78**:1349-1354.

Peveri, P., Walz, A., Dewald, B., and Baggiolini, M. (1988). A novel neutrophil-activating factor produced by human mononuclear phagocytes. *J. Exp. Med.* **167**:1547-1559.

Pinckard, R.N., McManus, L.M., Demopoulos, C.A., Halonen, M., Clark, P.O., Shaw, J.O., Kniker, W.T., and Hanahan, D.J. (1980). Molecular pathobiology of acetyl glyceryl ether phosphorylcholine (AGEPC): Evidence for the structural

identity with platelet-activating factor (PAF). *J. Reticuloendothel. Soc.* **28**:95s-103s.

Podd, F., Carpinelli, G., DiVito, M., Giannini, M., Proietti, E., Fiers, W., Gresser, I. and Bellardelli, F. (1987). Nuclear magnetic resonance analysis of tumour necrosis factor-α induced alterations of phospholipid metabolites and pH in friend leukemia cell tumors and fibrosarcomas in mice. *Cancer Res.* **47**:6481-6489.

Pollard, T.D. and Mooseker, M.S. (1981). Direct measurement of actin polymerisation rate constants by electron microscopy of actin filaments nucleated by isolated microvillus cores. *J. Cell. Biol.* **88**:654-659.

Pontremoli, S., Melloni, E., Michetti, M., Sacco, O., Salamino, F., Sparatore, B. and Horecker, B.L. (1986). Biochemical responses in activated human neutrophils mediated by protein kinase C and a Ca^{2+}-requiring proteinase. *J. Biol. Chem.* **261**:8309-8313.

Proctor, R.A. (1979). Endotoxin in vitro interactions with human neutrophils: depression of chemiluminescence, oxygen consumption, superoxide production, and killing. *Infect. Immun.* **25**:912-921.

Pryzwansky, K.B., Schliwa, M. and Porter, K.R. (1983). Comparison of three-dimensional organisation of unextracted and triton-extracted human neutrophilic polymorphonuclear leukocytes. *Eur. J. Cell. Biol.* **30**:112-125.

Quie, P.G., White, J.G., Holmes, B. and Good, R.A. (1967). *In vitro* bactericidal capacity of human polymorphonuclear leukocytes diminished activity in chronic granulomatous disease in childhood. *J. Clin. Invest.* **46**:668-679.

Radmark, O, Shimizu, T., Jornvall, M., and Samuelsson, B. (1984). Leukotriene A_4 hydrolase in human leukocytes: Purification and properties. *J. Biol. Chem.* **259**:12339-12345.

Renooij, W., and Snyder, R. (1981). Biosynthesis of 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (platelet activating factor and a hypotensive lipid) by cholinephosphotransferase in various rat tissues. *Biochim. Biophys. Acta.* **663**:545-556.

Repine, J.E., Eaton, J.W., Anders, M.W., Hoidal, J.R. and Fox, R.B. (1979). Generation of hydroxyl radical by enzymes, chemicals and human phagocytes *in vitro*: detection with the anti-inflammatory agent dimethyl sulfoxide. *J. Clin. Invest.* **64**:1642-1651.

Rest, R.F. and Spitznagel, J.K. (1977). Subcellular distribution of superoxide dismutases in human neutrophils: influence of myeloperoxidase on the measurement of superoxide dismutase activity. *Biochem. J.* **166**:145-153.

Rhyne, J.A., Mizel, S.B., Taylor, R.G., Chedid, M. and McCall, C.E. (1988). Characterization of human interleukin 1 receptor on human polymorphonuclear leukocytes. *Clin. Immunol. Immunopathol.* **48**:354-361.

Robb, R.J. (1986). Conversion of low-affinity interleukin 2 receptors to a high-affinity state following fusion of cell membranes. *Proc. Natl. Acad. Sci. USA* **83**:3992-3996.

Robb, R.J. and Rusk, C.M. (1986). High and low affinity receptors for interleukin 2: implications of pronase, phorbol ester, and cell membrane studies upon the basis for differential ligand affinities. *J. Immunol.* **137**:142-149.

Roberts, P.J., Cross, A.R., Jones, O.T.G. and Segal, A.W. (1982). Development of cytochrome b and an active oxidase system in association with maturation of a human promyelocytic (HL-60). cell line. *J. Cell. Biol.* **95**:720-726.

Robinson, J.M., Badwey, J.A., Karnovsky, M.L. and Karnovsky, M.J. (1985). Release of superoxide and change in morphology by neutrophils in response to phorbol esters: antagonism by inhibitors of calcium-binding proteins. *J. Cell. Biol.* **101**:1052-1058.

Rola-Pleszcynski, M., Borgeat, P., and Sirois, P. (1982). Leukotriene B_4 induces human suppressor lymphocytes. *Biochem. Biophys. Res. Commun.* **108**:1531-1537.

Roos, D., Hamers, M.N., van Zwieten, R. and Weening, R.S. (1983). Acidification of the phagocytic vacuole: a possible defect in chronic granulomatous disease? *Adv. Host. Def. Mech.* **3**:145-193.

