MORPHOLOGICAL RESPONSES OF NEUTROPHILS

IN SUSPENSION

TO PLASMA COMPONENTS AND CHEMOTACTIC FACTORS

DAMIEN GERARD HARKIN

Thesis submitted for the degree of
Doctor of Philosophy
in
The University of Adelaide
(Faculty of Medicine)

Awarded 1993

For my parents.

TABLE OF CONTENTS

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

6.7.92

PUBLICATIONS IN SUPPORT OF THESIS

The following publications contain aspects of the material presented in this thesis.

Bignold L. P., Harkin D. G. and Rogers S. D. (1992) Interleukin-8 and neutrophil leukocytes: adhesion, spreading, polarisation, random motility, chemotaxis and deactivation in assays using 'sparse-pore' polycarbonate (Nuclepore) membranes in the Boyden chamber. International Archives of Allergy and Applied Immunology, (in press).

Bignold L. P., Rogers S. D. and Harkin D. G. (1990) Effects of plasma proteins on the adhesion, spreading, polarisation in suspension, random motility and chemotaxis of neutrophil leukocytes on polycarbonate (Nuclepore) filtration membrane. European Journal of Cell Biology, 53, 27-34.

Harkin D. G. and Bignold L. P. (1990) Importance of extracellular divalent cations to polarisation of polymorphonuclear leukocytes induced by plasma. Cell Biology International Reports, 14 (12), 1099-1107.

Harkin D. G. and Bignold L. P. (1990) Human immunoglobulin type G induces polarisation of human neutrophil leukocytes in cell suspensions. Proceedings of the Australasian Society for Experimental Pathology, Immunology and Cell Biology, 68 (Supplement 1).

Harkin D. G. and Bignold L. P. (1991) Assessment of techniques for the analysis of neutrophil morphology. Proceedings of the Australasian Society for Experimental Pathology, Immunology and Cell Biology, 69 (Supplement 1), 7.

Harkin D. G. and Bignold L. P. (1991) Different patterns in the morphological responses of neutrophil leukocytes to chemotactic stimuli and plasma proteins. Proceedings of the Australasian Society for Experimental Pathology, Immunology and Cell Biology, 69 (Supplement 1), 8.

ACKNOWLEDGEMENTS

Sincere thanks are extended to Professor B. Vernon-Roberts and the staff of the Department of Pathology at the University of Adelaide for their helpful discussion and support throughout the course of this work. In particular, I am grateful to Dr S. Gadd for operating the fluorescence activated cell sorter, and to Mr D. Caville for expertly printing the photographs presented in this thesis.

Special thanks are extended to the numerous volunteers from the University of Adelaide, the Institute of Medical and Veterinary Science and the Royal Adelaide Hospital, for their generous donations of blood.

Many thanks go to my parents Marie and Denis and to my sisters Miriam, Louise and Claire, for their constant love and encouragement.

I am also deeply grateful to my fiancee Blazenka for her endless love and support, and for proof reading sections of this thesis during its preparation.

Finally, my most sincere thanks are extended to Dr L. P. Bignold, for supervising the work presented in this thesis and for providing generous guidance and encouragement throughout the course of the degree.

The work presented in this thesis was carried out with the support of an Australian Postgraduate Research Award scholarship.

SUMMARY

Neutrophil leukocytes develop polarised cytoplasmic extensions as a prerequisite for their emigration into inflamed tissues. This shape-change has been extensively studied in cell suspensions as a response of neutrophils to chemotactic factors such as the synthetic bacterial peptide N-formyl-methionyl-leucyl-phenylalanine (FMLP). However, neutrophils also polarise when suspended in fresh heparinised plasma and the details of this response have not been previously characterised.

This study examined the time course and degree of neutrophil polarisation in plasma and compared this response with those induced by FMLP, purified plasma proteins (particularly immunoglobulin type G) and chemotactic inflammatory mediators (the complement derived fragment C5a, interleukin-8, leukotriene B_4 and platelet activating factor). In addition, the possible roles of extracellular divalent cations (Ca^{2+} and Mg^{2+}), intracellular Ca^{2+} ions and actin microfilament distribution during responses to each stimulus were examined.

Neutrophils were isolated from human peripheral blood by a one-step Hypaque-Ficoll method and resuspended in Hanks' balanced salt solution buffered with 20 mM Hepes (HBSS-Hepes) containing test agents, at 37 °C. Polarisation was assessed by microscopic examination (Nomarski optics) and classification of glutaraldehyde (2.5% v/v in phosphate-buffered saline)-fixed cells into five morphological subtypes: spherical (unstimulated); type 1 cells, characterised by non-polarised extensions; type 2 cells, characterised by polarised extensions and round body; type 3 cells, characterised by polarised extensions and an oval body; and type 4 cells (fully polarised), characterised by polarised extensions including a tail (uropod). Optimal responses to each stimulus were defined as those exhibiting the greatest proportion of type 4 cells. Computerised morphometry and fluorescence activated cell sorter (FACS) analysis were examined as methods for assessing

polarisation, but neither technique was satisfactory since morphometry did not consistently distinguish between non-polarised (type 1 cells) and polarised cells (types 2 and 3) and FACS analysis only detected changes in cell size.

Standard preparations of heparinised (12.5 I.U./ml) plasma (10%, 50% and 90% v/v) induced immediate (within 30 seconds) polarisation responses which were apparent throughout the 60 minutes incubation, but cells rarely developed a type 4 morphology. In contrast, plasma anticoagulated with ethylene-bis-(oxyethyl-enenitrilo)-tetra-acetic acid (EGTA, 5 mM) or low concentrations of heparin (2.5 I.U./ml), standard plasma pre-treated for 5 minutes with soy bean trypsin inhibitor (0.25 mM) or additional Mg^{2+} ions (0.5 to 5 mM), and fresh serum, induced formation of many type 4 cells. Plasma anticoagulated with either ethylene-diamine-tetra-acetic acid (EDTA, 5 mM) or disodium hydrogen citrate (4 mg/ml) induced little or no change in cell shape.

Responses to standard preparations of heparinised plasma were reduced in the presence of chelating agents of extracellular cations (EDTA, EGTA, or disodium hydrogen citrate; 5 mM). These inhibitory effects of chelating agents did not occur if compensatory concentrations of either additional Ca^{2+} or Mg^{2+} ions (5 mM) were present.

Commercial preparations of IgG (0.005% and 0.0005% w/v) containing aggregates induced neutrophil polarisation, but this effect was delayed until 5 minutes. Reducing the proportion of aggregated IgG by ultra-centrifugation further delayed or abolished responses. Partial removal (60%) of the type III receptor for IgG on neutrophils did not affect the response of the cells to this protein. Fc and Fab fragments of IgG prepared by papain digestion did not induce polarisation.

Heat aggregated preparations of IgG (0.1%, 0.01% and 0.001% w/v) induced neutrophil polarisation with high proportions of type 4 cells at higher concentrations, but these responses were also generally delayed until 5 minutes. In contrast, supernatants from cells suspended in 0.01% heat aggregated IgG for 5 minutes induced an immediate (within 30 seconds) response in fresh cells. Responses to 0.1% and

0.01% (but not 0.001%) heat aggregated IgG were markedly reduced in the presence of EDTA, but all responses were unaffected by EGTA.

Optimal responses to each chemotactic factor were observed at 10 nM for FMLP and leukotriene B_4, 1 nM for C5a, 12.5 nM for interleukin-8, and 40 to 400 nM for platelet activating factor. These responses were rapid in onset (within 30 seconds), sustained for at least 60 minutes and were characterised by moderate to high numbers of type 4 cells. Furthermore, in contrast to the responses to plasma and IgG, responses to each chemotactic factor were generally enhanced in the presence of chelating agents of extracellular divalent cations.

Responses to plasma, IgG and chemotactic factors were generally abolished by pre-treating cells with 8-[diethylamino]-octyl 3,4,5-trimethoxybenzoate hydrochloride (TMB-8; 5×10^{-4} M for 10 minutes) an inhibitor of the release of intracellular Ca^{2+} ions. Furthermore, cells treated with plasma, IgG or chemotactic factors consistently displayed abundant F-actin within their cytoplasmic extensions when stained with the fluorescent F-actin probe rhodamine phalloidin.

The present studies demonstrate significant differences between the polarisation responses of neutrophils in suspension to heparinised plasma, IgG and chemotactic factors. This implies that motile responses of neutrophils in inflamed tissues may vary according to the cause and mediation of the inflammatory response. In addition, these findings suggest that plasma derived factors other than established chemotactic stimuli may regulate the emigration of neutrophils in vivo.

CHAPTER 1.

INTRODUCTION

1. 1. Neutrophil leukocytes

1. 1. 1. History and terminology

In the middle of the 18th century the first microscopists noted the existence of red (coloured) cells, or erythrocytes, and white (colourless) cells, or leukocytes, in human peripheral blood (Rolleston, 1934). Almost a century later, it was recognised that leukocytes could be classified as granular or non-granular according to the presence or absence of cytoplasmic granules (Rolleston, 1934). However, current classifications of white blood cells are based on observations made of cells stained with specialised dyes developed by Ehrlich during the later part of the 19th century (Rolleston, 1934). This technique has allowed the granular leukocytes, or granulocytes, of human blood to be classified into neutrophils, acidophils (eosinophils) and basophils according to the affinity of the cells' cytoplasmic granules for acidic and basic dyes. However, in certain species such as the rabbit and guinea pig, the granules within cells which corresponded to human neutrophils have an affinity for acidic dyes such as eosin. The terms 'pseudo-eosinophil' or 'heterophil' are therefore used as more general names for neutrophils (Klebanoff and Clark, 1978).

Studies of stained preparations of blood cells have also demonstrated the nuclei of granulocytes to consist of varying numbers of lobes. The term polymorphonuclear leukocyte or PMN has therefore been used as an additional collective term for these cells. However, since the majority of granulocytes in human peripheral blood are neutrophils

(approximately 95%), the terms 'PMN' and 'granulocyte' have been used synonymously with 'neutrophil' (Murphy, 1976; Klebanoff and Clark, 1978).

During the mid to late 19th century it became apparent that the primary function of neutrophils was to phagocytose bacteria and other inflammatory materials present in damaged and inflamed tissues (Metchnikoff, 1891). In view of this activity of neutrophils the term 'phagocyte' has also been used in reference to these cells (Klebanoff and Clark, 1978).

1. 1. 2. Histology and ultrastructure

Neutrophils constitute 60-70% of leukocytes found in normal peripheral blood of humans and are identified in blood smears as spherical cells with a segmented nucleus, and abundant pale-staining cytoplasmic granules (Junqueira and Caneiro, 1983). The diameter of neutrophils in these preparations is approximately 10 μm, but the diameter of neutrophils suspended in physiological media is closer to 7 μm (Schmid-Schonbein et al., 1980).

Histochemically, the neutrophil's granules are divided into a primary or azurophilic population containing lysozyme, myeloperoxidase, neutral proteases, acid hydrolases and cationic proteins, and a secondary or specific population containing lysozyme, alkaline phosphatase, collagenase, vitamin B12-binding protein and lactoferrin (Sandborg and Smolen, 1988). A tertiary population of granules containing gelatinase has also been identified, but is less well characterised (Dewald et al., 1982).

In cell sections examined by transmission electron microscopy, the neutrophil's plasma membrane appears as a 75-100 Å thick tri-laminar structure composed of two electron dense layers separated by a layer of lower electron density (Klebanoff and Clark, 1978). This plasma membrane is separated from underlying organelles by a cortical layer of actin microfilaments approximately 0.25 μm thick (Stossel, 1982; Bray et al., 1986; Sheterline and Rickard, 1989). Other components of the neutrophil's cytoskeleton include microtubules, composed of the protein tubulin (Malawista and

Bensch, 1967; Anderson, 1982), and intermediate filaments composed of the protein vimentin (Stossel, 1988). The neutrophil's microtubules and intermediate filaments are present in fewer numbers than microfilaments and are restricted primarily to the central region of the cell, but some of these structures penetrate into the cell's cortical layer of microfilaments (Stossel, 1988). The neutrophil's cytoplasmic granules are bound by a 70-90 Å thick trilaminar membrane and have approximate diameters between 0.3 and 0.8 μm (Klebanoff and Clark, 1978). The nucleus is located towards the centre of the cell and is composed of 2-5 lobules linked together by thin threads of chromatin. Each lobule is composed of a peripheral ring of condensed heterochromatin and a core of loosely arranged euchromatin with no nucleoli. Other organelles within the neutrophil's cytoplasm include a rudimentary Golgi apparatus and scarce quantities of rough endoplasmic reticulum, mitochondria and glycogen granules (Klebanoff and Clark, 1978).

1. 1. 3. Histogenesis

Neutrophils are formed in the bone marrow by the division and differentiation of hemopoietic stem cells. Several stages in the development of neutrophils have been identified from histological sections of bone marrow and blood smears. These stages are, in order of their development; myeloblast, promyelocyte, myelocyte, metamyelocyte, band-form and mature neutrophil (Bainton, 1988). At the myeloblast stage of development the cells are poorly differentiated and have the potential to give rise to neutrophils, eosinophils and basophils. Primary granules appear during the promyelocyte stage of development and secondary granules appear during the myelocyte stage. At the metamyelocyte stage the developing cells become incapable of performing further mitoses and their nuclei develop a deep indentation. The nuclei of developing cells are further deformed into a 'U'-shape during the band-form stage and then finally become segmented as they develop into mature neutrophils (Bainton, 1988).

1. 1. 4. Kinetics

Mature neutrophils occupy three compartments within the body; the bone marrow, the blood stream and connective tissues. The bone marrow and blood stream provide storage sites for neutrophils and the connective tissues provide sites of neutrophil destruction. The bone marrow contains greater then 90% of mature neutrophils present in the body, with the remainder being primarily located in the blood stream. In adults, approximately 10^{14} neutrophils enter the blood stream from the bone marrow daily, but this number may increase to almost 10^{18} during infection. Within the blood stream, neutrophils are approximately evenly distributed between a free or circulating pool, and a marginated pool adherent to the walls of blood vessels (Davis and Gallin, 1981; Malech, 1988). The concentration of neutrophils in the circulating pool of blood is normally between 3,000 and 5,000/μl and rarely exceeds 30,000/μl during infection. Radioactive tracer studies indicate that neutrophils have a half-life of 6 to 10 hours in the blood stream, but can survive for 1 to 2 days within connective tissues. The oral cavity and gastrointestinal tract are considered to be the major sites of neutrophil departure into connective tissues, but the spleen is also thought to serve as a site for the destruction of senescent neutrophils (Davis and Gallin, 1981; Malech, 1988).

1. 2. Activities of neutrophils during inflammation (Fig. 1. 1.)

1. 2. 1. The inflammatory response

When tissues of the body are damaged a complex series of cellular and biochemical events are initiated, known collectively as an inflammatory response (Gallin et al., 1988). These events are localised to within the affected area and usually protect damaged tissues against further injury as well as aiding their repair. At the anatomical level, inflammation has been recognised since the time of the Ancient Greeks by the classical

symptoms of redness, heat, swelling, pain and loss of tissue function. In more recent times, the invention of the microscope has enabled studies of inflammatory responses at the cellular level. These studies have revealed inflamed tissues to be characterised by changes in the diameter and permeability of blood vessels. Furthermore, inflamed tissues contain large numbers of leukocytes which have emigrated from the blood stream (Boyd and Sheldon, 1980).

The composition of leukocytes present in inflamed tissues varies according to the duration of the inflammatory response. During the primary or acute stage of an inflammatory response affected tissues are infiltrated by large numbers of neutrophils. In the presence of continued or repeated injury a chronic stage of inflammation may develop. Transition of inflamed tissues from an acute to a chronic stage is characterised by the appearance of monocytes and lymphocytes and the disappearance of neutrophils (Florey, 1962; Buckley, 1963; Boyd and Sheldon, 1980; Gallin et al., 1988).

1. 2. 2. Vascular events of inflammation

The vascular events associated with an inflammatory response are, in order of their appearance; (1) a transient constriction of arterioles, (2) dilatation of arterioles, including the opening of new microvascular beds, thus promoting (3) an increased blood flow through the tissue, (4) an increase in the permeability of blood vessels which promotes passage of fluid and plasma proteins into the extravascular tissues, and finally (5) a decrease in blood flow resulting in the accumulation of blood cells within the blood vessels (Florey, 1962; Buckley, 1963).

1. 2. 3. Margination

During the initial constriction of blood vessels and increase in blood flow associated with an inflammatory response, blood cells including neutrophils appear to be concentrated in a centrally flowing stream. However, during the subsequent dilatation of

blood vessels the blood flow decreases and neutrophils appear closer to the vessel wall where they come into contact with endothelial cells which line the inner wall of blood vessels. At this stage, neutrophils are initially round and roll across the surface of the endothelial cells, occasionally being washed away by the blood stream. With time, these rolling cells adhere more strongly and become flattened and elongated in the direction of blood flow. This activity of neutrophils has been called 'pavementing', but is more commonly referred to as 'margination' (Clark and Clark, 1935; Florey, 1962; Movat, 1983; Colditz, 1987).

1. 2. 4. Emigration

Following margination, neutrophils develop an extension of their cytoplasm, or pseudopod, and locomote across the endothelium in the direction which this extension projects. The cell biological aspects of neutrophil motility will be discussed in a later section (1. 4.). On reaching a junction between two endothelial cells, the neutrophil's pseudopod extends between these cells and directs migration of the cell through the endothelium and underlying basement membrane into the surrounding connective tissues (Florey, 1962). This process is referred to as emigration or diapedesis and is similar to that displayed by neutrophils entering the blood stream after their formation in the bone marrow (Malech, 1988). An issue which is not clear is whether or not emigration of neutrophils through the walls of blood vessels might be facilitated by the release of digestive enzymes by these cells.

1. 2. 5. Chemotaxis

Following emigration, neutrophils continue to locomote and accumulate at the site of tissue injury (Buckley, 1963). In addition, inflamed tissues and bacteria produce chemicals which cause an infiltration of neutrophils when injected into normal tissues (see section 1. 3.). These observations have supported the theory that chemical

concentration gradients of soluble factors produced and/or released from inflamed tissues stimulate and direct the motility of neutrophils in vivo towards damaged tissues. The directed migration of cells in response to a chemical concentration gradient is called chemotaxis and the chemicals which induce this response are referred to as chemoattractants or chemotactic factors (Keller et al., 1977). Certain products from bacteria and inflamed tissues have been shown to induce neutrophil chemotaxis in vitro, but it is difficult to assess the extent to which this response contributes to the accumulation of these cells in vivo (Bignold, 1988). The motile response of neutrophils to chemotactic factors in vitro will be discussed in a later section (1. 4. 6.).

1. 2. 6. Phagocytosis and intracellular degranulation

At the site of inflammation, neutrophils begin to phagocytose and destroy invading micro-organisms. This antimicrobial activity of neutrophils consists of three important stages: attachment, engulfment and destruction. During the attachment stage neutrophils recognise specialised plasma proteins called opsonins (IgG, see section 1. 3. 5.; C3b and C3bi, see section 1. 3. 6.) which coat the surface of bacteria and bind to specific sites on the neutrophil's surface. During the engulfment stage, bacteria are usually surrounded by the anterior pseudopod of the advancing cell and sealed off inside the cell as a phagocytic vacuole. During the destruction stage, the neutrophils cytoplasmic granules fuse with the phagocytic vacuole to produce a phagolysosome and discharge their contents into the vacuolar space via a process referred to as intracellular degranulation (Klebanoff and Clark, 1978). The neutrophil's primary and secondary granules both participate in this process and their contents promote the destruction of micro-organisms within the phagolysosome by a variety of mechanisms including proteolysis (Elsbach and Weiss, 1988) and catalysis of reactions leading to the production of toxic oxygen radicals (see section, 1. 2. 8.).

1. 2. 7. Extracellular degranulation

During phagocytosis, degranulation often occurs into developing phagocytic vacuoles before their complete enclosure thus resulting in the release of granule contents into the extracellular space. This event is known as extracellular degranulation and is observed during the phagocytosis of a variety of opsonised and particulate materials by neutrophils including bacteria (Klebanoff and Clark, 1988). In addition, the fungal metabolite cytochalasin B, which disrupts the structure of actin microfilaments (Godman and Miranda, 1978; Schroeder, 1978), facilitates the extracellular degranulation of neutrophils induced by phagocytic stimuli, and promotes extracellular degranulation in response to chemotactic factors (Stossel, 1988). Thus far, stimuli of extracellular degranulation by neutrophils have been found to either induce the release of both primary and secondary granules or only induce the release of secondary granules (Klebanoff and Clark, 1978).

1. 2. 8. Respiratory burst

Phagocytosis and degranulation by the neutrophil is accompanied by a dramatic increase in the cell's uptake and consumption of oxygen known as the 'respiratory burst'. During the respiratory burst, oxygen uptaken by the cell is converted into superoxide anions ($O_2^{\cdot -}$) hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radicals ($OH^{\cdot -}$), all of which are toxic to micro-organisms. Conversion of oxygen into these metabolites is catalysed by an NADPH-oxidase located on the inner surface of the plasma membrane. This enzyme is dormant in the resting neutrophil, but is activated following invagination of the cell's plasma membrane to form a phagocytic vacuole. Following degranulation, the enzyme myeloperoxidase released from primary granules reacts with hydrogen peroxide in the presence of halide ions to produce hypochlorous acid an additional compound with potent antimicrobial properties (Klebanoff, 1988).

1. 2. 9. Tissue damage

In addition to destroying invading bacteria, the neutrophil's activities of respiratory burst and extracellular degranulation are also toxic to surrounding host tissues. The destruction of tissues by neutrophils is demonstrated during a variety of inflammatory disorders including rheumatoid arthritis (Harris, 1988) and adult respiratory distress syndrome (Simon and Ward, 1988).

1. 2. 10. Release of chemotactic factors

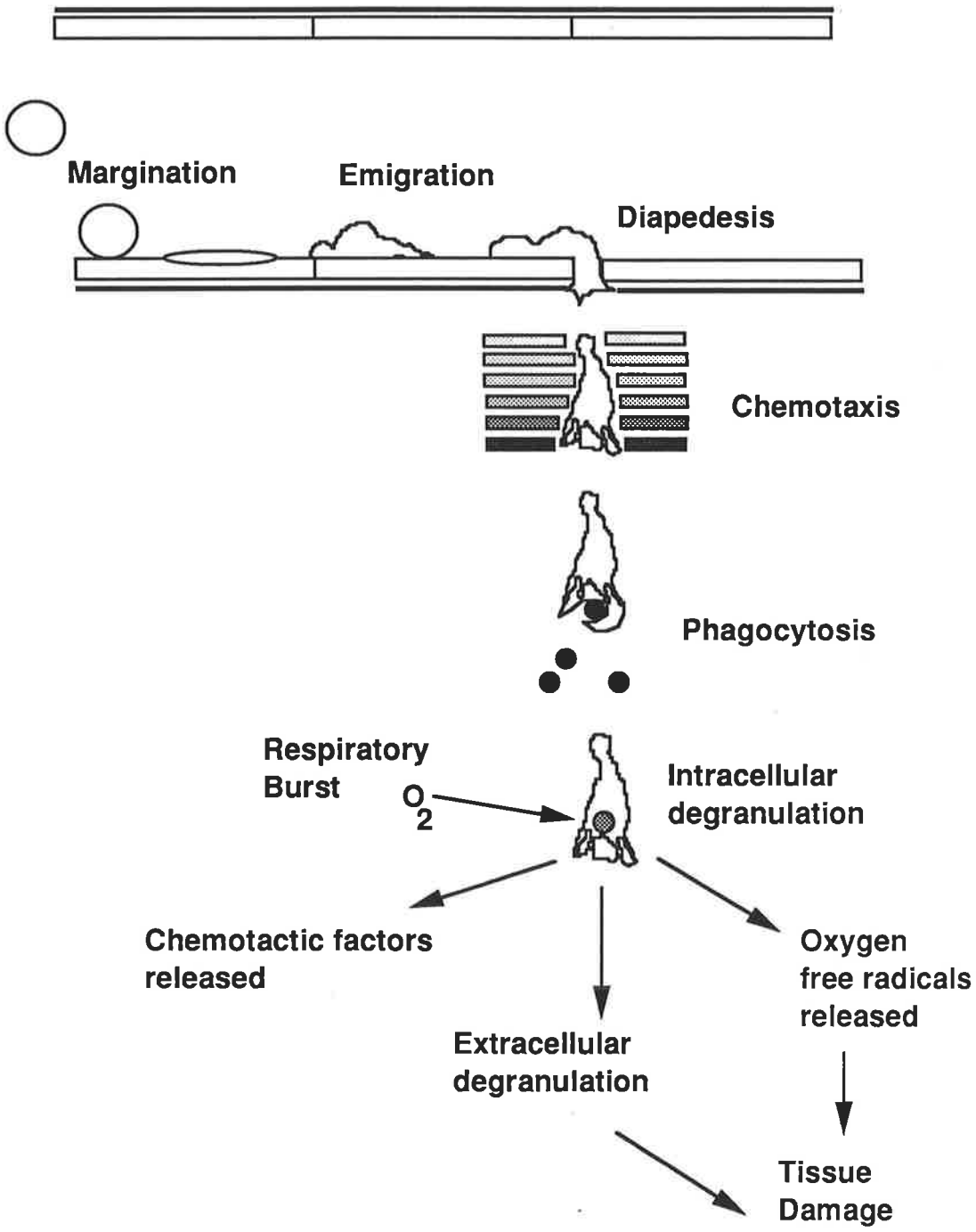
Several chemotactic and phagocytic stimuli have been reported to stimulate the production and release of additional chemotactic factors (leukotriene B_4, see section 1. 3. 8.; platelet activating factor, see section 1. 3. 9.) by neutrophils. This activity may contribute to the emigration of additional neutrophils to sites of inflammation in vivo.

1. 2. 11. Disorders related to defective activities of neutrophils

A large number of clinical and inflammatory disorders have been reported to be associated with impaired or defective activities of neutrophil (Curnette, 1988a; 1988b). Whether or not these defects are the primary cause or simply a result of these disorders is often not clear. Disorders of this type can be grouped according to the type of disease. For example, diabetes and thyroid disease are both associated with defective neutrophil function and can be classified as hormonal disorders (Klebanoff and Clark, 1978). Alternatively, disorders can be grouped according to the defective activity of neutrophils displayed. For example: lazy leukocyte syndrome, rheumatoid arthritis, leukemia, alcoholism and periodontal disease are associated with defective chemotaxis of neutrophils (Pinkerton et al., 1978; Klebanoff and Clark, 1978; Harris, 1988).

In most cases, the biochemical basis for defective activities of neutrophils observed during clinical and inflammatory disorders is not clear. Nevertheless, chronic

Fig. 1. 1. Activities of neutrophils during inflammation



granulomatous disease, hereditary myeloperoxidase deficiency and Chediak-Higashi syndrome, are three disorders which have been extensively studied at the molecular level. For example, chronic granulomatous disease (CGD) is characterised by recurrent and severe infections of the lymph nodes, skin, lung, liver and other organs, and is associated with a defect in the ability of neutrophils to destroy phagocytosed bacteria (Klebanoff and Clark, 1978). Detailed analysis of CGD has demonstrated this condition to be genetically transmitted disorder characterised by a deficiency in the NADPH oxidase required for the production of antimicrobial oxygen metabolites, as well as other enzymes required for the cell's oxidative metabolism (Curnette, 1988b).

1. 3. Regulation of neutrophil function by bacterial products, plasma proteins and inflammatory mediators

1. 3. 1. Bacterial products and their synthetic analogues

1. 3. 1. 1. N-formyl peptides

In 1975 Schiffmann et al. (1975) isolated peptides with chemotactic activity for neutrophils from culture supernatants of the bacterium E. coli . This discovery and subsequent studies have lead to the development of a group of synthetic N-formylated peptides with potent chemotactic properties for neutrophils (Showell et al., 1976). In particular, the peptide, N-formyl-methionyl-leucyl-phenylalanine (FMLP) has been an extensively studied stimulus of neutrophil motility, chemotaxis, degranulation and respiratory burst (Becker, 1990). Motile and chemotactic responses of neutrophils to FMLP have been reported to be optimal at a concentration of 10 nM (Keller, 1983; Keller et al., 1983). Higher concentrations (100 nM) of FMLP have been found necessary to stimulate optimal degranulation and respiratory burst responses in neutrophils (Lew et al., 1984; Jacob, 1988).

1. 3. 1. 2. Endotoxin

Endotoxins are lipopolysaccharides (LPS) liberated from bacteria (mainly from the cell wall) during their death and destruction (Raetz, 1990). The effects of these bacterial products on neutrophil kinetics are controversial since injections of LPS into the skin of animals provokes local tissue leukocytosis and intravenous administration of these compounds produces neutropenia (Cybulsky et al., 1988). Endotoxins have been reported to inhibit the accumulation of neutrophils at sites of inflammation and decrease the migration and chemotaxis of these cells in vitro (Hartiala et al., 1985; Bignold et al., 1991). Furthermore, while endotoxin is not directly chemotactic for neutrophils, these compounds are considered to stimulate the formation of chemotactic factors in plasma by activating the 'complement system' (section 1. 3. 6.)

1. 3. 2. The coagulation system of plasma

The coagulation or clotting system consists of a variety of soluble and cellular components including plasma proteases and their substrates, Ca^{2+} ions and platelets. Activation of these elements during inflammation results in the formation of a fibrous clot which plugs holes in the walls of damaged blood vessels thus preventing further loss of blood. A major product of coagulation and a primary component of the fibrous clot is the protein fibrin. The soluble elements remaining in blood following coagulation are known as serum.

1. 3. 2. 1. Activation (Fig. 1. 2.)

Coagulation can be activated by either an intrinsic pathway, for which all soluble factors are present within the blood vessel, or by an extrinsic pathway which requires the release of a tissue factor. The initial step in the intrinsic pathway involves the interaction between an inactive plasma protease called Hageman factor (also known as

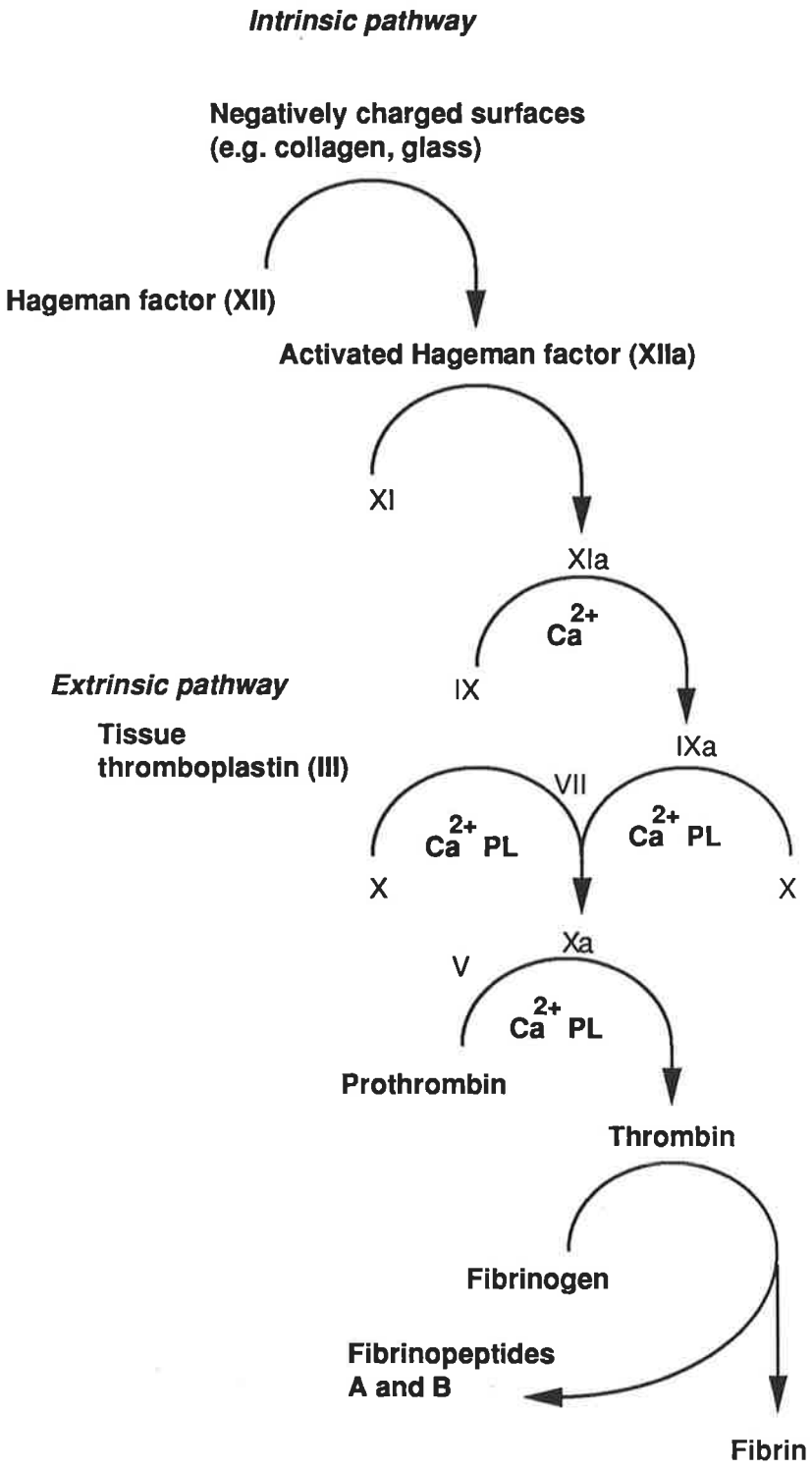
coagulation factor XII) with extravascular surfaces such as the collagen fibers of connective tissues (in vivo) and glass (in vitro). The interaction between Hageman factor and these surfaces produces an active form of the protease known as activated Hageman factor or coagulation factor XIIa. The series of subsequent proteolytic reactions, during which Ca^{2+} ions and platelets are required, produce the plasma protease thrombin from it's precursor molecule prothrombin. Thrombin then catalyses formation of fibrin from it's precursor, fibrinogen. The extrinsic pathway of coagulation is linked to the intrinsic pathway and is initiated by the release of the protease thromboplastin by tissues. Tissue thromboplastin (or coagulation factor III) activates the enzyme required for thrombin formation during the intrinsic pathway (Kaplan and Austen, 1975; O'Reilly, 1985).

1. 3. 2. 2. Anticoagulants

Anticoagulants are chemicals which are added to whole blood or plasma to inhibit the formation of fibrin clots. An anticoagulant which has been widely used in vitro and clinically is heparin (Leikin and Bessman,1956; Warkentin and Kelton, 1990). Heparin is found naturally within the secretory granules of mast cells but cannot be detected in normal plasma since it is rapidly degraded by macrophage when released into tissues (O'Reilly, 1985). Structurally, heparin consists of long, linear, polysaccharide chains attached to a central protein core, but commercial preparations of this anticoagulant consist only of various lengths of the molecule's side chains. These side chains are composed of an alternating sequence of negatively charged glucuronic and glucosamine subunits and combine with positively charged lysine residues on antithrombin III. Heparin-antithrombin III complexes inhibit coagulation by inactivating several coagulation factors including thrombin (O'Reilly, 1985). Chemicals which chelate Ca^{2+} ions have also been used to prevent coagulation. These anticoagulants include ethylene diamine tetra-acetic acid (EDTA), ethylenebis-(oxyethylene-nitrilo)-tetra-acetic acid (EGTA) and disodium hydrogen citrate (Platten et al., 1973).

Fig. 1. 2. The coagulation system of plasma

(adapted from Kaplan and Austen, 1975 and O' Reilly, 1985)



1. 3. 2. 3. Effects on neutrophils

While fresh plasma is not widely regarded as chemotactic for neutrophils, Wilkinson et al. (1969) and Stecher et al. (1971) have reported the appearance of chemotactic activity in plasma for neutrophils following coagulation. This activity was found to be greater when serum was produced in the presence of blood cells, particularly neutrophils. Furthermore, Lo et al. (1988) have demonstrated the appearance of a factor which induces neutrophil 'aggregation' (see section 1. 4. 2.) and adherence following the addition of thrombin to either whole blood or plasma. This observation is complicated by the fact that thrombin has been demonstrated to activate proteins of other systems in plasma including the complement component C5, a well established precursor of chemotactic factors (see section 1. 3. 6.; Sundsmo and Fair, 1985). Other components of the coagulation system which have been reported to activate neutrophils include activated Hageman factor which has been noted to stimulate aggregation and degranulation (Wachtfogel et al., 1986). In addition, fibrinopeptides A and B, released from fibrinogen during fibrin formation, are reported to be chemotactic for neutrophils (Stecher and Sorkin,1974; Skogen et al.,1988).