Roos, D., Weening, R.S., Voetman, A.A., Vanschaik, M.L.J., Bot, A.A.M., Meerhof, L.J. and Loos, J.A. (1979). Protection of phagocytic leukocytes by

endogenous glutathione: Studies in a family with glutathione reductase deficiency. *Blood* **53**:851-866.

Roos, D., Weening, R.S., Wyss, S.R. and Aebi, H.E. (1980). Protection of human neutrophils by endogenous catalase: studies with cells from catalase-deficient individuals. *J. Clin. Invest.* **65**:1515-1522.

Root, R.K. and Cohen, M.S. (1981). The microbicidal mechanisms of human neutrophils and eosinophils. *Rev. Infect. Dis.* 3(3):565-598.

Rosen, H., and Klebanoff, S.J. (1976). Chemiluminescence and superoxide production by myeloperoxidase-deficient leukocytes. *J. Clin. Invest.* **58**:50-60.

Rossi, F., and Zatti, M. (1964). Biochemical aspects of phagocytosis in polymorphonuclear leukocytes: NADH and NADPH oxidation by the granules of resting and phagocytosing cells. *Experientia* **20**:21-23.

Roubin, R., Elsas, P.P., Fiers, W. and Dessein, A.J. (1987). Recombinant human tumor necrosis factor (rTNF). enhances leukotriene biosynthesis in neutrophils and eosinophils stimulated with the Ca^{2+} ionophore A23187. *Clin. Exp. Immunol.* **70**(2):484-90.

Rouzer, C.A., and Cerami, A. (1980). Hypertriglyceridemia associated with *Trypanasoma brucei* infection in rabbits: role of defective triglyceride removal. *Mol. Biochem. Parasitol.* 2:31-38.

Rouzer, C.A., and Samuelsson, B. (1985). On the nature of the 5-lipoxygenase reaction in human leukocytes: Enzyme purification and requirement for multiple stimulatory factors. *Proc. Natl. Acad. Sci. USA* **82**:6040-6044.

Runge, M.S., Laue, T.M., Yphantic, D.A., Lifsics, M.R., Saito, A., Altin, M., Reinke, K., and Williams, R.C., Jr. (1981). ATP-induced formation of an associated complex between microtubules and neurofilaments. *Proc. Natl. Acad. Sci. USA* **78**:1431-1435.

Sagone, A.L. Jr., Decker, M.A., Wells, R.M. and DeMocko, C. (1980). A new method for the detection of hydroxyl radical production by phagocytic cells. *Biochim. Biophys. Acta.* **628**:90-97.

Saklatvala, J. (1986). Tumor necrosis factor alpha stimulates reorption and inhibits synthesis of proteoglycan in cartilage. *Nature (Lond.)* **322**:547-549.

Saklatvala, J., Sarswfield, S.J., and Townsend, Y. (1985). Pig interleukin-1: Purification of two immunologically different leukocyte proteins that cause cartilage resorption, lymphocyte activation, and fever. *J. Exp. Med.* **162**:1208-1222.

Salton, M.R.J. (1957). The properties of lysozyme and its action on microorganisms. *Bacterial Rev.* **21**:82-99.

Sato, A., and Yoshimatsu, S. (1925). The peroxidase reaction in endemic encephalitis. A new diagnostic and prognostic method. *Amer. J. Dis. Child.* **29**:310-311.

Sawyer, D.T. and Valentine, J.S. (1981). How super is superoxide? *Acc. Chem. Res.* **14**:393-400.

Scatchard, G. (1949). The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* **51**:660-672.

Scheurich, P., Thoma, B., Ucer, U., Pfizenmaier, K. (1987). Immunoregulatory activity of recombinant human tumor necrosis factor (TNF)-alpha: induction of TNF receptors on human T cells and TNF-alpha-mediated enhancement of T cell responses. *J. Immunol.* **138**:1786-1790.

Schiffmann, E., Corcoran, B.A., and Wahl, S.M. (1975). *N*-formylmethionyl peptides as chemoattractants for leukocytes. *Proc. Natl. Acad. Sci. USA* **72**:1059-1062.

Schliwa, M., and van Blerkom, J. (1981). Structural interaction of cytoskeletal components. *J. Cell. Biol.* **90**:222-235.

Schliwa, M., Pryzwansky, K.B. and Euteneuer, U. (1982). Centrosome splitting in neutrophils: an unusual phenomenon related to cell activation and motility. *Cell* **31**:705-717.

Schmitt, M., Painter, R.G., Jesaitis, A.J., Preissner, K., Sklar, L.A., and Cochrane, C.G (1983). Photoaffinity labelling of the *N*-formyl peptide receptor binding site of intact human polymorphonuclear leukocytes. A label suitable for following the fate of the receptor-ligand complex. *J. Biol. Chem.* **258**:649-654.

Schneider, M., Pennington, R., Talmadge, J.E. (1987). Effect of rH TNF, rM IFN-γ, rH IL-2 and rM CSF-GM on stem cell activity in normal and leukopenic mice. *Fed. Proc.* **46**:1510a.

Schreiber, R.D., Pangburn, M.K., Bjornson, A.B., Brothers, M.A., and Müller-Eberhard, H.J. (1982). The role of C3 fragments in endocytosis and extracellular cytotoxic reactions by polymorphonuclear leukocytes. *Clin. Immunol. Immunopathol.* **23**:335-357.

Schultz, J. and Kaminker, K. (1962). Myeloperoxidase of the leukocyte of normal human blood. I. Content and localization. *Arch. Biochem. Biophys.* **96**:465-467.

Segal, A.W., and Peters, T.J. (1976). Characterization of the enzyme defect in chronic granulomatous disease. *Lancet* **1**:1363-1365.

Segal, A.W., Cross, A.R., Garcia, R.C., Borregard, N., Valerius, N.H., Soothill, J.F., and Jones, O.T. (1983). Absence of cytochrome $b\text{-}_{245}$ in chronic granulomatous disease; a multicenter European evaluation of its incidence and relevance. *N. Engl. J. Med.* **308**:245-251.

Segal, A.W., Jones, O.T.G., Webster, D. and Allison, A.C. (1978). Absence of a newly described cytochrome b from neutrophils of patients with chronic granulomatous disease. *Lancet* 2:446-449.

Seligmann, B.E., Fletcher, M.P. and Gallin, J.I. (1982). Adaptation of human neutrophil responsiveness to the chemo-attractant N-formylmethionyl-

leucylphenylalanine. Heterogeneity and/or negative cooperative interaction of receptors. *J. Biol. Chem.* **257**:6280-6286.

Sha'afi, R.I., Volpi, M. and Naccache, P.H. (1986). Differences between the effects of f Met-Leu-Phe and leukotriene B4 on phosphoinositol turnover and their relationship to calcium mobilization and protein kinase C activation. In: *Prostaglandins, Leukotrienes and Lipoxins.* edited by J. Martin, p.241, Plenum Press, New York.

Sha'afi, R.I., White, J.R., Molski, T.F.R., Shefcyk, J.,Volpi, M., Naccache, P.N. and Feinstein, N.B. (1983). Phorbol 12-myristate 13-acetate activates rabbit neutrophils without an apparent rise in the level of intracellular free calcium. *Biochem. Biophys. Res. Commun.* **114**:638-645.

Shalaby, M.R., Aggarwal, B.B., Kinderknecht, E., Svedersky, L.P., Finkle, B.S. and Palladino Jr., M.A. (1985). Activation of human polymorphonuclear neutrophil functions by interferon-γ and tumor necrosis factors. *J. Immunol.* **135**:2069-2073.

Shalaby, M.R., Palladino, M.A., Jr., Hirabayashi, S.E., Ecssalu, T.E., Lewis, G.D., Shepard, H.M. and Aggarwal, B.B. (1987). Receptor binding and activation of polymorphonuclear neutrophils by tumor necrosis factor-alpha. *J. Leukocyte Biol.* **41**:196-204.

Shaw, D.R. and Griffin, F.M., Jr. (1981). Phagocytosis requires repeated triggering of macrophage phagocytic receptors during particle ingestion. *Nature (Lond.)* **289**:409-411.

Shaw, J.O., Pinckard, R.N., Ferrigni, K.S., McManus, L.M., and Hanahan, D.J. (1981). Activation of human neutrophils with 1-*O*-hexadecyl/octadecyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine (platelet-activating factor). *J. Immunol.* **127**:1250-1255.

Shear, M.J., and Andervont, H.B. (1936). Chemical treatment of tumors; separation of hemorrhage-producing fraction of *E. coli* filtrate. *Proc. Soc. Exp. Biol. Med.* **34**:323-325.

Shefcyk, J.R., Yassin, M., Volpi, M., Molski, T.F.P., Naccache, P.H., Munoz, J.J., Becker, E.L., Feinstein, M.B., and Sha'afi, R.I. (1985). Pertussis but not cholera toxin inhibits the stimulated increase in actin association with the cytoskeleton in rabbit neutrophils: role of the "G proteins" in stimulus-response coupling. *Biochem. Biophys. Res. Commun.* **126**:1174-1181.

Shirai, T., Yamaguchi, H., Ito, H., Todd, C.W., and Wallace, R.B. (1985). Cloning and expression in *Escherichia coli* of the gene for human tumor necrosis factor. *Nature (Lond.)* **313**:803-806.

Showell, H.J., Freer, R.J., Zigmond, S.H., Schiffmann, E., Aswanikumar, S., Corcoran, B. and Becker, E.L. (1976). The structure-activity relations of synthetic peptides as chemotactic factors and inducers of lysosomal secretion for neutrophils. *J. Exp. Med.* **143**:1154-1169.

Showell, H.J., Naccache, P.H., Vitkauskas, G., Williams, D., Sha'afi, R. and Becker, E.L. (1978). Time and concentration- dependent desensitization of the secretion of lysosomal enzymes in incubating rabbit neutrophils with formyl-

methionyl-leucyl-phenylalanine (FMLP) before adding cytochalasin B (CB). *Fed. Proc.* **37**:1565a.