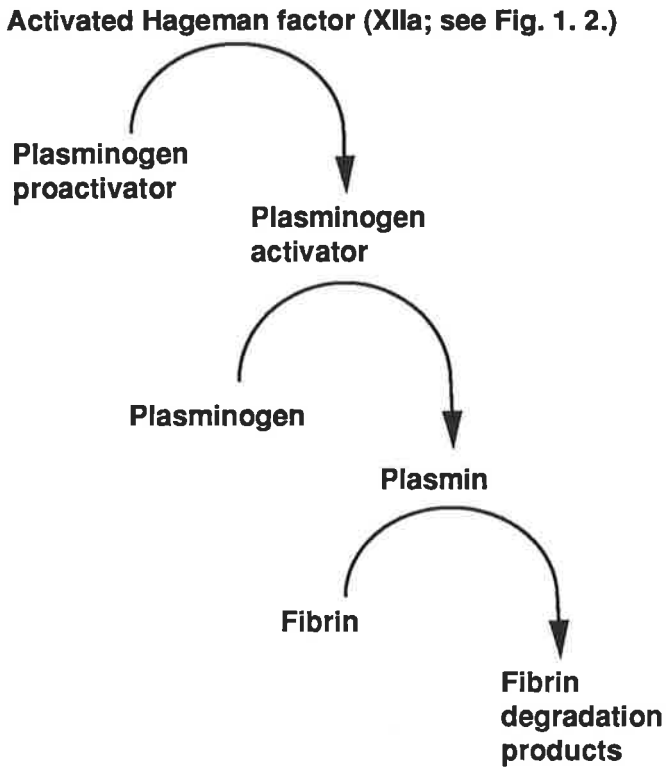
Studies with anticoagulants have also indicated a role for coagulation products during neutrophil activation. Platten et al. (1973) have reported the ability of anticoagulants to inhibit the motility of neutrophils in plasma. In this study, Ca^{2+} chelating anticoagulants were reported to reduce migration of neutrophils by inhibiting the formation of chemotactic factors. In contrast, heparin was reported to exert its inhibitory effects via a direct interaction with the cells.

1. 3. 3. The fibrinolytic system of plasma

The fibrinolytic system in plasma is linked to the coagulation system and when activated results in the formation of the plasma protease plasmin which degrades fibrin.

Fig. 1. 3. The fibrinolytic system of plasma

(adapted from Kaplan and Austen, 1975)



1. 3. 3. 1. Activation (Fig. 1. 3.)

Activated Hageman factor initiates fibrinolysis by converting an inactive plasma protease called plasminogen proactivator into it's active form, plasminogen activator. Finally, plasminogen activator catalyses the formation of plasmin from it's precursor molecule, plasminogen.

1. 3. 3. 2. Effects on neutrophils

Small peptides produced from the digestion of fibrin by plasmin are reported to be chemotactic for neutrophils (Stecher and Sorkin,1974; Skogen et al.,1988). In addition, plasminogen activator has been reported to be chemotactic for neutrophils (Kaplan et al., 1973).

1. 3. 4. The kinin system of plasma

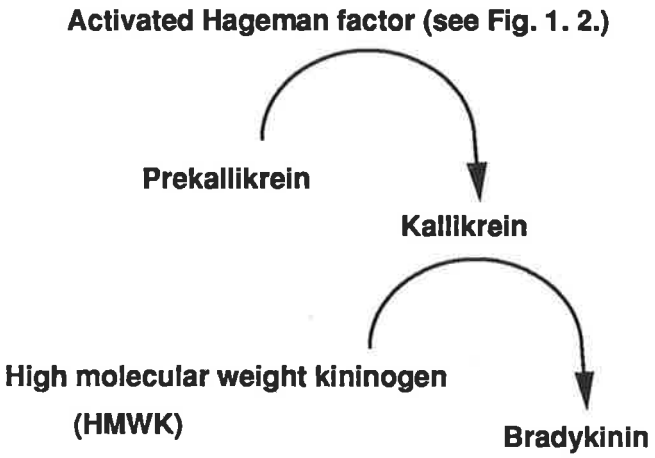
The kinin system consists of a family of plasma proteases which are activated during an inflammatory response to produce bradykinin. Bradykinin is a peptide of nine amino acids and a potent stimulus of the vascular events of acute inflammation including increased vascular permeability and vasodilation. Furthermore, bradykinin causes pain when injected into skin (Kaplan and Austen, 1975).

1. 3. 4. 1. Activation (Fig. 1. 4.)

The kinin system is activated in a similar manner to the coagulation and fibrinolytic systems by the formation of activated Hageman factor. Activated Hageman factor converts an additional plasma protein known as prekallikrein into it's active proteolytic form called kallikrein. Bradykinin is then formed from the proteolytic digestion of an additional protein known as high-molecular-weight kininogen (HMW-

Fig. 1. 4. The kinin system of plasma

(adapted from Kaplan and Austen, 1975)



kininogen) by kallikrein (Kaplan and Austen, 1975; Colman, 1986; Gustafson and Colman, 1987).

1. 3. 4. 2. Effects on neutrophils

Bradykinin is not considered to affect neutrophils, but purified kallikrein is reported to be chemotactic for these cells (Kaplan et al., 1972; Goetzl and Austen, 1974). In these studies, the ability of kallikrein to catalyse bradykinin formation and it's ability to promote neutrophil chemotaxis were both found to be blocked in the presence of soybean trypsin inhibitor, a known inhibitor of kallikrein's enzymatic activity. It has been subsequently suggested that kallikrein may activate neutrophils by digesting an unknown protein present on the cell's surface (Colman, 1986). This structure has yet to be identified, but Gustafson et al. (1989) have demonstrated neutrophils to bind high molecular weight kininogen which is known to exist as a complex with prekallikrein. In contrast to above studies, Wiggins et al. (1981) did not find purified kallikrein to be directly chemotactic for neutrophils in rabbits, but did find kallikrein to catalyse the formation of a chemotactic factor from the fifth component of complement (C5a; see section 1. 3. 6.). The issue of whether or not kallikrein is chemotactic for neutrophils is therefore controversial. Other reported effects of kallikrein on neutrophils include aggregation (Schapira et al., 1982, Colman, 1986) and degranulation (Wachtfogel et al., 1983; Colman, 1986). Nevertheless, other workers have reported purified preparations of human kallikrein to have no effect on the degranulation or respiratory burst of neutrophils (Kozin and Cochrane, 1988).

1. 3. 5. The immunoglobulins

1. 3. 5. 1. General activities and structure

Immunoglobulins, or antibodies are a group of glycoproteins produced by lymphocytes and plasma cells in response to foreign substances or antigens (Roitt et al, 1985). These glycoproteins bind to their appropriate antigen with various results including neutralisation of toxins, activation of plasma proteases (e. g. complement system; see section 1. 3. 6.) and stimulation of a variety of cells involved with inflammatory and immune responses including neutrophils.

The basic structural unit of immunoglobulins consist of two identical heavy polypeptide chains and two identical light polypeptide chains which are linked together by disulphide bonds (Fig. 1. 5. a.; Roitt et al, 1985). The amino acid sequences of these polypeptides are highly variable at their N-terminus (V regions) providing the diversity necessary to form antibodies of different specificity for forming immune complexes with different antigens. The C-terminal ends of heavy and light chains are relatively constant (C regions) except for minor genetically determined variations which do not affect the function of the antibody. These constant regions are required for interaction with other plasma proteins (complement system; see section, 1. 3. 6) and receptors located on the surface of cells. Some immunoglobulins are linked to form polymeric structures by an additional polypeptide sequence known as a J chain which binds to the C-terminal ends of their heavy chains.

Proteolytic digestion of immunoglobulins by enzymes such as papain (Fig. 1. 5. b.) or pepsin result in the separation of the molecule's antigen binding and cell binding regions (Gorevic et al., 1985). The fragments containing the immunoglobulin's antigen binding region are referred to as Fab fragments (antigen binding fragment) and the fragments containing the immunoglobulin's cell binding regions are referred to as Fc fragments (crystalline fragment).

Five classes of immunoglobulin have been identified in humans; immunoglobulin type G (IgG), type A (IgA), type M (IgM), type D (IgD) and type E (IgE). However, only IgG and IgA have been demonstrated to interact with human neutrophils (Lawrence et al., 1975; Alexander et al., 1979; Kurlander and Batker, 1982; Walsh and Kay, 1986; Albrechtsen et al., 1988).

Fig. 1. 5. a. Basic structure of immunoglobulins

(adapted from Roitt et al., 1985)

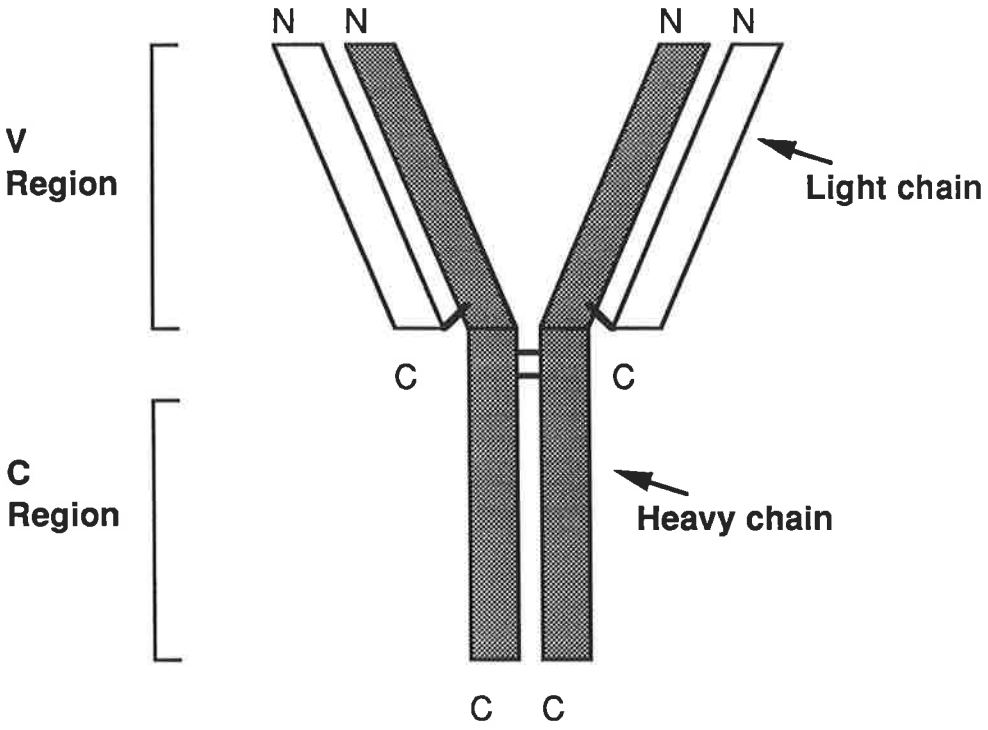
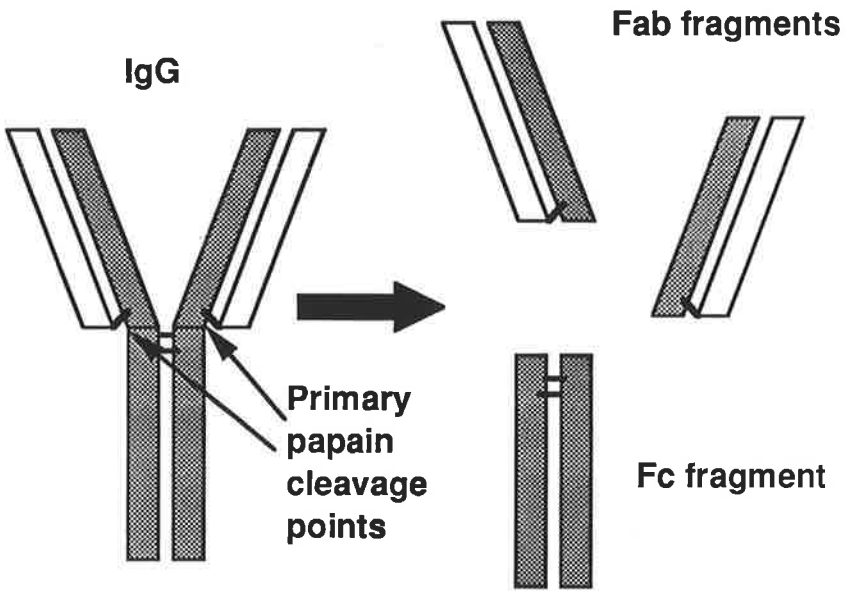


Fig. 1. 5. b. Papain digestion of IgG

(adapted from Roitt et al., 1985)



1. 3. 5. 2. Immunoglobulin type A

Immunoglobulin type A (IgA) constitutes 15-20% (w/v) of total serum immunoglobulins and exists mostly in a monomeric form with a molecular weight of 160 kDa. However, IgA is the predominant immunoglobulin found in body secretions where it is found as an dimer of approximately 385 kDa.

IgA has been reported to activate phagocytosis, degranulation and the respiratory burst in neutrophils (Gorter et al., 1987; Spiegelberg, 1988). With regard to neutrophil motility, IgA has been reported to inhibit migration towards chemotactic stimuli (Van Epps and Williams, 1976; Kemp et al., 1980; Schroder and Christophers, 1983; Moy et al., 1990).

1. 3. 5. 3. Immunoglobulin type G

Immunoglobulin type G (IgG), constitutes 70-75% (w/v) of total serum immunoglobulin and exists in a monomeric form in plasma. Four subclasses of these immunoglobulin have been characterised (IgG1, 2, 3 and 4) with an average molecular weight of approximately 160 kDa. During inflammation, IgG opsonises the surfaces of bacteria and inflammatory debris and immune complexes of IgG activate complement via the classical pathway (see section 1. 3. 6. 1.) thus producing the chemotactic factor C5a (Boyden, 1962; Laster and Gleich, 1971).

Polymeric molecules of IgG either in the form of immune complexes, aggregates (e.g. prepared by heating IgG monomers) or bound to surfaces such as bacteria or cells, bind to specific receptors located on the surface of neutrophils (see section 1. 2. 11. 1. 2.) and stimulate phagocytosis, degranulation and the respiratory burst (Hawkins, 1971; Henson, 1971a; 1971b; Henson et al., 1972; Smith et al., 1986; Blackburn and Heck, 1988).

With regard to chemotactic activity, Yamamoto et al. (1973) and Hayashi et al. (1974) have isolated a chemotactic factor from inflamed tissues called leucoegressin

which they believe to be a product derived from the limited proteolysis of IgG by proteases released during extracellular degranulation by neutrophils. Further evidence for chemotactic activity of structurally modified IgG has also been provided by Wilkinson (1980) who demonstrated this activity to correlate with the protein's degree of denaturation. The chemotactic activity of denatured IgG may not require interaction with the neutrophil's usual receptors for this protein (see section 1. 2. 11. 1. 2.), because serum albumin which does not usually bind to receptors on the neutrophil's surface, has also been reported to be chemotactic for these cells following denaturation (Wilkinson and McKay, 1971). Chemotaxis of neutrophils towards unmodified IgG has also been reported in humans (Kemp et al.,1980; Sibille et al., 1987) and rats (Arashi et al, 1989), but these findings are complicated by the results of other studies which suggest neutrophils treated with IgG release a chemotactic factor (Wilkinson, 1973; Zigmond and Hirsch, 1973). In addition, the effects of IgG on neutrophil motility are complicated by the ability of this immunoglobulin to alter the adhesion and spreading of neutrophils in contact with substrata (Keller et al., 1979a; Wilkinson, 1980; Kemp and Brown, 1980; Wilkinson et al., 1984; Hashimoto and Hurd, 1981).

1. 3. 6. The complement system of plasma

The term "complement" was introduced by Ehrlich at the turn of the century to describe a heat labile component of plasma which assisted antibodies in the killing of micro-organisms (Morgan, 1989). Complement is now known to consist of a group of proteins which when activated produce a "membrane attack complex" which assembles on the surface of bacteria promoting osmotic lysis of the organism (Ruddy, 1986; Morgan, 1989). Furthermore, peptides derived from the third component of complement (C3) known as C3b and C3bi, opsonise bacteria and tissue debris thus preparing this material for phagocytosis by neutrophils.

By-products of complement activation include a group of biologically active peptides referred to as anaphylatoxins. Anaphylatoxins are derived from the third, fourth

and fifth components of complement (C3, C4 and C5) and are known as C3a, C4a and C5a (Müller-Eberhard, 1988). These peptides produce vascular changes similar to those observed during an inflammatory response such as vasodilation and increased vascular permeability (Robbins et al., 1984). However, the formation of anaphylatoxins in human serum has been difficult to demonstrate owing to the presence of "anaphylatoxin inactivator" a plasma protease which rapidly abolishes the activities of both C3a and C5a by removing arginyl residues from the C-terminal ends of these peptides (Müller-Eberhard, 1988).

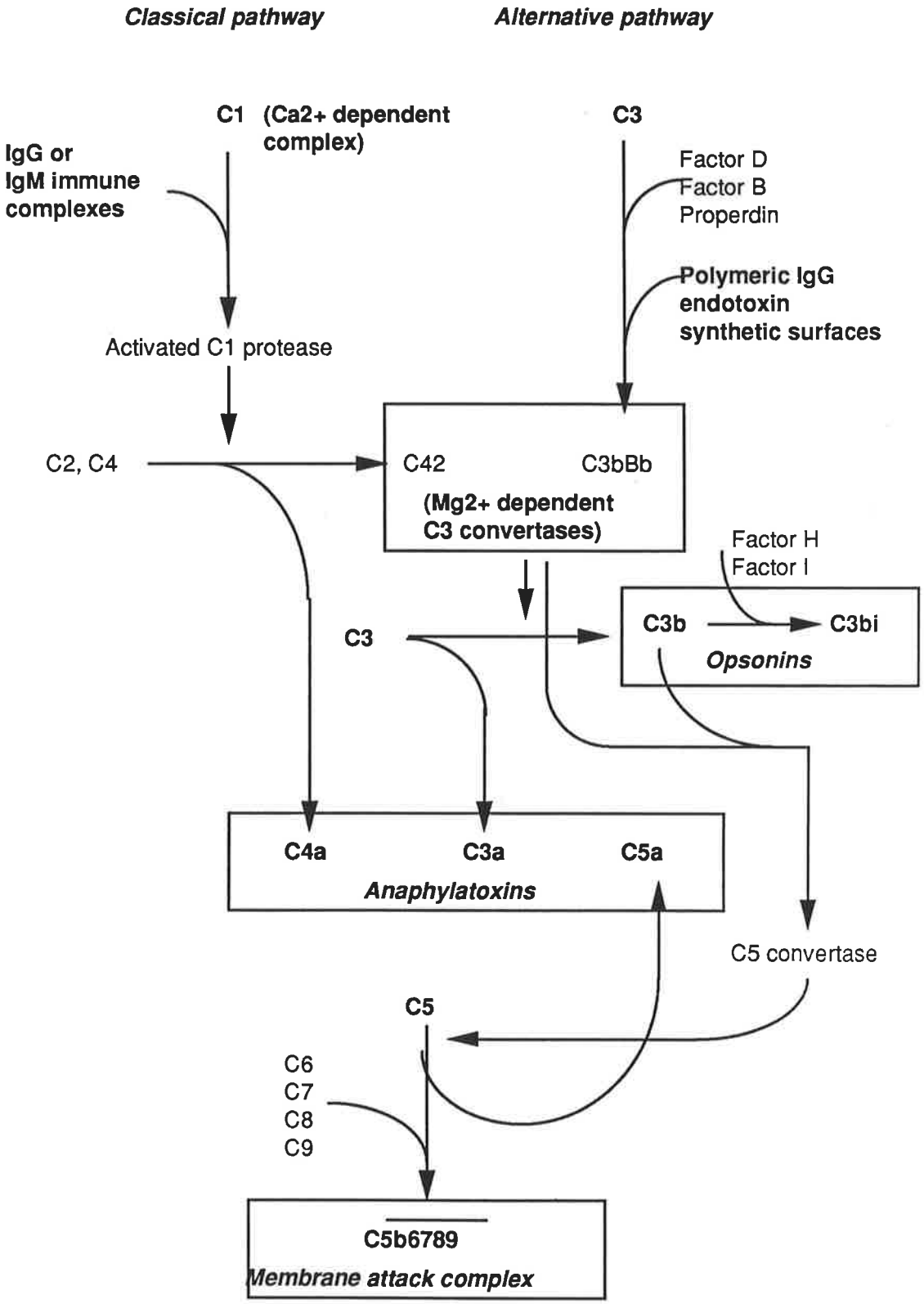
1. 3. 6. 1. Activation (Fig. 1. 6.)

Complement activation occurs either via a 'classical', immunological pathway or via an 'alternative' pathway. The initial step in the classical pathway involves interaction of a Ca^{2+} dependent complex known as C1 with immune complexes of IgG or IgM type antibodies. The initial step in the alternative pathway involves interaction of the third component of complement (C3) with either aggregated immunoglobulin or certain polymeric structures such as those found in bacteria (endotoxins; see section 1. 3. 1. 2.), fungi and viruses (Pangburn and Müller-Eberhard, 1985). While the primary steps of these two pathways differ, both result in the formation of a Mg^{2+} dependent enzyme known as a C3 convertase which then initiates activation of the remaining components of complement to produce the membrane attack complex and anaphylatoxins.

Both the classical and alternative pathways of complement activation are inhibited by the cation chelating agent EDTA which has a high affinity for Mg^{2+} ions as well as Ca^{2+} ions (Dawson et al., 1986). Nevertheless, since the alternative pathway of complement activation proceeds in the absence of Ca^{2+} ions, only the classical pathway is inhibited by EGTA since this chelating agent binds preferentially to Ca^{2+} ions and has a relatively poor affinity for Mg^{2+} ions (Kabat and Mayer, 1961; Fine et al., 1972; Cradock et al., 1977; Dawson et al., 1986).

Fig. 1. 6. The complement system of plasma

(adapted from Müller-Eberhard, 1988)



While complement activation is most widely studied in relation to inflammatory responses in tissues, C5a has been detected in the plasma of patients undergoing extracorporeal circulation during dialysis (Craddock, 1977) or open heart surgery (Chenoweth, 1981). Evidence from these studies suggests that certain synthetic surfaces encountered by plasma while outside the body activate complement via the alternative pathway.

1. 3. 6. 2. Effects on neutrophils

As opsonins, the complement fragments C3b and C3bi have the biological activity of promoting phagocytosis of micro-organisms by neutrophils (Goldstein, 1988). In addition, these peptides have been demonstrated to induce degranulation and respiratory burst responses in neutrophils (Goldstein, 1988).

Early evidence for the role of complement during neutrophil chemotaxis was provided by Boyden (1962), who described the appearance of a heat-stable chemotactic factor in serum following the addition of immune complexes. Subsequent studies indicated that the agent responsible for this chemotactic activity is the anaphylatoxin C5a (Hugli and Morgan, 1984; Goldstein, 1988). Concentrations of highly purified C5a as low as 0.1 nM have been reported to be chemotactic for neutrophils (Goldstein, 1988). However, concentrations of C5a higher than 1.0 nM have been reported to decrease motility of neutrophils by a process referred to as "desensitisation" (see section 1. 3. 12.). Further studies of the effects of C5a on neutrophil motility have demonstrated low concentrations (0.1 nM) of this peptide to reduce the adherence of neutrophils to cultured endothelial cells and stimulate chemotaxis, whereas higher concentrations (>1 nM) increased the adhesion of neutrophils and decreased their chemotaxis (Charo et al., 1986). In contrast to C5a, C3a and C4a do not possess potent chemotactic activity for neutrophils (Goldstein, 1988).

In a manner typical of other potent chemotactic factors such as FMLP, C5a has also been demonstrated to stimulate degranulation and respiratory burst responses in

neutrophils, which are enhanced by pretreating the cells with cytochalasin B (Hugli and Morgan, 1984; Goldstein, 1988).

1. 3. 7. Cytokines

1. 3. 7. 1. Historical perspective

Over the past 50 years researchers in the fields of immunology, virology, hematology and cell biology have been concerned with the identification and characterisation of polypeptide glycoproteins which regulate the activity of cells. Depending on the research discipline, these molecules have been referred to as interleukins or lymphokines (immunology), interferons (virology), colony-stimulating factors (hematology) or growth factors (cell biology). While having different origins of discovery it has become apparent that these classes of regulatory proteins often overlap. In view of this, the term cytokine is now often used to encompass all of these regulatory peptides (Bignold, 1989; West, 1990; Nathan and Sporn, 1991).

1. 3. 7. 2. Major cytokines

The cytokines relevant to neutrophils which have been most extensively studied are: the interleukins, 1, 2, 4, 6 and 8 (see 1. 3. 4. 3), tumour necrosis factors, interferon-gamma, granulocyte-macrophage colony stimulating factor and transforming growth factor ß (West, 1990).

1. 3. 7. 2. 1. Interleukin-1

Interleukin-1 (IL-1) was discovered in the 1940s as a substance produced in inflamed tissues, which caused fever when injected into animals and humans ("endogenous pyrogen"; reviewed by Dinarello, 1988). Initially, IL-1 was considered

to be primarily a product of monocytes when stimulated with LPS, but other cells including fibroblasts, endothelial cells and neutrophils can also produce this cytokine (West, 1990). Analysis of the structure of IL-1 has revealed two closely related forms of this molecule each with a molecular weight of approximately 17 kDa. The complete activities of this cytokine are broad and include the activation of T lymphocytes. In relation to neutrophils, IL-1 stimulates the production of these cells in the bone marrow and promotes their release into the blood stream. In addition, IL-1 increases the adhesive properties of cultured endothelial cells for neutrophils. An ability of IL-1 to affect emigration was demonstrated when this cytokine was found to induce accumulation of neutrophils when injected into the peritoneal cavity of mice. Nevertheless, this response was blocked by an inhibitor of protein synthesis. Furthermore, the ability of IL-1 to induce motility and chemotaxis of neutrophils in vitro is controversial as both positive (Bignold et al., 1990a) and negative (West, 1990) effects have been reported .

1. 3. 7. 2. 2. Interleukin-2

Interleukin-2 (IL-2) was discovered in the late 1970s as a factor which promoted the growth of activated human T lymphocytes in culture (reviewed by Greene, 1988). Activated T lymphocytes produce IL-2 as a 15 kDa protein which stimulates the production of other cytokines by helper T lymphocytes (West, 1990). Interleukin-2 is not chemotactic for neutrophils, but is reported to increase adhesion of these cells (West, 1990). The effects of IL-2 on the bactericidal activities performed by neutrophils are unknown.

1. 3. 7. 2. 3. Interleukin-4

Interleukin-4 (IL-4), like IL-2, is a product of activated T lymphocytes and stimulates activities of both T and B lymphocytes. This cytokine is not chemotactic for

neutrophils, but the effects of IL-4 on the other activities of this cell are unknown (West, 1990).

1. 3. 7. 2. 4. Interleukin-6

Interleukin-6 (IL-6) is a 26 kDa protein produced by T lymphocytes and fibroblasts with antiviral activity, which inhibits proliferation of fibroblasts and regulates synthesis of acute phase proteins by the liver (West, 1990). IL-6 is not chemotactic for neutrophils, but does stimulate extracellular degranulation by these cells (Borish et al., 1989). Furthermore, IL-6 has been reported to enhance the respiratory burst of neutrophils induced by chemotactic stimuli (Borish et al., 1989).

1. 3. 7. 2. 5. Tumour necrosis factors

Tumour necrosis factors (TNFs) are a family of cytokines produced by monocytic cells and were originally characterised by their ability to cause lysis of tumour cells. This family consists of TNF-alpha (cachectin) and TNF-beta (lymphotoxin). These peptides have a molecular weight of approximately 17 kDa and are 30% homologous in their amino acid sequence (Ruddle, 1987). In addition to destroying tumour cells, TNFs have a variety of other effects including cachexia (weight loss, TNF-alpha) and fibroblast mitogenesis (TNF-alpha and beta; West, 1990). With regard to neutrophils, TNFs have been demonstrated to enhance the adhesion of these cells to cultured endothelium (Remick et al., 1987). The ability of TNFs to induce neutrophil chemotaxis is controversial as positive responses (Ming et al., 1987) and negative responses (Bignold et al., 1990a) have been reported for both forms of this cytokine. Neutrophil phagocytosis and respiratory burst are stimulated by TNF-alpha (Tennenberg and Solomkin, 1990; West, 1990), but the effects of TNF-beta on these activities is not clear (West, 1990).

1. 3. 7. 2. 6. Interferon-gamma

Interferon-gamma (IFN gamma) is produced by T lymphocytes and belongs to a family of glycoproteins characterised by antiviral, immunoregulatory and antiproliferative functions (Figari et al., 1987). The structure of IFN gamma consists of two peptide subunits of 20 kDa and 25 kDa. In contrast to other IFNs, the actions of IFN gamma are primarily immunomodulatory rather than antiviral and include the activation of monocytic cells (Larsen and Henson, 1983). Neither adhesion nor chemotaxis of neutrophils are directly stimulated by IFN-gamma (Ribeiro et al., 1990; West, 1990), but this cytokine does stimulate the antimicrobial activities of these cells (Nathan, 1987; West, 1990).

1. 3. 7. 2. 7. Granulocyte-macrophage colony stimulating factor

Granulocyte-macrophage colony stimulating factor (GM-CSF) was originally detected because of it's ability to stimulate the formation of granulocytes and monocytes in bone marrow cultures (Metcalf, 1988). A variety of cells including, lymphocytes, monocytes, endothelial cells and fibroblasts produce GM-CSF as a 22 kDa glycoprotein (West, 1990). The effects of GM-CSF on mature neutrophils are: enhanced respiratory burst induced by chemotactic factors, increased phagocytosis of micro-organisms and reduced migration towards chemotactic factors (Golde and Gasson, 1988). GM-CSF has also been reported to stimulate neutrophil chemotaxis (Wang et al., 1987), but this finding has yet to be confirmed.

1. 3. 7. 2. 8. Transforming growth factor ß

Transforming growth factor ß (TGFß) is produced by fibroblasts and consists of two peptides joins together by disulphide bonds to produce a 25 kDa dimer. The general actions of this cytokine include fibroblast chemotaxis and inhibition of T and B

lymphocyte proliferation induced by other cytokines (Postlethwaite and Kang, 1988; West, 1990). Little is known of the effects of this cytokine on neutrophils (West, 1990).

1. 3. 7. 3. Interleukin-8

In recent years, a 6-8 kDa glycoprotein produced primarily by macrophage has been found to be a potent stimulus of neutrophil chemotaxis (Yoshimura et al., 1987; Van Damme, 1988). Terms used for this cytokine include monocyte-derived neutrophil chemotactic factor/peptide (Djeu et al., 1990), neutrophil activating peptide/factor 1 (Detmers, 1990) and interleukin 8 (IL-8; Larsen et al., 1989; Baggiolini et al., 1989). IL-8 consists of 72 amino acids with four cysteine residues and has considerable homology with heparin-binding proteins such as platelet factor 4 and b-thromboglobulin (Baggiolini et al., 1989). This structure, unlike that of several other endogenous chemotactic factors is insensitive to digestion by plasma proteases (Peveri et al., 1988).

In contrast to IL-1, subcutaneous injections of IL-8 produce neutrophil infiltrations (Van Damme et al., 1988; Foster et al., 1989; Larsen et al., 1989) which are independent of protein synthesis (Colditz et al., 1989) thus suggesting a direct chemotactic activity of this cytokine in vivo. Similar responses are observed for lymphocytes, but not monocytes (Larsen et al., 1989; Yoshimura et al., 1987). In vitro studies have demonstrated IL-8 to be chemotactic for neutrophils at concentrations between 1 and 100 nM (Yoshimura et al., 1987; Van Damme, 1988; Smith et al., 1991). Other effects of IL-8 on neutrophils include activation of the respiratory burst (Thelen et al., 1988; Colditz, et al., 1989), degranulation (Colditz, et al., 1989), and increased adhesive properties (Detmers et al., 1990).

1. 3. 8. Metabolites of arachidonic acid

1. 3. 8. 1. Major metabolites (Fig. 1. 7.)

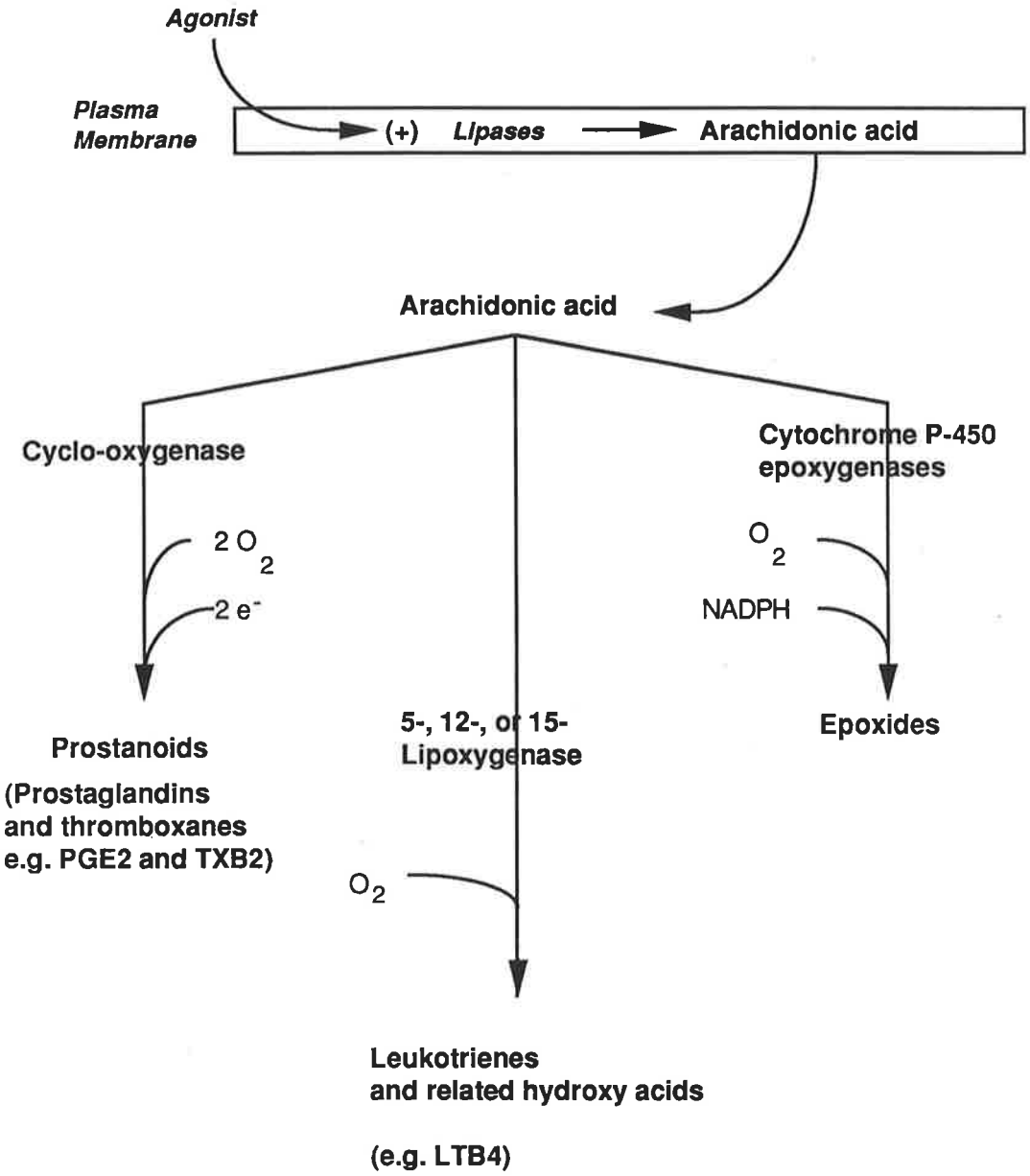
Arachidonic acid (5,8,11,14-eicosatetraenoic acid) is a 20 carbon chain, fatty acid found within the plasma membrane of mammalian cells. Phospholipases, activated following stimulation of cells by agonists, release arachidonic acid into the cytoplasm where it is metabolised into one or more oxygenated derivatives. These derivatives of arachidonic acid are classified into three major categories, (1) the 'prostanoids' (prostaglandins and thromboxanes), which are produced via the action of the enzyme cyclo-oxygenase, (2) the 'leukotrienes' (and related hydroxy acids) which are formed via the action of 5-, 12-, or 15-lipoxygenase, and (3) the 'epoxides' which are formed via the action of a cytochrome P-450 epoxygenases (Smith, 1989). A different subset of the arachidonate metabolites are synthesized and secreted by different cell types according to the availability of the enzymes required for their formation. The major arachidonate metabolite secreted by neutrophils is the leukotriene, 5[S],12[R]-dihydroxy-6,-14-cis-8,10 trans-eicosatetraenoic acid, more commonly referred to as leukotriene B_4 (LTB_4). Neutrophils synthesize and secrete LTB_4 in response to a variety of chemotactic stimuli such as FMLP and C5a, as well as immune complexes of IgG (Naccache et al., 1989; Ford-Hutchinson, 1990). Neutrophils have also been reported to secrete the prostanoids prostaglandin E_2 (PGE_2) and thromboxane B_2 (TXB_2), but these metabolites are produced in much smaller quantities that LTB_4 (Naccache et al., 1989).