Showell, H.J., Williams, D., Becker, E.L., Naccache, P.H. and Sha'afi, R. (1979). Densensitization and deactivation of the secretory responsiveness of rabbit neutrophils induced by the chemotactic peptide, formyl-methionyl-leucyl-phenylalanine. *J. Reticuloendothel. Soc.* **25**:139-150.

Silberstein, D.S., Owen, W.F., Gasson, J.C., DiPersio, J.F., Golde, D.W., Bina, J.C., Soberman, R., Austen, K.F., and David, J.R. (1986). Regulation of human eosinophil function by granulocyte-macrophage colony-stimulating factor. *J. Immunol.* **137**:3290-3294.

Silverstein, S.C., Steinman, R.M., and Cohn, Z.A. (1977). Endocytosis. *Ann. Rev. Biochem.* **46**:669-722.

Simchowitz, L., Fischbein, L.C., Spilberg, I., and Atkinson, J.P. (1980). Induction of a transient elevation of intracellular levels of adenosine-3',5'-cyclic monophosphate by chemotactic factors: an early event in human neutrophil activation. *J. Immunol.* **124**:1482-1491.

Simon, M.F., Chap, H., and Douste-Blazy, L. (1986). Selective inhibition of human platelet phospholipase A_2 by buffering cytoplasmic calcium with the fluorescent indicator quin 2. Evidence for different calcium sensitivities of phospholipase A_2 and C. *Biochim. Biophys. Acta.* **875**:157-164.

Sisson, J.H., Prescott, S.M., McIntyre, T.M., and Zimmerman, G.A. (1987). Production of platelet-activating factor by human polymorphonuclear leukocytes. Correlation of synthesis with release, functional events, and leukotriene B_4 metabolism. *J. Immunol.* **138**:3918-3926.

Sklar, L.A. (1986). Ligand-receptor dynamics and signal amplification in the neutrophil. In: *Advances in Immunology. Vol.39.* edited by F.J. Dixon, pp. 95-143, Academic Press, New York.

Sklar, L.A., Finney, D.A., Oades, Z.A., Jesaitis, A.J., Painter, R.G., and Cochrane, C.G. (1984). The dynamics of ligand-receptor interactions. Real-time analysis of association, dissociation and internalization of an *N*-formyl peptide and its receptors on the human neutrophil. *J. Biol Chem.* **259**:5661-5669.

Sklar, L.A., Omann, G.M. and Painter, R.G. (1985). Relationship of actin polymerization and depolymerization to light scattering in human neutrophils: dependence on receptor occupancy and intracellular calcium. *J. Cell. Biol.* **101**:1161-1166.

Sklar. L.A., Bokoch, G.M., Button, D., and Smolen, E.J. (1987). Regulation of ligand-receptor dynamics by guanine nucleotides: real time analysis of interconverting states for the neutrophil formylpeptide receptor. *J. Biol. Chem.* **262**:135-139.

Smith, C.D., Lane, B.C., Kusaka, I., Verghese, M.W., and Snyderman, R. (1985). Chemoattractant receptor-induced hydrolysis of phosphatidylinositol 4,5-bisphosphate in human polymorphonuclear leukocyte membranes. *J. Biol. Chem.* **260**:5875-5878.

Smith, R.J., and Bowman, B.J. (1982). Stimulation of human neutrophil degranulation with 1-*O*-octadecyl-2-*O*-acetyl-*sn*-glyceryl-3-phosphorylcholine: Modulation by inhibitors of arachidonic acid metabolism. *Biochem. Biophys. Res. Commun.* **104**:1495-1501.

Smith, R.J., Iden, S.S., and Bowman, B.J. (1984). Activation of the human neutrophil secretory process with 5(*S*),12(*R*)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetranoic acid. *Inflammation* **8**:365-384.

Smolen, J.E., Korchak, H.M. and Weissmann, G. (1981). The roles of extracellular and intracellular calcium in lysosomal enzyme release and superoxide anion generation by human neutrophils. *Biochim. Biophys. Acta.* **677**:512-520.

Snyderman, R., Gewurz, H., and Mergenhagen, S.E. (1968). Interactions of the complement system with endotoxic lipopolysaccharide: Generation of a factor chemotactic for polymorphonuclear leukocytes. *J. Exp. Med.* **128**:259-275.

Snyderman, R., Pike, M.C., and Altmann, L.C. (1975). Abnormalities of leukocyte chemotaxis in human disease. *Ann. N. Y. Acad. Sci.* **256**:386-401.

Snyderman, R., Shin, H.S., Phillips, J.K., Gewurz, H. and Mergenhagen, S.E. (1969). A neutrophil chemotactic factor derived from C5 upon interaction of guinea pig serum with endotoxin. *J. Immunol.* **103**:413-422.

Snyderman, R. (1985). Regulatory mechanisms of a chemoattractant receptor on human polymorphonuclear leukocytes. *Rev. Infect. Dis.* **7**:390-394.

Soberman, R.J., Harper, T.W., Betteridge, D., Lewis, R.A., and Austen, K.F. (1985). Characterisation and separation of arachidonic acid 5-lipoxygenase and linoleic acid ω-6 lipoxygenase (arachidonic acid 15-lipoxygenase of human polymorphonuclear leukocytes. *J. Biol. Chem.* **60**:4508-4515.