1. 3. 8. 2. Inflammatory effects

Arachidonate metabolites are considered to be an important source of inflammatory mediators during inflammation. For example, the prostaglandin PGE_2 causes vasodilation and enhances the increased vascular permeability of blood vessels induced by histamine and bradykinin. In addition, the leukotriene LTB_4 induces emigration of neutrophils into tissues (Larsen and Henson, 1983; Ford-Hutchinson, 1990) and causes increased vascular permeability in the presence of PGE_2. The effects of LTB_4 on neutrophils and other cell types have implicated this arachidonate metabolite

Fig. 1. 7. Arachidonic acid metabolism in cells

(adapted from Smith, 1989)



in the development of asthma, gout, rheumatoid arthritis, and inflammatory diseases of the skin and bowel (Chilton, 1990; Ford-Hutchinson, 1990; Lewis et al., 1990).

A role for archachidonate metabolites during inflammatory processes is supported by studies of the mechanism of action of anti-inflammatory drugs such as corticosteroids. Corticosteroids such as cortisol inhibit arachidonate metabolism by blocking the action of phospholipases required for release of arachidonic acid into the cytoplasm (Marcus, 1988).

1. 3. 8. 3. Effects of LTB_4 on neutrophils in vitro

Since LTB_4 has been shown to stimulate emigration of neutrophils in vivo, most studies of the effects of arachidonate metabolites on neutrophils in vitro have concerned this inflammatory mediator. In studies of neutrophil motility, LTB_4 has been found to induce optimal chemotaxis at a concentration of approximately 100 nM (Ford-Hutchinson et al.,1980; Goetzl and Pickett, 1980; Hopkins et al., 1984). Higher concentrations of LTB_4 have been demonstrated to induce neutrophil degranulation, but this response was lower than those induced by either FMLP or C5a (Goetzl and Pickett, 1980). In addition, LTB_4 has also been reported to stimulate the respiratory burst of neutrophils, but again this response is considerably lower than that induced by other chemotactic factors such as FMLP (Naccache et al., 1989). Furthermore, LTB_4 has been demonstrated to stimulate adhesion of neutrophils to endothelial cells and nylon wool (Zimmermann et al., 1987; Naccache et al., 1989).

1. 3. 9. Platelet activating factors

1. 3. 9. 1. Origin and chemistry

During the early 1970s activated leukocytes were demonstrated to release a factor which induced platelet aggregation and degranulation. While the structure of this

chemical was not known it was named platelet activating factor in accordance with it's biological activity. Further analysis demonstrated PAF to be a group of 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine phospholipids containing alcohol side chains of varying length at the sn-1 position of the molecule (Snyder, 1989; Chilton and Lichtenstein, 1990). Platelet activating factor is now known to be produced by a variety of cells including neutrophils, platelets and endothelial cells and is considered to stimulate increases in vascular permeability associated with inflammation as well as during allergic responses such as asthma (Hanahan, 1986; Zimmerman et al., 1987; Pinckard et al., 1988).

1. 3. 9. 2. Effects on neutrophils

Platelet activating factor induces an accumulation of neutrophils when injected into tissues in vivo and stimulates neutrophil migration and chemotaxis in vitro (Shaw et al., 1981; Hopkins et al., 1984). Concentrations of PAF required for a maximum chemotactic response in neutrophils are reported to be approximately equal to 200 nM. Furthermore, PAF has been reported to stimulate other activities of the neutrophil including adhesion (Zimmermann et al., 1987), superoxide production (Shaw et al., 1981), degranulation (Shaw et al., 1981) and LTB_4 production (Lin et al., 1982). In addition to these activities PAF has been shown to prime responses of neutrophils to other stimuli (Braquet et al., 1989).

1. 3. 10. Histamine

Histamine (2-[4-imidazoyl]ethylamine or 5 ß-aminoethylimidazole), released from mast cells and basophils during an inflammatory response, increases the permeability of blood vessels and causes contraction of smooth cells in the walls of blood vessels and air-ways of the lung (Siraganian, 1988). Histamine is not chemotactic for neutrophils, but has been demonstrated to increase the motility of these cells (Anderson

et al., 1977). Nevertheless, the neutrophil's degranulation and respiratory burst responses to chemotactic factors are are generally reduced by histamine (Anderson et al., 1977; White and Kaliner, 1988; Reibman et al., 1990).

1. 3. 11. Stimulus-response coupling mechanisms in neutrophils (Fig. 1. 8.)

Considerable effort has been expended on identifying the mechanisms by which chemotactic and phagocytic stimuli activate responses in neutrophils. On the basis of these studies the following model for stimulus-response coupling in neutrophils has been established (Snyderman and Uhing, 1988).

1. 3. 11. 1. Membrane receptors

The primary step in the neutrophil's stimulus-response coupling pathway is the binding of chemotactic and phagocytic stimuli to receptor proteins located on the outer membrane of these cells (Lew, 1990). Specific membrane receptors have been characterised on neutrophils for several stimuli including FMLP (Williams et al., 1977), IgG (Anderson, 1989; Unkeless, 1989; Anderson et al., 1990; Huizinga et al., 1990), C5a (Huey and Hugli, 1985), IL-8 (Besemer et al., 1989); LTB_4 (Goldman and Goetzl, 1982; 1984; Lin et al., 1984) and PAF (Hwang et al., 1983; Valone and Goetzl, 1983).

1. 3. 11. 1. 1. Receptors for FMLP

The membrane receptor for FMLP has been partially characterised as a glycoprotein with a molecular weight of 60-70 kDa, with high and low affinity binding states. The FMLP receptor-ligand complex is internalised following it's formation and replaced by receptors released from internal stores (Snyderman and Uhing, 1988). In addition, the number of receptors for FMLP on the neutrophil's surface has been reported

to be upregulated following addition of FMLP, C5a, LTB_4 or PAF (Snyderman and Uhing, 1988; Norgauer et al., 1991). Furthermore, an asymmetric distribution of these receptors is seen on the cell's surface during locomotion (Sullivan et al., 1984).

1. 2. 11. 1. 2. Receptors for IgG

Immunoglobulin type G (IgG) has been demonstrated to bind to three different types of receptor proteins on human neutrophils; a 72 kDa type I receptor, a 40 kDa type II receptor and a 50-70 kDa type III receptor (Anderson, 1989; Unkeless, 1989; Anderson et al., 1990; Huizinga et al., 1990). The type I receptor (FcRI) is a high affinity binding site for monomeric IgG which is not normally found on neutrophils but is expressed after overnight incubation with the cytokine interferon gamma (Perussia et al., 1983; Petroni et al., 1988; Erbe et al., 1990). This receptor has been demonstrated to mediate phagocytosis and respiratory burst (Shen et al., 1987; Van de Winkel and Anderson, 1990; Ackerley et al., 1991). The type II (FcRII) and III (FcRIII) receptors bind to aggregates or immune complexes of IgG and do not require cellular activation for their surface expression. Approximately 10,000-20,000 type II receptors and 100,000-200,000 type III receptors, for IgG are present on the neutrophil's surface (Tosi and Berger, 1988; Huizinga et al., 1989). The type II receptor mediates phagocytosis, degranulation and respiratory burst (Huizinga et al., 1989; Van de Winkel and Anderson, 1990) and preferentially accumulates at the uropod of polarised cells (Petty et al., 1989). The type III receptor for IgG on human neutrophils belongs to a unique class of membrane proteins which are joined to the cell surface via a phosphoinositol (PI) linkage and therefore do not contain a transmembrane domain (Low, 1989; Skubitz and Lakatua, 1990). The role of this IgG receptor has been studied by removing it from the neutrophil's surface via digestion of it's PI linkage with enzymes such as phosphoinositol specific phospholipase C (PIPLC) or elastase (Tosi and Berger, 1988). This receptor has been reported to be more important than the type II receptor for the reception of immune complexes but less likely to transduce an activation signal

across the cell membrane (Tosi and Berger, 1988). In this model the type III receptor binds immune complexes and then presents them to the less abundant type II receptors which activate responses of the cell. Nevertheless, the type III receptor has been found to mediate increases in cytosolic calcium ions (Kimberly et al., 1990) and degranulation (Van de Winkel and Anderson, 1990).

1. 3. 11. 1. 3. Receptors for C5a

Studies have identified the C5a receptor on the surface of human neutrophils as a polypeptide with an approximate molecular weight of 42 to 48 kDa (Huey and Hugli, 1985; Goldstein, 1988).

1. 3. 11. 1. 4. Receptors for IL-8

Kinetic studies of the binding of radiolabelled IL-8 to neutrophils have indicated the existence of high and low affinity binding sites for IL-8 on these cells, but the relative roles of these receptors are not clear (Besemer et al., 1989). Little is known of the structure of IL-8 receptors on neutrophils.

1. 3. 11. 1. 5. Receptors for LTB_4

High and low affinity receptors have been identified for LTB_4 (Goldman and Goetzl, 1984). The high affinity binding site for LTB_4 has been characterised as a 60 kDa protein which is predominantly localised on the cell surface. While the high affinity receptor for LTB_4 regulates chemotaxis, the low affinity receptor regulates degranulation and respiratory burst. Down regulation of the high affinity receptor has been observed following stimulation, but is not related to internalisation (Goldman and Goetzl, 1984; Boggs et al., 1991).

1. 3. 11. 1. 6. Receptors for PAF

A variety of radioligand binding studies and experiments with antagonists indicate that PAF binds to specific sites on the surfaces of neutrophils and other cells, but the structure of these receptors is not clear (Hwang et al., 1983; Valone and Goetzl, 1983; Snyder, 1989).

1. 3. 11. 2. Guanine nucleotide-binding proteins

Guanine nucleotide-binding proteins (G-proteins) are a family of integral membrane proteins which bind GTP nucleotides following their interaction with receptor-ligand complexes. Evidence for the involvement of G-proteins during stimulus-response coupling pathways in neutrophils has been largely obtained by using pertussis toxin a product of the bacterium Bordetella pertussis which catalyses ribosylation of the GTP binding site of certain G-proteins thus rendering them inactive. Such studies have indicated a requirement for G-proteins during the motile and bactericidal responses of neutrophils to a number of stimuli including FMLP, IgG, C5a, LTB_4, PAF (Bokoch, 1990; Christiansen, 1990) and IL-8 (Thelen et al., 1989). Furthermore, a G-protein has reported to be linked to the membrane receptor for FMLP on neutrophils (Lad et al., 1985; Khachatrian et al., 1990). In addition to regulating responses of neutrophils, other evidence suggests that these G-proteins regulate the binding affinity of the chemotactic receptors (Pike, 1990).

1. 3. 11. 3. Phospholipid metabolism

One activity of G-proteins which is considered to take place following their stimulation by receptor-ligand complexes is the activation of phospholipid metabolism within the plasma membrane (Berridge, 1986; Bokoch, 1990, Snyderman and Uhing, 1988; Traynor-Kaplan, 1990). More specifically, activated G-proteins stimulate the

membrane associated enzyme, phospholipase C to breakdown the membrane phospholipid, phosphatidyl-inositol 4, 5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol-1,4,5,-triphosphate (IP_3). In neutrophils, DAG has been shown to activate the enzyme protein kinase C (PKC) by promoting it's translocation to the plasma membrane in the presence of Ca^{2+} ions (Tauber et al., 1990; Christiansen, 1990) and IP_3 stimulates the release of Ca^{2+}ions from cytoplasmic stores (see section 1. 3. 11. 5.).

1. 3. 11. 4. Protein kinase C

Early studies of PKC showed that it was the cellular receptor for the tumour promoting agent phorbol myristate acetate (PMA) which activates degranulation and respiratory burst responses of neutrophils (White et al., 1984). While PMA and the cellular activator of PKC, DAG (see above) do not stimulate neutrophil locomotion, both induce an increase and redistribution of F-actin within neutrophils and lymphocytes (Roos et al., 1987; Zimmermann et al., 1988a; Keller et al., 1989). In addition, a number of actin binding proteins including myosin are phosphorylated during their activation, but the role of PKC in these reactions is still not clear (Omann et al., 1987). Furthermore, PKC has been reported to regulate the activity of membrane receptors for chemotactic factors (O'Flaherty et al., 1986). The role of PKC during neutrophil activation has been studied using a number of inhibitors of the enzyme such as H-7 (1-[5-isoquinolinylsulfonyl]-2 methylpiperazine; Gaudry, 1988), staurosporine (Dewald et al., 1989) and others (Pontremoli et al., 1986; Sha'afi et al., 1986; Kramer et al., 1989). While these inhibitors are not totally specific for PKC they have been used widely for this purpose (Kawamoto and Hidaka, 1984).

1. 3. 11. 5. Calcium ions

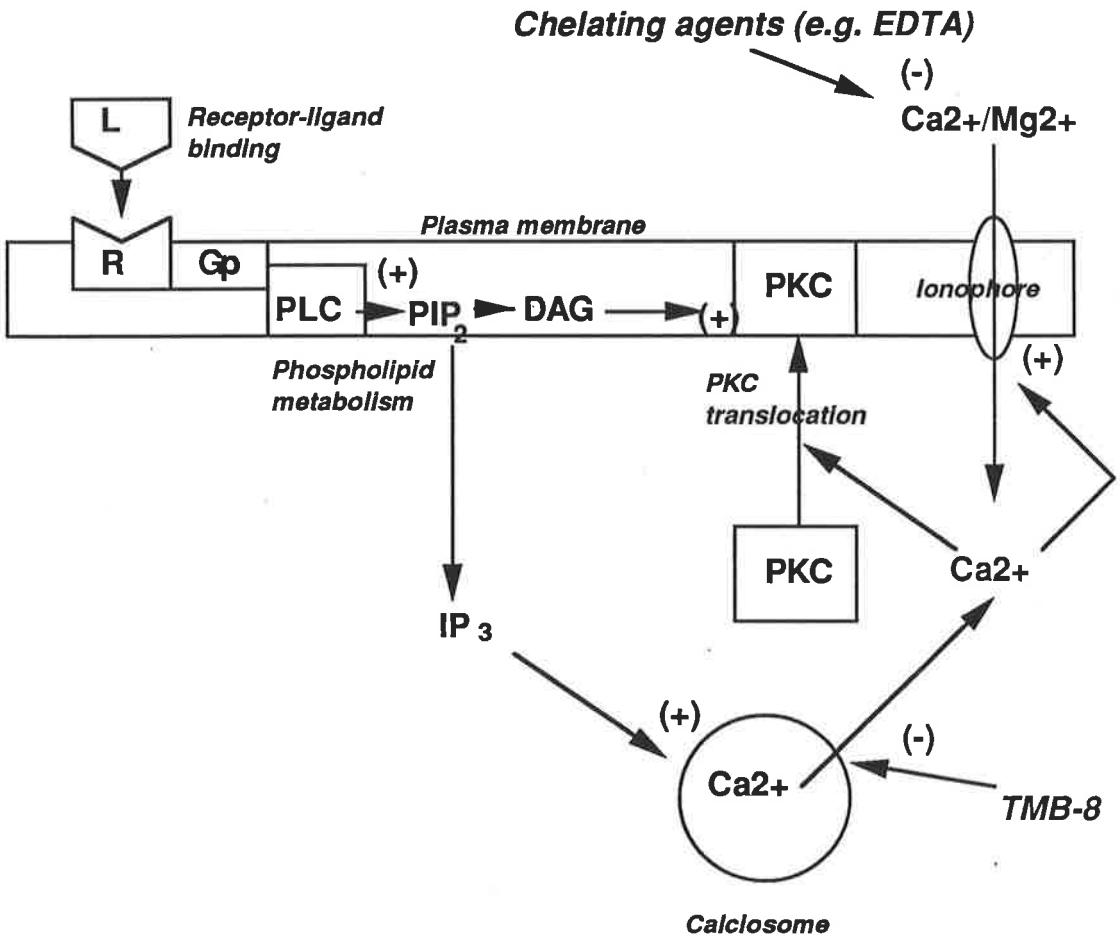
The intracellular concentration of divalent calcium ions ($[Ca^{2+}_i]$) in unstimulated neutrophils is approximately 100 nM (Hallett and Campbell, 1983). This

concentration is approximately 10,000 times smaller than that found in plasma and extracellular fluid (Marshall, 1976), and is similar to that found in most other mammalian cells (Campbell, 1986). In the neutrophil, this concentration is maintained primarily by active transport of Ca^{2+} ions out of the cell by a Mg^{2+}-ATP dependent pump located in the plasma membrane (Harlan et al., 1977). In addition, Ca^{2+} ions are associated with the plasma membrane (Naccache et al., 1979) and are uptaken and stored by poorly defined membrane bound organelles called 'calciosomes' (Krause and Lew, 1987). Furthermore, the neutrophil contains ion channels within it's plasma membrane which regulate influxes of extracellular Ca^{2+} ions into the cell (Andersson et al., 1986). These channels are operated by changes in $[Ca^{2+}_i]$ and are therefore unlike those activated by changes in the electrical potential difference across the plasma membrane (Westwick and Poll, 1986; Krause and Welsh, 1990).

When neutrophils are stimulated by chemotactic factors, an increase in $[Ca^{2+}_i]$ is observed. This increase occurs within seconds of the stimulus being applied and returns to basal levels within minutes. During this time the $[Ca^{2+}_i]$ may increase to levels greater than 1 μM (Westwick and Poll, 1986). The mechanism by which these changes in $[Ca^{2+}_i]$ are regulated has been extensively studied and the following model established (Dedman, 1986; Cooke et al., 1989; Di Virgilio et al., 1989). Inositol trisphosphate (IP_3) released from the plasma membrane following stimulation (see section 1. 3. 11. 3.) signals release of Ca^{2+} ions from calciosomes resulting in an initial increase in $[Ca^{2+}_i]$. This increase in $[Ca^{2+}_i]$ then opens Ca^{2+} channels present within the plasma membrane allowing entry of extracellular Ca^{2+} into the cell, thus increasing the $[Ca^{2+}_i]$ of the cell further. Finally, the Mg^{2+}-ATP dependent Ca^{2+} pump located in the neutrophil's plasma membrane is activated producing an efflux of Ca^{2+} ions which returns the $[Ca^{2+}_i]$ towards resting levels (Korchak et al., 1984). The time scale of these changes in $[Ca^{2+}_i]$ suggest that Ca^{2+} ions may act as a secondary messenger inside the neutrophils to regulate activation of the cell's motile and batericidal responses to external stimuli (Lew et al., 1984). This opinion has been supported by studies using TMB-8, a chemical which inhibits the release of Ca^{2+} ions from intracellular stores.

Fig. 1. 8. Stimulus-response coupling mechanisms in neutrophils

(adapted from Snyderman and Uhing, 1988)



Treatment of cells with TMB-8 inhibits both the rise in [Ca^{2+}_i] and subsequent responses of neutrophils induced by chemotactic factors (Smolen et al., 1981).

The role of extracellular Ca^{2+} has been studied by a variety of methods which include addition of cation chelators such as EDTA or EGTA to the incubation medium. In general, most responses of neutrophils to stimuli are found to be enhanced in the presence of extracellular Ca^{2+} ions (Hallett and Campbell, 1984; Westwick and Poll,1986; Borish et al., 1987; Sandborg and Smolen,1988; Snyderman and Uhing, 1988; Gallin and Sheehy, 1988; Gallin and McKinney,1989; Di Virgilio et al.,1990).

1. 3. 11. 6. Cyclic nucleotides

Chemotactic factors (FMLP, C5a, LTB4, PAF) and immune complexes of IgG all induce small but consistent increases in the intracellular concentration of cyclic adenosine 3',5'-monophosphate (cAMP) of neutrophils (Reibman et al., 1990). These responses begin within 5 seconds, peak between 10-20 seconds then return to baseline levels by 60-120 seconds (Smolen and Weissman, 1981). The time course of this response suggests that increases in intracellular cAMP may regulate neutrophil activities in a manner similar to that described in other cells (Cheung, 1981). However, studies of the relationship between rises intracellular cAMP concentration and the subsequent responses of the cell have failed to produce consistent evidence for a secondary-messenger role of these nucleotides (Reibman et al., 1990; Sandborg and Smolen, 1988). Nevertheless, increased levels of cAMP have been demonstrated to inhibit degranulation of neutrophils (Reibman et al., 1990).

Increases in the intracellular concentration of cyclic guanosine 3', 5'-monophosphate (cGMP) have also been demonstrated in neutrophils following addition of chemotactic factors such as FMLP. However, less literature is available of the possible role of these nucleotides than for cAMP. Nevertheless, Reibman et al. (1990) have proposed that cGMP may have a reciprocal effects to cAMP within the neutrophil since cGMP has been shown to enhance degranulation by these cells.

1. 3. 11. 7. Membrane potential

An electrical potential difference exists across the plasma membrane of cells created by differences in the ionic composition of intracellular and extracellular fluids. In the neutrophil, measurements of the cell's resting membrane potential have varied between -53 and -67 mV. The intracellular ions which contribute to this value are Na+ (25 mM), K+ (120 mM), Cl- (80 mM) and Ca^{2+} (approximately 100 nM; Gallin and Sheehy, 1988; Gallin and McKinney; 1989; 1990).

Stimulation of neutrophils with FMLP results in a depolarisation of the membrane potential followed by a repolarisation which is complete within 8-10 minutes. While the ionic basis of this response is yet to be resolved a Na+/K+ pump has been characterised in the cell membrane (Simchowitz et al., 1982). This pump is inhibited by ouabain and appears to be sensitive to intracellular Na+ ions. There is little reported evidence to suggest a relationship between membrane depolarisation and neutrophil motility and findings for the bactericidal activities of the cell have been inconsistent (Gallin and McKinney, 1989; 1990).

1. 3. 11. 8. Intracellular pH

Chemoattractants induce a rapid acidification of the neutrophil's cytoplasm followed by alkalinisation (Swallow et al., 1990). One view is that acidification may result from activation of the cell's NADPH-oxidase (Sandborg and Smolen, 1988). Alkalinisation is considered to be mediated via an amiloride sensitive Na+/H+ pump which exports H+ ions out of the cell in return for an influx of Na+ ions. Evidence suggests that chemotactic factors turn on this pump (Naccache et al., 1977a; 1977b) via the activation of protein kinase C (Swallow et al., 1990).

Rearrangements of the neutrophil's cytoskeleton and migration have been correlated with changes in intracellular pH. For example a decrease in pH has been shown

to be necessary but insufficient for actin polymerisation (Molski and Sha'afi, 1987; Tonetti et al., 1990). Nevertheless, increases in pH have been demonstrated to enhance actin polymerisation and cell migration (Swallow et al., 1990). In particular, amiloride has been shown to inhibit the chemotaxis of neutrophils towards FMLP (Simchowitz and Cragoe, 1986). Little evidence is available to suggest that acidification initiates the respiratory burst, but a decrease in pH has been found to impair this response to FMLP (Swallow et al., 1990).

1. 3. 12. Deactivation of neutrophil functions by bacterial products and inflammatory mediators

Neutrophil deactivation or desensitisation can be defined as the reduced responsiveness of these cells to stimuli after prior exposure to the either the same (direct deactivation; Boggs et al., 1991) or other stimulus (cross-deactivation; Ward and Becker, 1968; Wright, 1981; Bignold et al., 1992). For example, Ternowitz et al. (1987) have reported direct deactivation of the neutrophils chemotactic response to LTB_4 following prior exposure to this agent, and cross-deactivation of the chemotactic response to LTB_4 by prior exposure to FMLP.

Possible explanations for the mechanisms of deactivation in neutrophils include two main categories. The first category deal with the possibility that components of the neutrophil's signal transduction pathway such as membrane receptors, intracellular Ca^{2+} ions and protein kinase C might be consumed during the response to the initial stimulus and thus unavailable during responses to subsequent stimuli. The second category of explanations for deactivation deal with the possibility that inhibitors may be released during the initial response of a cell which then prevent or reduce the responsiveness of the cell to subsequent stimuli (Wright, 1981).

1. 4. Cell biological aspects of the motility of neutrophils

1. 4. 1. The motile behaviour of neutrophils

The motile behaviour displayed by neutrophils in vivo is also displayed in vitro and is similar to that displayed by other mammalian cells including lymphocytes (De Bruyn, 1944; Haston and Shields, 1984), macrophage, cultured endothelial cells and fibroblasts (Singer and Kupfer, 1986) and tumour cells (Zimmermann and Keller, 1987). In addition, the motility of these mammalian cells is often termed amoeboid owing to it's similarity to that displayed by amoebae (De Bruyn, 1944; Devreotes and Zigmond, 1988). The characteristic features of amoeboid movement are the requirement for a substratum, on which the cell migrates, and contractile or crawling-like movements of the cytoplasm. The crawling-like movements of the cytoplasm during amoeboid movement divide cells into three regions, (1) an anterior region characterised by a broad extension of the cytoplasm or pseudopod, (2) a body region which remains relatively free of extensions, and (3) a tail region characterised by a bulbous posterior extension or uropod (Lewis, 1934; De Bruyn, 1946). Furthermore, a narrow region of constricted cytoplasm, or contraction ring, has often been observed to form immediately behind the neutrophil's anterior pseudopod which passes down the length of the cell as a wave of contraction (Shields and Haston, 1985). This motile morphology is essential for amoeboid movement of cells across substrata and is often referred to as 'polarised' owing to the front-tail distribution of cytoplasmic extensions (Zigmond et al., 1981; Keller et al., 1983). The adoption of this polarised morphology by cells is referred to as 'polarisation' (Bignold et al., 1990b; see also section 1. 5.).

1. 4. 2. Adhesion, spreading and aggregation

Theories of neutrophil migration propose that adhesion to a substratum provides the traction required to convert the contractile movements of the cytoplasm into locomotion of the cell. Nevertheless, studies have shown that the degree of cell adhesion to

a substratum affects it's rate of migration (Wilkinson, 1982). When adhesion is increased the rate of neutrophil migration is reduced. Conversely, when the degree of adhesion is too low the cell can not obtain the necessary traction required for migration.

Neutrophils adhere to a variety of artificial substrates including plastics and glass (Keller, 1983; Smith and Hollers, 1980). The major factor affecting motility of neutrophils on surfaces such as glass is the presence of proteins such as serum albumin in the incubation medium (Keller et al, 1983). In the absence of protein, adhesion is liable to be excessive and accompanied by a pronounced spreading of the cell across the surface (Bessis and de Boisfleury, 1976; Rogers and Bignold, 1990). Other plasma proteins, such as immunoglobulins, increase adhesion and spreading, while fibronectin decreases adhesion (Bignold et al, 1990b). This effect of fibronectin on neutrophil adhesion is the reverse of its effect on adhesion of fibroblasts (Singer and Kupfer, 1986).

The morphological characteristics of adherent cells, for example, during locomotion on synthetic surfaces such as glass, involve mainly the neutrophil's anterior pseudopod which appears as a flattened veil-like structure or lamellipodium, and appears to pull the remainder of the cell along behind itself (Ramsey, 1972b). In contrast, the body of the cell appears to have less contact with the underlying substratum. The uropod or tail of locomoting neutrophils has often been regarded as very adhesive for the underlying substratum. This opinion is partly based on the appearance of long filamentous structures (retraction fibers) which stretch out from the rear of the cell and eventually break leaving behind traces of cellular material (Ramsey, 1972b; Bessis and de Boisfleury, 1976; Zigmond and Sullivan, 1979).

Studies of the mechanism of adhesion of neutrophils to cells and artificial surfaces have lead to the development of two major theories. Firstly, the inhibitory effects of cation chelating agents such as EDTA on adhesion have suggested that divalent cations such as Ca^{2+} and Mg^{2+} may provide cross-bridges between negatively charged glycoproteins located on the surface of neutrophils and negatively charged sites on substrata (Jones, 1970). More recently, monoclonal antibodies to the neutrophil's surface have been used

to inhibit adhesion of these cells to cultured endothelial cells (Tonnesen et al., 1989). These findings have been interpreted as evidence for the existence of specific adhesion or receptor sites located on the surfaces of cells called 'adhesion molecules' which regulate cell-cell adhesion (Butcher, 1990; Edelman and Crossin, 1991). One adhesion molecule which has been identified is an antigen common to the surfaces of neutrophils and monocytes called Mo-1 or Mac-1. This structure corresponds to a molecule known as the complement type 3 receptor (CR3), otherwise referred to as the C3bi receptor (Gallin, 1988). While proteins such as adhesion molecules have been identified as major contributors to neutrophil adhesion the mechanism of how these and other surface molecules regulate cell migration is not clear. In particular a current area of interest is how molecules on the cells surface may serve as a link between the cytoskeleton and substratum (Bignold, 1987b).

In addition to adhering to endothelial cells, neutrophils treated with chemotactic factors have been demonstrated to adhere to each other via a process referred to as 'aggregation' (O'Flaherty et al.,1977). This activity, along with spreading and chemotaxis, is impaired in patients with leukocyte adhesion protein deficiency; a condition in which neutrophils lack the C3bi receptor on their surface (Gallin, 1988).

1. 4. 3. Intrinsic motility

When neutrophils are allowed to settle onto artificial substrata in the absence of any apparent stimulus, most cells develop a continuous veil of extended cytoplasm around their periphery which causes them to spread. Nevertheless, in some instances these adherent cells adopt a polarised morphology and begin to locomote in the direction of the extended cytoplasm (Zigmond, 1977; Boyles and Bainton, 1979). These observations have lead to the notion that motility may be an intrinsic or spontaneous activity of neutrophils in contact with substrata.

1. 4. 4. Random motility

Random motility refers to the direction of neutrophil migration and has been defined in two ways (Keller et al., 1977). Neutrophil motility may be considered to be random if cells within a population of adherent cells have different directions of migration. Alternatively, random motility may be considered as an aspect of the locomotion of individual cells. In this instance, the motility of the cell is considered random when it is composed of short passages of movement in random directions.

1. 4. 5. Chemokinesis

A major area of interest to studies of migration of neutrophils has been the indentification of factors which may alter the rate or direction of locomotion. One important finding of these studies is that chemical agents may alter the rate of neutrophil migration without affecting the direction of cell migration (Keller and Zimmermann, 1987; Keller et al., 1990b). This motile response of neutrophils is referred to as 'chemokinesis' and the agents which stimulate this activity are known as 'chemokinetic' factors. While some chemokinetic stimuli appear to enhance migration by inducing formation of a polarised morphology others appear to do so by affecting the cell's degree of adhesion to substrata.

1. 4. 6. Chemotaxis

While neutrophil motility is often regarded as an intrinsic activity of the cell, it is more readily demonstrated as a response of the cell to chemotactic factors. When a concentration gradient of chemotactic factor is applied to a population of adherent neutrophils the cells polarise in the direction of increasing concentration and commence a sustained pattern of migration in this direction (Zigmond, 1977; Allan and Wilkinson, 1978; Zigmond et al., 1981). If the position of the chemotactic source is changed the direction of chemotaxis is altered accordingly (Ramsey, 1972a). If the concentration of

chemotactic factor is dramatically altered the neutrophil stops migration, adopts a rounded morphology and then repolarises before commencing migration again (Zigmond and Sullivan, 1979). In addition, under non gradient conditions polarisation and migration are induced but the pattern of migration is referred to as random. These observations suggest that neutrophils possess a mechanism for sensing gradients of chemotactic factors. There is much debate over whether neutrophils sense changes in chemotactic factor concentration over time (temporal model) or by comparing concentrations in the enviroment at different points along the length of their polarised morphology (spatial model; Lauffenburger et al., 1987).

The most widely accepted method for assaying chemotaxis of neutrophils is referred to as the Boyden chamber technique (Boyden, 1962; Wilkinson, 1982). The apparatus used in this method consists of upper and lower chambers separated by a membrane with pores large enough to allow the passage of a migrating neutrophil. When a chemotactic factor is added to the lower chamber a concentration gradient is established by diffusion into the upper chamber. Neutrophils are then applied to the upper chamber and the extent of chemotaxis assessed by the proportion of cells migrating through the porous membrane. In order to differentiate between chemotactic and chemokinetic responses it is necessary to compare the extent of migration in the presence of a concentration gradient with that obtained when the stimulating factor is added to both the upper and lower chamber (Frosch and Czarnetski, 1987).

1.4.7. Contact guidance and haptotaxis

In addition to chemotactic factors the direction of cell motility has also been reported to be controlled by the architecture and/or adhesive properties of the underlying substratum. This concept was first discussed by Weiss (1961) who introduced the term 'contact guidance' for his theory that cell orientation and migration are determined by differences in the number and relative strength of contacts made with substrata at varying points on the cell's surface. In a subsequent article, Carter (1965)

reported directed migration of fibroblasts towards increasing concentrations of metal particles coating a substratum. Carter suggested that this directed migration was caused by the metal particles acting as a gradient of adhesion sites and called this phenomenon 'haptotaxis'. In addition, Carter suggested that chemotactic factors may act in a similar manner by stimulating cell adhesion, but Keller et al. (1979b) have reported chemotaxis of neutrophils in the absence of increased adhesion. Nevertheless, studies by Wilkinson et al. (1982) and Wilkinson and Lackie (1983) have shown that neutrophil chemotaxis across a substratum of collagen or fibrin fibres is enhanced when the fibres are aligned in the axis of chemotactic gradient. The role that contact with substrata plays in directing migration of neutrophils therefore remains controversial.

1. 4. 8. Role of extracellular divalent cations

In general, studies have found that responses of neutrophils to a variety of stimuli are reduced in the absence of extracellular divalent cations. In particular, the motility and chemotaxis of neutrophils appears to be sensitive to the availability of these cations. While both extracellular Ca^{2+} and Mg^{2+}ions have been shown to be required for optimal chemotaxis (Becker and Showell, 1972; Gallin and Rosenthal, 1974; Wilkinson, 1975; Boucek and Snyderman,1976) spontaneous motility of neutrophils has been observed in the presence of Mg^{2+}ions alone (Becker and Showell, 1972). Different roles for extracellular Ca^{2+} and Mg^{2+} have also been reported by Marasco et al. (1980) who observed that more rapid migration took place in the absence of Ca^{2+} ions with nM concentrations of Mg^{2+} ions being sufficient to support migration.

One theory on the requirement for external divalent cations during neutrophil migration is that these ions regulate the cell's degree of adhesion to substrata. This theory is supported by the fact that chelating agents of Ca^{2+} and Mg^{2+} such EDTA have been shown to reduce the sticking of neutrophils to endothelial cells during acute inflammation (Jones,1970; Wilkinson, 1982). Similar effects of chelating agents have been found *in vitro* for the adhesion of neutrophils to artificial surfaces such as nylon and

glass (Bryant et al., 1966; Kvarstein,1969; Pfister et al., 1988). In these systems Mg^{2+} ions appear to be more important for cell adhesion than Ca^{2+} ions (Bryant et al., 1966; Kvarstein, 1969).

1. 4. 9. Role of intracellular calcium ions

The role of intracellular Ca^{2+} ions during neutrophil motility has been studied using a variety of techniques. Inhibition of intracellular Ca^{2+} ions using the fluorescent chelator quin-2 has been found to inhibit neutrophil chemotaxis (Elferink and Deierkauf, 1985; Meshulam et al., 1986), but increasing the intracellular concentration of cations by addition of membrane ionophores has lead to conflicting findings. While Wilkinson (1975) and Zigmond et al. (1988) reported chemotaxis to be inhibited by ionophores in the presence of external calcium ions, Estensen et al. (1976) reported enhanced chemotaxis by a mechanism which required both external Ca^{2+} and Mg^{2+} ions. Other studies have attempted to study the distribution of intracellular Ca^{2+} ions during neutrophil migration. Cramer and Gallin (1979) and Sawyer et al. (1985) have reported localisation of intracellular Ca^{2+} to the pseudopod of migrating neutrophils but Marks and Maxfield (1990a; 1990b) have recently been unable to support this finding. Furthermore, Perez et al. (1989) have reported that neutrophils do not require alterations in their intracellular calcium concentrations during chemotaxis.