Soberman, R.J., Sutyak, J.P., Okita, R.T., Wendelborn, D.F., Roberts, II, L.J. and Austen, K.F. (1988). The identification and formation of 20-aldehyde leukotriene B. *J. Biol. Chem.* **263**(17):7996-8002

Southwick, F.S. and Stossel, T.P. (1981). Isolation of an inhibitor of actin polymerization from human polymorphonuclear leukocytes. *J. Biol. Chem.* **256**:3030-3036.

Southwick, F.S. and Stossel, T.P. (1983). Contractile proteins in leukocyte function. *Sem. Hematol.* **20**(4):305-321.

Souza, L.M., Boone, T.C., Gabrilove, J., Lai, P.H., Zsebo, K.M., Murdock, D.C., Chazin, V.R., Bruszewski, J., Lu, H., Chen, K.K., Barendt, J., Platzer, E., Moore, M.A.S., Mertelsmann, R., and Welte, K. (1986). Recombinant human granulocyte colony-stimulating factor: effects on normal and leukemic myeloid cells. *Science* 232:61-65.

Spagnuolo, P.J., and Ellner, J.J. (1980). Comparative stimulation of granulocyte adherence and chemotaxis by bacterial products. *Infect. Immun.* **27**:519-524.

Spielberg, S.P., Boxer, L.A., Oliver, J.M., Allen, J.M. and Schulman, J.D. (1979). Oxidative damage to neutrophils in glutathione synthetase deficiency. *Br. J. Haematol.* **42**:215-223.

Spitznagel, J.K., Dalldorf, F.G., Leffell, M.S., Folds, J.D., Welsh,I.R.H., Cooney, M.H. and Martin, L.E. (1974). Character of azurophil and specific granules purified from human polymorphonuclear leukocytes. *Lab. Invest.* **30**(6):774-785.

Springer, G.F., and Adye, J.C. (1975). Endotoxin-binding substances from human leukocytes and platelets. *Infect. Immun.* **12**:978-986.

Stendahl, O., and Lindgren, S. (1976). Function of granulocytes with deficient mycloperoxidase-mediated iodination in a patient with generalised pustular psoriasis. *Scand. J. Haemat.* **16**:144-153.

Stetson Jr, C.A. (1951). Similarities in the mechanisms determining the Arthus and Shwartzman phenomenon. *J. Exp. Med.* **94**:347-357.

Stolpen, A.H., Guinan, E.C., Fiers, W., Pober, J.S. (1986). Recombinant tumor necrosis factor and immune interferon act singly and in combination to reorganize human vascular endothelial cell monolayers. *Am. J. Pathol.* **123**:16-24.

Stossel, T.P. (1988) The mechanical responses of white blood cells. In: *Inflammation: Basic Principles and Clinical Correlates.* edited by J.I. Gallin, I.M. Goldstein and R. Snyderman. pp. 325-442, Raven Press, New York.

Strominger, J.L. and Ghuysen, J.-M. (1967). Mechanisms of enzymatic bacteriolysis. Cell walls of bacteri are solubilised by action of either specific carbohydrases or specific peptidases. *Science* **156**:213-221.

Sullivan, R., Griffin, J.D., Simons, E.R., Schafer, A.I., Meshulam, T., Fredette, J.P., Maas, A.K., Gadenne, A-S., Leavitt, J.L. and Melnick, D.A. (1987). Effects of recombinant human granulocyte and macrophage colony-stimulating factors on signal transduction pathways in human granulocytes. *J. Immunol.* **139**:3422-3430.

Sullivan, S.J. and Zigmond, S.H. (1980). Chemotactic peptide receptor modulation in polymorphonuclear leukocytes. *J. Cell. Biol.* **85**:703-711.

Sumimoto, H., Takeshige, K., and Minakami, S. (1985). NAD^+-dependent conversion of 20-OH-LTB_4 by a cell-free system of human polymorphonuclear leukocytes. *Biochem. Biophys. Res. Commun.* **132**:864-870.

Takeshige, K. and Minakami, S. (1981). Involvement of calmodulin in phagocytic respiratory burst of leukocytes. *Biochem. Biophys. Res. Commun.* **99**:484-490.

Territo, M.C., and Golde, D.W. (1976). Granulocyte function in experimental human endotoxemia. *Blood* **47**:539-544.

Tharp, M.D., and Kasper, C.S. (1987). Mast cell mediated cytotoxicity is inhibited in part by anti-TNF antibodies. *Clin. Res.* **35**:466A.

Thelen, M., Peveri, P., Kernan, P., von Tscharner, V., Walz, A., and Baggiolini, M. (1988). Mechanism of neutrophil activation by bNAF, a novel monocyte derived peptide agonist. *FASEB J.* **11**:2702-2706.

Thorne, K.J.I., Oliver, R.C. and Barrett, A.J. (1976). Lysis and killing of bacteria by lysosomal proteinases. *Infect. Immun.* **14**:555-635.

Thorne, K.J.I., Oliver, R.C. and Lackie, J. (1977). Changes in the surface properties of rabbit polymorphonuclear leukocytes, induced by bacteria and bacterial endotoxin. *J. Cell Sci.* **27**:213-225.