1. 4. 10. Role of cytoskeleton

The apparent contractile nature of the neutrophil's crawling-like movements during locomotion suggested to researchers that their migration may be powered by interactions between polymers of actin and myosin similar to those observed in muscle cells (Stossel, 1988).

Actin can exist in either a monomeric form (G-actin) or in polymeric form (F-actin). In muscle cells the majority of actin is polymerised, but in non-muscle cells such as the neutrophil, most exists as G-actin (Sheterline and Rickard, 1989). However, when neutrophils are exposed to the chemotactic peptide FMLP the F-actin content of the cell is doubled within 30-45 seconds before decreasing to an intermediate level by 10 minutes (Wallace et al., 1984; Howard and Oresajo, 1985; Wang et al., 1985). These increases are accompanied by the redistribution of F-actin to within the cytoplasm of pseudopodia formed during polarisation (Oliver et al., 1978; Haston, 1987; Stossel, 1989). In addition to actin, pseudopodia of polarised neutrophils contain an assortment of actin binding proteins which have been demonstrated to alter the degree of actin filament polymerisation and cross-linking in vitro. In particular, gelsolin binds to and cleaves actin filaments in the presence of micromolar concentrations of Ca^{2+} ions (Stossel, 1988). Other evidence for the involvement of microfilaments during neutrophil migration comes from use of the fungal metabolite cytochalasin B which inhibits the assembly of F-actin filaments. Cells pretreated with cytochalasin B remain non-polarised and immobile when exposed to chemotactic factors (Hartwig and Stossel, 1976; Lichtman et al., 1976).

A myosin-like protein has been isolated from the cytoplasm of neutrophils (Stossel and Pollard, 1973; Senda et al., 1975) and has been shown to cause contraction of leukocyte cytoplasmic extracts in the presence of ATP in vitro, but the existence of actino-myosin contractions during neutrophil locomotion have yet to be unequivocally demonstrated. Furthermore, a myosin defective mutant of the slime mould Dictyostelium which displays amoeboid movement during the unicellular stage of it's life cycle, has been demonstrated to extend pseudopodia, but the polarity of these extensions is lost (Fukui et al., 1990).

Evidence for the involvement of microtubules during neutrophil migration and chemotaxis has been largely based on the effects of the microtubule disrupting agent colchicine (Malawista and Bensch, 1967; Becker, 1990). These studies suggest that microtubules are required for directing the migration of neutrophils towards

chemotactic stimuli, but not for polarisation and commencement of migration (Allan and Wilkinson, 1978). In addition, chemotactic factors have been demonstrated to induce an increase in microtubule length parallel to the direction of cell migration (Anderson et al., 1982). Keller et al. (1984) have reported colchicine to induce neutrophil polarisation (see section 1. 5. 5. 2.) and chemokinesis at concentrations which correlated with those required for microtubule disassembly. Nevertheless, these motile responses were lower than those activated by chemotactic factors.

1. 5. Studies of neutrophil polarisation in cell suspensions

1. 5. 1. The morphology of neutrophils in suspension

1. 5. 1. 1. Spherical and polarised

Although adoption of a polarised morphology is associated with the motility of neutrophils across substrata, this morphology is also displayed by neutrophils floating in cell suspensions. Early studies by Lichtman et al. (1976) suggested that neutrophils were always polarised when suspended in either plasma or artificial physiological media such as Hanks' balanced salt solution (HBSS), but subsequent studies (Smith et al., 1979; Keller et al., 1983; Shields and Haston, 1985; Haston and Shields, 1986; Roos et al., 1987; Bignold and Ferrante, 1988; Keller et al., 1990a; 1990b) have indicated that neutrophils remain spherical in suspension unless a chemotactic factor or other stimulus of neutrophil motility is present. Neutrophil polarisation in suspension is therefore an indication that the motile apparatus of these cells has been activated. Furthermore, assays of this shape change in suspension provide a method for distinguishing between chemokinetic factors which stimulate the neutrophil's motile apparatus and those which influence the cell's degree of adhesion to substrata.

1. 5. 1. 2. Non-polarised

Activators of protein kinase C such as DAG and PMA stimulate neutrophils to develop multiple extensions of the cytoplasm which are distributed randomly over the surface of the cell (Zimmermann et al., 1988a; Roos et al., 1988). Heavy water (D_2O) has been reported to induce a similar change in neutrophil shape, but the cytoplasmic projections were noted to be shorter and more densely packed than those induced by PMA or DAG (Zimmermann et al., 1988b). While the significance of this morphology is not clear, similar shapes are induced by chemotactic factors under certain conditions (section 1. 5. 2. 1.). Furthermore, neutrophils with a non-polarised morphology in suspension are immobile when allowed to settle onto a substratum (Keller, 1983).

1. 5. 1. 3. Zeiosis (Blebbing)

Bessis and de Boisfleury (1976) have documented the ability of leukocytes to develop small spherical extensions of the cytoplasm or "spherulations" under pathological conditions. These structures have subsequently been referred to as bubbles or blebs and the process by which they are formed is known as zeiosis (Godman and Miranda, 1978) or blebbing (Schroeder, 1978).

The significance of this morphology to neutrophil polarisation is that cells treated with cytochalasin B develop a blebbed morphology rather than a polarised morphology when exposed to the chemotactic peptide FMLP (Bignold and Ferrante, 1988). It has been suggested that this morphology might therefore be related to the marked degranulation induced by this combination of chemicals (Bignold and Ferrante, 1988).

1. 5. 2. Polarisation responses of neutrophils to FMLP and other stimuli

1. 5. 2. 1. Response to FMLP

The morphological response of neutrophils in suspension to the synthetic N-formyl peptide FMLP has been studied in more detail than responses to other stimuli. The results from these studies can be summarised as follows. At 30 seconds after addition of the peptide most cells display cytoplasmic extensions which are randomly distributed over their entire surface (Smith et al., 1979; Haston and Shields, 1986). After a few minutes these extensions are localised to one side of the cell while the body of the cell remains approximately round (Haston and Shields,1986) Over the next 30 minutes the cells increase in length as their bodies become elongated or oval (Yuli and Snyderman,1984; Howard and Osesajo, 1985; Shields and Haston, 1985; Haston and Shields, 1985; 1986). During elongation the rear of the cell often tapers off into a bulbous uropod (Keller, 1983; Keller et al., 1983). An optimal increase in cell length is seen at a concentration 10 nM which is approximately equal to the dissociation constant for the FMLP receptor. This optimal response is associated with a maximal proportion of polarised cells with uropods and correlates with concentrations required for optimal motility across substrata (Keller, 1983; Keller et al., 1983). Higher concentrations of FMLP (100 nM and 1000 nM) produce smaller increases in cell length and are associated with higher proportions of cells which have multiple and randomly distributed extensions (i.e. non-polarised cells; Keller, 1983; Keller et al., 1983; Shields and Haston,1985; Bignold and Ferrante, 1988). These cells have been referred to by different workers as 'non-polarised' (Keller, 1983; Keller et al., 1983), 'poorly polarised' (Shields and Haston, 1985) or 'short polarised' (Bignold and Ferrante, 1988). Nevertheless, the time course of these variations according to FMLP concentration have yet to be investigated.

The morphological response of neutrophils in suspension to FMLP has also been demonstrated to be reversible and cross-deactivated (see section 1. 3. 12.) by previous exposure to C5a (Smith et al., 1979).

1. 5. 2. 2. Response to plasma

The earliest studies to report neutrophil polarisation in cell suspensions observed this response when the cells were isolated from peripheral blood and resuspended in anticoagulated plasma, but similar levels of polarisation were observed in artificial physiological media (Lichtman et al., 1976; Keller and Cottier, 1981). However, Bignold and Ferrante (1988) have more recently demonstrated high levels of neutrophil polarisation in 100% heparinised plasma (>90%) while responses in a control medium of Hanks' balanced salt solution remained low (>10% polarised). Furthermore, Bignold and Ferrante (1988) have reported the polarised morphology of neutrophils suspended in plasma to be similar to that induced by FMLP. Nevertheless, time course and concentration-response studies of neutrophil polarisation in plasma have yet to be reported. In addition, while plasma is known to be a source of chemotactic factors (see section, 1. 3.), the stimuli responsible for inducing neutrophil polarisation in plasma are not clear since this medium has not been established as a chemotactic agent in vitro.

1. 5. 2. 3. Responses to inflammatory mediators

The morphological response of neutrophils to C5a has been demonstrated to be similar to that induced by FMLP both in the time course and type of shape changes induced by this peptide (Smith et al., 1979; Haston and Shields, 1985; Shields and Haston, 1985). Optimal increases in the mean length of neutrophils are reported to occur during the response to 1 nM C5a (Haston and Shields, 1985; Shields and Haston, 1985). Higher concentrations of C5a (100 nM) produce smaller increases in the mean length of cells (Shields and Haston, 1985). However, detailed time courses of the morphological changes in relation to concentration of this factor have not been reported. The response to C5a is reversible and is not deactivated (see section 1. 3. 12.) by prior stimulation with either C5a or FMLP (Smith et al., 1979).

The morphological response of neutrophils towards IL-8 has been examined by Thelen et al. (1988) but no direct observation of the cells' shape was made during this

study. Instead, the degree of light transmission through cell suspensions was used as an index of cell shape (see section 1. 5. 3. 3.). The degree of light transmission rose sharply within seconds of the addition of 3 nM IL-8 reaching a maximum level by 30 seconds, but returned to basal levels by 90 seconds.

Although LTB_4 and PAF are recognised as potent chemotactic stimuli for neutrophils, no studies appear to have been made of the morphological responses of neutrophils in suspension to either of these inflammatory mediators.

1. 5. 2. 4. Responses to microtubule disrupting agents and temperature changes

Microtubule disrupting agents such as colchicine induce polarisation of neutrophils in suspension (Keller et al., 1984), but several aspects of this response are different to that induced by FMLP. Unlike the rapid response to FMLP responses to colchicine are not seen until 3-5 minutes after stimulation and the extensions produced have a bubbled or zeiotic appearance.

Low temperatures (4 °C) have been reported to inhibit polarisation of neutrophils in suspension (Lichtman et al.,1976; Keller and Cottier, 1981), but cells stored at these temperatures have been reported to spontaneously polarise when placed at 37 °C (Keller et al., 1984). This response is considered to be due to disruption of microtubules which has been demonstrated to take place at low temperatures (Keller et al., 1984).

1. 5. 2. 5. Response to H-7

The protein kinase C inhibitor H-7 (see section, 1. 3. 11. 4.) has been recently reported by Keller et al. (1990a;1990b) to polarise neutrophils, and to stimulate motility of the cells, but not to be chemotactic. However, cells treated with H-7 are reported to have a different polarised morphology to that induced by FMLP as they lack a uropodal tail.

1. 5. 3. Role of extracellular divalent cations

The advantages of studying neutrophil polarisation in suspension have provided researchers with the opportunity to examine whether the effect of extracellular divalent cations on neutrophil motility (see section 1. 4. 8.) are related to activities of the cell other than adhesion. However, the results concerning the requirements of polarisation for extracellular cations is inconclusive. On the one hand, Keller and Cottier (1981) reported EDTA to inhibit polarisation of neutrophils suspended in plasma. On the other hand, Lichtman et al. (1976) reported neutrophil polarisation in plasma to be unaffected by chelating agents, and Smith et al. (1979), Marasco et al. (1980) and Shields and Haston (1986) found the morphological responses of neutrophils in suspension to chemotactic factors such as FMLP and C5a to be unaffected by exclusion of Ca^{2+} and Mg^{2+} ions, EDTA or calcium channel antagonists.

1. 5. 4. Role of intracellular divalent cations

Little is known of the requirements for intracellular Ca^{2+} ions during neutrophil polarisation in cell suspensions. For example, the effects of TMB-8 (an inhibitor of the release of Ca^{2+} ions from intracellular stores; see section 1. 3. 11. 5.) on the neutrophil's morphological responses to plasma and chemotactic stimuli of polarisation have not been examined in detail.

1. 5. 5. Role of microfilaments (F-actin)

Neutrophils in cell suspensions polymerise actin following the addition of FMLP (Howard and Oresajo, 1985). During this response, F-actin accumulates within the cytoplasm of pseudopodia (Haston, 1987) in a similar manner to that observed in neutrophils migrating across substrata (Oliver et al., 1978). However, Southwick et al.

(1989) have reported adhesion to substrata to also induce actin polymerisation. Studies of the effects of chemotactic factors on actin polymerisation and distribution in cell suspensions therefore prevent substratum induced responses from affecting the results obtained.

A technique which has been used to observe the distribution of F-actin microfilaments within neutrophils in suspension is to stain these cells following fixation with fluorescent derivatives of bicyclic peptides isolated from the mushroom Amanita phalloides known as phallotoxins. These fluorescent phallotoxins bind to large and small filaments of polymerised actin (F-actin), but do not bind to monomeric actin (G-actin). Using this method, several studies have documented the F-actin distribution within unstimulated (spherical) neutrophils and neutrophils polarised in response to FMLP (Howard and Oresajo, 1985; Haston, 1987; Roos et al., 1987). In these studies, unstimulated spherical neutrophils displayed a generally uniform distribution of F-actin within their cytoplasm. In contrast, the F-actin within cells treated with FMLP was preferentially located within pseudopodia. However, no studies have been made of the F-actin distribution within neutrophils polarised in response to plasma or chemotactic inflammatory mediators.

1. 5. 6. Techniques for measuring neutrophil polarisation

1. 5. 6. 1. Visual classification

The simplest and most widely used method for measuring neutrophil polarisation in suspension has been to count the number of cells with a 'polarised' morphology and express this value as a percentage of total cells observed. The criteria which have been used to define cells as 'polarised' have been based on the early descriptions by Lewis (1934) and De Bruyn (1944; 1946) of the amoeboid morphology of neutrophils while migrating across substrata (Lichtman et al., 1976; Keller and Cottier, 1981; Haston and Shields, 1986; Lopez et al., 1986; Bignold, 1986; Bignold, 1987a; Lord and Roath,

1990). Others have defined polarised cells as those whose morphology deviated from a spherical outline (Haston and Shields, 1985) or cells which are longer in one axis than the other with defined anterior and posterior ends (Haston and Wilkinson, 1988).

A more detailed visual classification method has been introduced by Keller (1983) and his colleagues (Keller et al., 1983) to distinguish between different morphological subtypes of polarised and non-polarised cells. In this method of Keller et al., neutrophils are classified into four morphological subtypes: (1) spherical cells, (2) cells polarised with a uropod, (3) cells polarised without a uropod, and (4) cells with non-polarised cytoplasmic extensions. More recent studies from Keller's laboratory have modified this system of classification to differentiate between polarised cells with spherical ('spherical smooth cells with unifocal projections') and elongated ('polarised') bodies (Roos et al, 1987; Zimmermann et al., 1988a; 1988b). Furthermore, Roos et al. (1987) have differentiated between spherical cells with 'smooth' and 'rough' surfaces. These more detailed methods of classification enabled Keller and his colleagues to detect the variations in the morphological response of neutrophils to different concentrations of FMLP as reviewed earlier (see section 1. 5. 2. 1.; Keller, 1983; Keller et al., 1983). Other authors have also introduced additional categories of polarised cells to describe these variations according to FMLP concentration ("long polarised", "short polarised"; Bignold and Ferrante, 1988).

Continuous cinema- or video microscopy methods have been used to follow the time course of polarisation responses in individual cells, but it is difficult to retain particular cells or groups of cells in a microscopic field of focus for any length of time in cell suspensions. Furthermore, these studies are limited to observations of small numbers of cells within a population. Time course studies of morphological responses of neutrophils in suspension have therefore been performed by removing and fixing samples of cell suspension at various times after addition of a stimulus and then assessing the morphology of cells when mounted between slide and coverslip (Roos et al., 1987).

1. 5. 6. 2. Morphometry

A number of different morphometric techniques have been used to quantify changes in neutrophil shape. These techniques were developed to provide a more objective measurement of neutrophil polarisation than that provided by visual classification methods. In early studies time and concentration dependent changes in neutrophil shape were recorded by measuring cell length (Keller, 1983; Haston and Shields, 1985; Shields and Haston, 1985). An adaptation of this technique has been to measure the ratio of the major and minor axes or length and width of cells (Donabedian et al., 1987). Other studies have measured changes in neutrophil shape with the assistance of computer programs which analyse the shape of digitised images of cells (Howard and Oresajo, 1985). These semi-automated techniques enable the calculation of parameters such as area, perimeter and ellipticity which are difficult to quantitate manually. Ellipticity is calculated from the area and perimeter of a cell and is expressed as a shape factor or F value between 1.0 and 0.0 with spherical cells having a theoretical F value of 1.0. Using this parameter Howard and Oresajo (1985) have demonstrated an increase in the ellipticity of neutrophils when exposed to FMLP over a 10 minute period.

1. 5. 6. 3. Flow cytometry and photometry

A different approach to assessing changes in neutrophil shape has been to examine the interaction between individual cells with electric currents in a 'Coulter counter'. Using this apparatus the volume of each cell is proportional to the electrical resistence which it introduces as it enters the path of an electric current flowing through an ionic solution. Using this technique, O'Flaherty et al. (1977) and Hsu and Becker (1975a; 1975b) have reported an increase in the mean volume of neutrophils in suspension after the addition of FMLP.