Torti, F.M., Dieckmann, B., Beutler, B., Cerami, A., and Ringold, G.M. (1985). A macrophage factor inhibits adipocyte gene expression: an *in vitro* model of cachexia. *Science* **229**:867-869.

Tracey, K., Lowry, S., Beutler, B., Cerami, A., Albert, J.D., and Shires, G.T. (1986). Cachectin/tumor necrosis factor mediates changes of skeletal muscle plasma membrane potential. *J. Exp. Med.* **164**:1368-1373.

Tsujimoto, M., Yokota, S., Vilcek, J. and Weissmann, G. (1986). Tumor necrosis factor provokes superoxide anion generation from neutrophils. *Biochem. Biophys. Res. Comm.* **137**(3):1094-1100.

Turk, J., Henderson, W.R., Klebanoff, S.J. and Hubbard, W.C. (1983). Iodination of arachidonic acid mediated by eosinophil peroxidase, myeloperoxidase and lactoperoxidase. Identification and comparison of products. *Biochim. Biophys. Acta* **751**(2):189-200.

Umei, T., Takeshige, K. and Minakami, S. (1986). NADPH binding component of neutrophil superoxide-generating oxidase. *J. Biol. Chem.* **261**:5229-5232.

Vadas, M.A., Lopez, A.F. and Williamson, D.J. (1985). Selective enhancement of the expression of granulocyte functional antigens 1 and 2 on human neutrophils. *Proc. Natl. Acad. Sci. USA.* **82**:2503-2507.

Vadas, M.A., Nicola, N.A. and Metcalf, D. (1983). Activation of antibody-dependent cell-mediated cytotoxicity of human neutrophils and eosinophils by separate colony-stimulating factors. *J. Immunol.* **130**:795-799.

Valerius, N.H., Stendahl, O., Hartwig, J.H., and Stossel, T.P. (1981). Distribution of actin-binding protein and myosin in polymorphonuclear leukocytes during locomotion and phagocytosis. *Cell* **24**:195-202.

van Gelder, B.F. and Slater, E.C. (1962). The extinction coefficient of cytochrome C. *Biochim. Biophys. Acta.* **58**:593-595.

Varigos, G., Morstyn, G., and Vadas, M.A. (1982). Bullous pemphigoid blister fluid stimulates eosinophil colony formation and activates eosinophils. *Clin. Exp. Immunol.* **50**:555-562.

Vel, W.A.C., Namavar, F., Verweij, A., Marian, J.J., Pubben, A.N.B. and MacLaren, D.M. (1984). Killing capacity of human polymorphonuclear leukocytes in aerobic and anaerobic conditions. *J. Med. Microbiol.* **18**:173-180.

Verghese, M.W., Smith, C.D., and Snyderman, R. (1985). Involvement of a guanine nucleotide regulatory protein in chemoattractant reseptor mediated polyphosphoinositide metabolism, Ca^{++} mobilisation and cellular responses by leukocytes. *Biochem. Biophys. Res. Commun.* **127**:450-457.

Voetman, A.A. and Roos, D. (1980). Endogenous catalase protects human blood phagocytes against oxidative damage by extracellularly generated hydrogen peroxide. *Blood* **56**:846-852.

Volpi, M., Molski, T.F.P., Naccache, P.H., Feinstein, M.B. and Sha'afi, R.I. (1985a). Phorbol-12-myristate 12-acetate potentiates the action of the calcium ionophore in stimulating arachidonic acid release and production of phosphatidic acid in rabbit neutrophils. *Biochem. Biophys. Res. Comm.* **128**:594-600.

Volpi, M., Naccache, P.H., Molski, T.F.P., Shefcyk, J., Huang, C-K., Marsh, M.L., Munoz, J., Becker, E.L. and Sha'afi, R.I. (1985b). Pertussis toxin inhibits f Met-leu-phe but not phorbol ester-stimulated changes in rabbit neutrophils: Role of G proteins in excitation response coupling. *Proc. Natl. Acad. Sci. USA* **82**:2708-2712.

Walker, R.I. and Willemze, R. (1980). Neutrophil kinetics and the regulation of granulopoiesis. *Rev. Infect. Dis.* **2**:282-292.

Walter, R.J. and Marasco, W.A. (1987). Direct visualization of formylpeptide receptor binding on rounded and polarized human neutrophils: cellular and receptor heterogeneity. *J. Leuk. Biol.* **41**:377-391.

Wang, A.M., Creasey, A.A., Ladner, M.B., Lin, L.S., Strickler, J.,Van Arsdell, J.N., Yamamoto, R., and Mark, D.F. (1985). Molecular cloning of the complementary DNA for human tumor necrosis factor. *Science* **228**:149-154.

Wang, J.M., Bersani, , and Mantovani, A. (1987). Tumor necrosis factor is chemotactic for monocytes and polymorphonuclear leukocytes. *J. Immunol.* **138**:1469-1474.

Wang, J.M., Colella, S., Allavena, P. and Mantovani, A. (1987). Chemotactic activity of human recombinant granulocyte-macrophage colony-stimulating factor. *Immunol.* **60**:439-444.

Ward, P.A., and Becker, E.L. (1968). The deactivation of rabbit neutrophils by chemotactic factor and the nature of the activable esterase. *J. Exp. Med.* **127**:693-709.

Ward, P.A., and Newman, L.J. (1969). A neutrophil chemotactic factor from human C'5. *J. Immunol.* **102**:93-99.

Ward, P.A., and Schlegal, R.J. (1969). Impaired leukotactic responsiveness in a child with recurrent infections. *Lancet*, ii, 344-347.

Ward, P.A., Cochrane, C.G., and Müller-Eberhard, H.J. (1965). The role of serum complement in chemotaxis of leukocytes in vitro. *J.Exp. Med.* **122**:327-346.

Webster, R.O., Hong, S.R., Johnston, R.B., Jr., and Henson, P.M. (1980). Biological effects of the human complement fragments C5a and C5a des Arg on neutrophil function. *Immunopharmacology* 2:201-219.

Weening, R.S., Weever, R. and Roos, D. (1975). Quantitative aspects of the production of superoxide radicals by phagocytozing human granulocytes. *J. Lab. Clin. Med.* **85**:245-252.

Wegner, A. (1982). Treadmilling of actin at physiological salt concentrations. An analysis of the critical concentrations of actin filaments. *J. Mol. Biol.* **161**:607-615.

Weisbart, R.H., Golde, D.W., Gasson, J.C. (1986). Biosynthetic human GM-CSF modulates the number and affinity of neutrophil f Met-Leu-Phe receptors. *J. Immunol.* **137**:3584-3587.

Weiss, J., Elsbach, P., Olsson, D. and Odeberg, H. (1978). Purification and characterization of a potent bactericidal and membrane active protein from the granules of human polymorphonuclear leukocytes. *J. Biol. Chem.* **253**:2664-2672.

Weiss, P. (1961). Guiding principles in cell locomotion and cell aggregation. *Exp. Cell. Res. (suppl.)* **8**:260-281.

Weissmann, G., Zurier, R.B., Spielen, P.J. and Golstein, I.M. (1971). Mechanisms of lysosomal enzyme release from leukocytes exposed to immune complexes and other particles. *J. Exp. Med.* **134**:149-165s.

Welte, K., Platzer, E., Lu, L., Gabrilove, J.L., Levi, E., Mertelsmann, R., and Moore, M.A.S. (1985). Purification and biochemical characterization of human pluripotent hematopoietic colony-stimulating factor. *Proc. Natl. Acad. Sci. USA* **82**:1526-1530.

Wertz, P.W. and Mueller, G.C. (1978). Rapid stimulation of phospholipid metabolism in bovine lymphocytes by tumor-promoting phorbol esters. *Cancer Research* **38**(9):2900-2904.

Wilkinson, P.C. (1980). Leukocyte locomotion and chemotaxis: Effects of bacteria and viruses. *Rev. Infect. Dis.* **2**(2):293-318.

Wilkinson, P.C. and Lackie, J.M. (1983). The influence of contact guidance on chemotaxis of human neutrophil leukocytes. *Exp. Cell. Res.* **145**:255-264.

Wilkinson, P.C., Lackie, J.M., Forrester, J.V., and Dunn, G.A. (1984). Chemokinetic accumulation of human neutrophils on immune complex-coated substrata: analysis at a boundary. *J. Cell. Biol.* **99**:1761-1768.

Wilkinson, P.C., Shields, J.M., and Haston, W.S. (1982). Contact guidance of human neutrophil leukocytes. *Exp. Cell Res.* **140**:55-62.

Williams, J.D., Lee, T.H., Lewis, R.A., and Austen, K.F. (1985). Intracellular retention of the 5-lipoxygenase pathway product, leukotriene B_4, by human neutrophils activated with unopsonized zymosan. *J. Immunol.* **134**:2624-2630.

Williams, L.T., Snyderman, R., Pike, M.C. and Lefkowitz, R.J. (1977). Specific receptor sites for chemotactic peptides on human polymorphonuclear leukocytes. *Proc. Natl. Acad. Sci. USA* **74**:1204-1208.

Williamson, D.J., Begley, C.G., Vadas, M.A., and Metcalf, D. (1988). The detection and initial characterisation of colony-stimulating factors in synovial fluid. *Clin. Exp. Immunol.* **72**:67-73.

Wilson, M.E. (1985). Effects of bacterial endotoxins on neutrophil function. *Rev. Infect. Dis.* **7**(3):404-418.

Wilson, M.E., Jones, D.P., Munkenbeck, P., Morrison, D.C. (1982). Serum - dependent and -independent effects of bacterial lipopolysaccharides on human neutrophil oxidative capacity in vitro. *J. Reticuloendothel. Soc.* **31**:43-57.

Winterbourn, C.C., Garcia, R.C. and Segal, A.W. (1985). Production of the superoxide adduct of myeloperoxidase (compound III). by stimulated human neutrophils and its reactivity with hydrogen peroxide and chloride. *Biochem. J.* **228**:583-592.

Wollheim, F.A., Heingard, D., Palladino, M., Saxne, T., and Talal, N. (1987). Tumour necrosis factor α in synovial fluid. Difference between rheumatoid arthritis and reactive arthritis. *Arthiritis Rheum.* **30**:S129.

Wong, G.G., Witek, J.S., Temple, P.A., Wilkens, K.M., Leary, A.C., Luxenberg, D.P., Jones, S.S., Brown, E.L., Kay, R.M., Orr, E.C., Shoemaker, C., Golde, D.W., Kaufman, R.J., Hewick, R.M., Wang, E.A. and Clark, S.C. (1985). Human GM-CSF. Molecular Cloning of the cDNA and purification of the natural and recombinant proteins. *Science* **228**:810-815.

Worthen, G.S., Seccombe, J.F., Clay, K.L., Guthrie, L.A. and Johnston Jr., R.B. (1988). The priming of neutrophils by lipopolysaccharide for production of intracellular platelet-activating factor. Potential role in mediation of enhanced superoxide secretion. *J. Immunol.* **140**:3553-3559.

Wu, N.C. and Schultz, J. (1975). The prosthetic group of myeloperoxidase. *FEBS Lett.* **60**:141-144.

Wykle, R.L., Olson, S.C., and O'Flaherty, J.T. (1986). Biochemical pathways of platelet-activating factor synthesis and breakdown. *Adv. Inflam. Res.* **11**:71-81.

Yahara, I., Harada, F., Sekita, S., Yoshihira, K. and Natori, S. (1982). Correlation between effects of 24 different cytochalasins on cellular structures and cellular events and those on actin *in vitro*. *J. Cell. Biol.* **92**:69-78.

Yamaguchi, T., Sato, K., Shimada, K., and Kakinuma, K. (1982). Subcellular localization of O_2^- generating enzyme in guinea pig polymorphonuclear leukocytes: fractionation of subcellular particles by using a Percoll density gradient. *J. Biochem.* **91**:31-40.

Yamazaki, I. and Yokota, K.N. (1973). Oxidation states of peroxidase. *Mol. Cell. Biochem.* 2:39-52.

Yancey, K.B., O'Shea, J., Crushed, T., Brown, E., Takahashi, T., Frank, M.M. and Lawley, T.J. (1985). Human C5a modulates monocyte Fc and C3 receptor expression. *J. Immunol.* **135**:465-470.

Yang, S.F. (1969). Further studies on ethylene formation from ketomethylthiobutyric acid or β-methylthiopropionaldehyde by peroxidase in the presence of sulfite and oxygen. *J. Biol. Chem.* **244**:4360-4365.

Yin, H.L., Albrecht, J., and Fattoum, A. (1980). Identification of gelsolin, a Ca^{2+}-dependent regulatory protein of actin gel-sol transformation: its intracellular distribution in a variety of cells and tissues. *J. Cell. Biol.* **91**:901-906.

Yoon, F.S., Boxer, L.A., Mayo, L.A., Yang, A.Y. and Wicha, M.S. (1987). Human neutrophil laminin receptors: activation-dependent receptor expression. *J. Immunol.* **138**:259-265.

Yoshimura, T. and Sone, S. (1987). Different and synergistic actions of human tumor necrosis factor and interferon-gamma in damage of lipsome membranes. *J. Biol. Chem.* **262**(10):4597-4601.

Yuli, I., Tomonaga, A. and Snyderman, R. (1982). Chemoattractant receptor functions in human polymorphonuclear leukocytes are divergently altered by membrane fluidizers. *Proc. Natl. Acad. Sci. USA* **79**:5906-5910.

Zeya, H.I. and Spitznagel, J.K. (1966). Antimicrobial specificity of leukocyte lysosomal cationic proteins. *Science* **154**:1049-1051.

Zigmond, S.H. (1974). Mechanisms of sensing gradients by polymorphonuclear leukocytes. *Nature* **249**:450-452.

Zigmond, S.H. and Sullivan, S.J. (1979). Sensory adaptation of leukocytes to chemotactic peptides. *J. Cell. Biol.* **82**:517-527.

Zimmerman, G.A., and McIntyre, T.M. (1988). Neutrophil adherence to human endothelium *in vitro* occurs by CDw18 (Mo1, MAC-1/LFA-1/GP150,95). glycoprotein-dependent and -independent mechanisms. *J. Clin. Invest.* **81**:531-537.

Zucali, J.R., Dinarello, C.A., Oblon, D.J., Gross, M.A., Anderson, L., and Weiner, R.S. (1986). Interleukin 1 stimulates fibroblasts to produce granulocyte-

macrophage colony-stimulating activity and prostaglandin E_2. *J. Clin. Invest.* **77**:1857-1863.

Zucker-Franklin, D. and Hirsch, J.G. (1964). Electron microscopic studies on the degranulation of rabbit peritoneal leucocytes during phagocytosis. *J. Exp. Med.* **120**:569-576.

Zurier, R.B., Hoffstein, S. and Weismann, G.G. (1973). Cytochalasin B: Effect on lysosomal enzyme release from human leukocytes. *Proc. Natl. Acad. Sci. USA* **70**:844-848.