The interactions between cell suspensions and beams of light has also been used to assess changes in neutrophil shape. Yuli and Snyderman (1984) adapted a platelet

aggregometer to simultaneously measure perpendicular light scattering as well as light transmission within a suspension of neutrophils. Rapid changes in both parameters were detected but they could not be correlated with changes in cell morphology. Nevertheless, other workers have assumed that these photometric parameters provide an index of neutrophil shape changes (Faucher and Naccache,1987; Thelen et al., 1988; Wymann et al., 1989). Advances in this field have lead to the development of the fluorescence activated cell sorter or FACS. This apparatus detects both the emission and deflection of light by cells and has been used in conjunction with fluorescent probes to monitor a variety of neutrophil activities including phagocytosis, respiratory burst and expression of surface proteins (Fletcher and Seligmann, 1985; Lopez et al., 1986; Bassoe, 1989; Belloc, 1990). The outer morphology of cells is assessed using a parameter for measurement of deflected light known as 0° angle or forward scatter (FSC). Forward scatter detects light which is reflected off the front of the cell and provides an index of the the cell's size. An additional parameter known as 90° angle or side scatter (SSC) detects light which is deflected at right angles to the light source by organelles within the cytoplasm and therefore provides details about the cell's ultrastructure. While some workers have reported increases in FSC following the addition of chemotactic stimuli to suspensions of neutrophils (Fletcher and Seligmann, 1985; Lopez et al., 1986; Meshulam et al., 1986) others have noted decreases (McNeil et al.,1985; Donabedian et al., 1987). While the reason behind this difference in results is not clear only the findings of Lopez et al. (1986) were correlated with observed changes in cell shape which they determined to be polarisation. In addition, increases in FSC have been reported during the response to other stimuli which induce pseudopod formation such as PMA (Fletcher and Seligman, 1985; Roos et al., 1987). Decreases in SSC have been observed folowing stimulation of neutrophils but appear to be associated with degranulation rather than changes in cell shape (Fletcher and Seligmann, 1985; Meshulam et al., 1986).

1. 6. Outline of studies

From the foregoing, it is apparent that the adoption of a polarised morphology is an essential aspect of the motile and bactericidal activities of neutrophils in inflammatory lesions and in vitro. However, several important issues regarding this change in neutrophil shape remain unresolved. In particular, the factors responsible for the polarisation of neutrophils suspended in heparinised plasma are unknown. Furthermore, the polarisation responses of neutrophils in suspension to chemotactic inflammatory mediators have been poorly documented.

A detailed study was therefore made of the morphological responses of neutrophils in suspension to plasma and it's components, especially the immunoglobulin IgG. In addition, the morphological responses of neutrophils to C5a, IL-8, PAF and LTB_4 are examined since each of these inflammatory mediators have been well established as potent chemotactic stimuli for neutrophils. Furthermore, the responses of neutrophils to plasma and inflammatory mediators are compared with that induced by the synthetic chemotactic factor, FMLP.

The opportunity was also taken to begin studies of particular cell biological aspects of neutrophil polarisation induced by plasma and chemotactic factors. In particular, the roles of extracellular divalent cations (Ca^{2+} and Mg^{2+}), intracellular Ca^{2+} ions and the cytosolic distribution of microfilaments (F-actin) were examined because of the substantial literature concerning the involvement of these factors during related activities of the neutrophil especially adhesion, motility and chemotaxis. Considerable work was also carried out during the studies for this thesis with a preparation supplied as "H-7" by Sigma Co, with a view to examining the time courses and cation dependency of the neutrophil polarisation induced by this agent. However, the preparation did not polarise cells, and was later found to have been a chemically incorrect formulation of the compound (see Appendix).

Early in the course of these studies, it was realised that reported methods of assessing neutrophil polarisation in suspension required examination for their

suitability to the present work. Therefore, studies of several of these methods, particularly visual classification, were undertaken before the work on plasma and inflammatory mediators.

CHAPTER 2.

MATERIALS AND METHODS

2. 1. Materials

2. 1. 1. Chemicals

1. Acetic acid

   Ajax Chemicals, Auburn, New South Wales, Australia.

2. Blue dextran

   Pharmacia, Uppsala, Sweden.

3. Calcium chloride ($CaCl_2.2H_2O$)

   Ajax Chemicals, Auburn, New South Wales, Australia.

4. Coatasil siliconising solution

   Ajax Chemicals, Auburn, New South Wales, Australia.

5. Cohn fractions II, IV and V

   Sigma Chemical Company, St. Louis, Missouri, USA.

6. Cysteine HCl

   Was provided as a gift from Dr P. M. Bartold, Department of Pathology, University of Adelaide.

7. Dimethylsulfoxide (DMSO)

   Ajax Chemicals, Auburn, New South Wales, Australia.

8. Disodium hydrogen citrate

   By-products and Chemicals Pty. Ltd., NSW, Australia.

9. Ethanol

   Ajax Chemicals, Auburn, New South Wales, Australia.

10. Ethylene-diamine-tetra-acetic acid (EDTA)

    Sigma Chemical Company, St. Louis, Missouri, USA.

11. Ethylene-bis-(oxyethylenenitrilo)-tetra-acetic acid (EGTA)

    Sigma Chemical Company, St. Louis, Missouri, USA.

12. Fibronectin

    Sigma Chemical Company, St. Louis, Missouri, USA.

13. Ficoll 400

    Pharmacia LKB Biotechnology AB, Uppsala, Sweden.

14. Formyl-methionyl-leucyl-phenylalanine (FMLP)

    Sigma Chemical Company, St. Louis, Missouri, USA.

15. 25% glutaraldehyde, EM grade

    TAAB Laboratories Equipment Ltd., Reading, Berckshire, England.

16. Gentian violet

    Sigma Chemical Company, St. Louis, Missouri, USA.

17. Glycerol

Ajax Chemicals, Auburn, New South Wales, Australia.

18. Glycine

Sigma Chemical Company, St. Louis, Missouri, USA.

19. H-7 (1-[5-isoquinolinylsulfonyl]-2 methylpiperazine)

Sigma Chemical Company, St. Louis, Missouri, USA.

20. Hepes (N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid])

Sigma Chemical Company, St. Louis, Missouri, USA.

21. Human immunoglobulin type G (IgG; lyophilised)

Sigma Chemical Company, St. Louis, Missouri, USA.

22. Human immunoglobulin type G (20% solution)

Commonwealth Serum Laboratories (CSL), Melbourne, Australia.

23. Human gamma globulin

Sigma Chemical Company, St. Louis, Missouri, USA.

24. Human serum albumin, 20% solution (HSA)

Commonwealth Serum Laboratories, Melbourne, Australia.

25. Interleukin-8 (IL-8)

Provided as a gift by Dr I. J. D. Lindley of Sandoz Forschungsinstitut, Vienna, Austria.

26. Leukotriene B_4 (LTB_4)

Provided as a gift by Merck-Frosst Company, Quebec, Canada.

27. Lysophosphatidyl palmitoyl

Sigma Chemical Company, St. Louis, Missouri, USA.

28. Magnesium chloride ($MgCl_2.2H_2O$)

Ajax Chemicals, Auburn, New South Wales, Australia.

29. Magnesium sulphate ($MgSO_4.7H_20$)

Ajax Chemicals, Auburn, New South Wales, Australia.

30. 65% Meglumine diatrizoate (Angiografin)

Schering Pty. Ltd., Alexandria, NSW, Australia.

31. Monoclonal antibodies and fluorescein conjugated polyclonal antibodies

Kindly provided by Dr. S. Gadd of the Department of Pathology,
University of Adelaide.

32. Papain

Hopkin and Williams, Essex, England.

33. Paraformaldehyde

E. Merck, Darmstadt, Germany.

34. Phenol red

Sigma Chemical Company, St. Louis, Missouri, USA.

35. Phosphoinositol specific phospholipase C (PIPLC)

Immunotech SA, Marseille, Cedex, France.

36. Platelet activating factor (PAF)

Provided as a gift by Dr E. Bates of the Department of Immunology, Adelaide Children's Hospital, Adelaide, SA, Australia.

37. Rhodamine phalloidin

Molecular Probes Inc., Eugene, Oregon, USA.

38. Sodium azide

BDH Chemicals Ltd. Poole, England.

39. Sodium borohydride

Sigma Chemical Company, St. Louis, Missouri, USA.

40. Sodium diatrizoate

Sigma Chemical Company, St. Louis, Missouri, USA.

41. Sodium ethylmercurithiosalicylate (Thimerosal)

Sigma Chemical Company, St. Louis, Missouri, USA.

42. Sodium heparin

Fisons Pty. Ltd., Thornleigh, NSW, Australia

43. Soybean trypsin inhibitor

Sigma Chemical Company, St. Louis, Missouri, USA.

44. TMB-8 (8-[diethylamino]-octyl 3,4,5-trimethoxybenzoate hydrochloride)
Aldrich Chemical Co., Milwaukee, Wisconsin, USA.

45. Tris hydrochloride
Sigma Chemical Company, St. Louis, Missouri, USA.

46. Trypan blue
Sigma Chemical Company, St. Louis, Missouri, USA.

2. 1. 3. Buffers and reagents

1. Blood separation medium (specific gravity 1.114).

To 10 ml of double distilled water the following were added:

Ficoll 400	1.64 g
Sodium diatrizoate	1.13 g
65% Meglumine diatrizoate (Angiografin)	3.5 ml

This solution was then adjusted to a final volume of 20 ml with double distilled water and stored at 4 °C.

2. Hanks' balanced salt solution (HBSS) and HBSS-Hepes

To 500 ml of double distilled water the following were added:

NaCl	8.0 g
KCl	0.4 g
$CaCl_2$*	0.14 g
$MgSO_4.7H_2O$	0.2 g
Na_2HPO_4	0.48 g
$NaHCO_3$	0.36 g
KH_2PO_4	0.06 g
dextrose-D-glucose	1.0 g
phenol red	1 "rice grain"

* $CaCl_2$ was dissolved separately in 10 ml double distilled water before being added to the remainder of the solution.

This mixture was then; adjusted to a pH of 7.4, diluted to a final volume of 1000 ml, sterilised by passage through a Millipore filter and stored in glass bottles at 4 °C. Fresh stocks of HBSS were prepared every month.

For each experiment a new bottle of HBSS was opened and Hepes buffer (see below) added to a concentration of 20 mM producing HBSS-Hepes.

During preliminary studies, experiments often had to be discarded due to higher than acceptable numbers (see section, 2. 2. 7.) of cells displaying extensions in HBSS-Hepes. It was considered that this response of the cells may have been caused by an unknown contaminant present in the storage tanks for distilled water being used. In subsequent studies, all media were prepared using freshly distilled water and the percentages of cells displaying extensions while suspended in HBSS-Hepes were routinely ≤2% of the total number of cells counted.

3. Hepes buffer

To prepare 0.2 M Hepes buffer, 0.95 g of Hepes were dissolved in 10 ml double distilled water, the pH adjusted to 7.2 with 6 M NaOH and the volume adjusted to 20 ml with double distilled water. Hepes buffer was prepared fresh each week and stored at 4 °C.

4. Phosphate buffered saline (PBS)

To 400 ml double distilled water the following were added:

NaCl	4.0 g
KCl	0.1 g
$Na_2HPO_4.2H_2O$	0.71g
KH_2PO_4	0.1 g

The pH of the combined mixture was adjusted to 7.4 and the volume adjusted to 500 ml with double distilled water.

5. Glutaraldehyde (2.5% v/v) in 0.2 M phosphate buffer, pH 7.2.

Two 0.4 M phosphate solutions were prepared in the following manner:

Solution A: 6.24 g of $NaH_2PO_4.2H_20$ were dissolved in 100 ml of double distilled water.

Solution B: 8.52 g of Na_2HPO_4 were dissolved in 150 ml of double distilled water.

Fifty-six ml of solution A were combined with 144 ml of solution B and 40 ml of 25% glutaraldehyde EM grade. The pH of the combined solution was adjusted to 7.2 and the volume adjusted to 400 ml with double distilled water. This fixative solution was stored at 4 °C and prepared fresh every two to three months.

6. Formalin (8% v/v) in PBS.

Formalin was prepared fresh by dissolving 25 g of paraformaldehyde in 100 ml double distilled water at 60 °C. Solubility was aided by the addition of approximately 12 drops of 1 N NaOH. A stock solution of 8% formalin was prepared by dilution with phosphate buffered saline, pH 7.4 (PBS).

7. Tris buffered saline (TBS; 0.1 M, pH 7.8)

To 200 ml double distilled water were added the following:

NaCl	2 g
KCl	0.05 g
Tris HCl	3 g

This solution was then adjusted to a pH of 7.8 with HCl and adjusted to a final volume of 250 ml with double distilled water.

8. Tris buffered saline for Sephacryl 300 column chromatography of plasma proteins.

To 900 ml of double distilled water the following were added:

Tris HCl	12.11 g
NaCl	29.22 g

This solution was; adjusted to a pH of 8, adjusted to a final volume of 1 litre with double distilled water, degassed and sterilised by autoclaving and stored at 4 °C. Thimerosal (0.005% w/v) or sodium azide (0.01% w/v) were added to the buffer to prevent microbial growth.

2. 2. Methods

2. 2. 1. Isolation of neutrophils from human peripheral blood

Human blood was obtained by venepuncture and each 9 ml immediately added to a polycarbonate tube (Disposable products Pty. Ltd. South Australia) containing 125 I.U lithium heparin dissolved in 1 ml of 200 mM Hepes buffer (pH 7.2). Neutrophils were separated from this blood using the density gradient centrifugation method of Bignold and Ferrante (1987). Briefly, 5 ml of heparinised, buffered blood were layered onto 3 ml of a 'blood separation medium' with a specific gravity of 1.114 (see section 2. 1. 2.) and centrifuged at 500 x g for 15 minutes in a polycarbonate test tube with an inner diameter of approximately 1.4 cm and tapered end (Disposable products Pty. Ltd. South Australia). The plasma and mononuclear cell layers were then removed by Pasteur pipette and the remaining polymorphonuclear layer centrifuged for a further 15 minutes at 500 x g to improve the sedimentation of contaminating erythrocytes. The polymorphonuclear layer was then removed by Pasteur pipette and washed by adding to 3 ml HBSS-Hepes and centrifuging at 500 x g for 5 minutes. Washed cells were resuspended in fresh HBSS-Hepes and maintained at 37 °C throughout subsequent handling. The concentration of cells was adjusted to final stock concentration of 10^7/ml, after staining a sample of cell suspension with gentian violet in 7% (v/v) acetic acid and counting numbers of cells per µl in a hemocytometer. Cells in these preparations were greater than 95% polymorphonuclear leukocytes. Greater than 99% of cells in stock suspensions were found to be viable by their inability to uptake the stain Trypan blue.

Techniques for the lysis of contaminating erythrocytes were generally omitted as they have been reported to reduce the morphological responsiveness of neutrophils to polarisation stimuli (Bignold, 1987). Nevertheless, hypotonic lysis was used to improve the availability of enzyme during digestion of neutrophils with PIPLC (section 2. 2. 14). In these studies hypotonic lysis was perfomed by resuspension of

the washed polymorphonuclear layer pellet for 10 seconds in 900 µl of double distilled water immediately followed by the addition of 100 µl of a 10 x concentration of sterile PBS followed by 9 ml HBSS-Hepes. A similar technique was used during studies of the effects of erythrocyte lysis on changes in neutrophil shape assessed by FACS analysis (section, 3. 2. 2.).

2. 2. 2. Preparation of plasma and sera

Fresh autologous plasma was routinely obtained as the upper cell free layer formed during density gradient centrifugation of whole blood (see section 2. 2. 2.) This preparation of plasma consisting of 90% v/v plasma, 20 mM Hepes buffer and 12.5 units/ml lithium heparin will subsequently be referred to as 'standard heparinised plasma'.

During studies of the effect of heparin concentration and cation chelating anticoagulants on the ability of plasma to induce polarisation of neutrophils in suspension, plasmas were prepared in the following manner. Anticoagulants were appropriately diluted with Hepes buffer to a final volume of 200 µl in a 5 ml-plastic blood collection tube (Disposable products Pty. Ltd., South Australia). Each tube was filled with 1.8 ml blood gently agitated and then centrifuged at 500 x g for 20 minutes. Each plasma was then obtained as the upper cell free fluid following centrifugation.

Serum was obtained by addition of 9 parts human blood to 1 part Hepes buffer in a blood collection tube without added anticoagulant. This blood was allowed to clot at room temperature for 30 minutes, centrifuged at 500 x g for 20 minutes and serum obtained as the upper cell free layer of fluid.

2. 2. 3. Stock solutions of FMLP and inflammatory mediators

N-formyl-methionyl-leucyl-phenylalanine (FMLP) was prepared as a 10^{-3} M solution in dimethylsulfoxide and stored in 5 µl aliquots at -20 °C. Interleukin-8 (IL-8) was supplied as a 0.4 mg/ml solution in buffered saline and stored at -20 °C in 5 µl aliquots. Leukotriene B_4 (LTB_4) was supplied as a 0.1 mg/ml (3×10^{-4} M) solution in methanol, diluted to a concentration of 3×10^{-5} M with ethanol and stored at -70 °C in 5 µl aliquots. Stock solutions of FMLP, IL-8 and LTB_4 for use in experiments were prepared by dilution of aliquots with HBSS-Hepes.

Platelet activating factor (PAF) was supplied as a 2 mg/ml solution in chloroform and stored at -20 °C in 10 µl aliquots. For each experiment a 40 µM stock of PAF was prepared by taking one 10 µl aliquot, evaporating the chloroform under nitrogen gas and dissolving the residue in 900 µl HBSS-Hepes with 0.25% Human serum albumin. Additional dilutions were perfomed in HBSS-Hepes.

2. 2. 4. Stock solutions of IgG and other plasma proteins

Stocks of human IgG, gamma globulin and Cohn fractions were prepared by dissolving in HBSS-Hepes.

Ultracentrifuged stock solutions of IgG were prepared by centrifugation at 200,000 g for 4 hours at 4 °C in a Beckman ultracentrifuge (model TL-100) according to the method of Kemp and Brown (1980). The concentration of IgG in the remaining supernatant was measured in a Perkin Elmer spectrophotometer (model Lambda 5) at 280 nm using the relationship that 1.35 absorbance units at 280 nm corresponds approximately to 1 mg/ml IgG (Harlow and Lane, 1988). Final stock concentrations of ultracentrifuged IgG were then prepared by dilution with HBSS-Hepes.

Heat aggregated IgG (HAIgG) was prepared according to the method of various authors (Henson et al., 1972; Kemp and Brown, 1980; Smith et al., 1986) by heating a 1% solution of IgG in HBSS-Hepes at 63 °C for 20 minutes. Insoluble aggregates were removed by centrifugation at 8,000 g for 5 minutes in an Eppendorf microfuge (model 5413). Final stock concentrations of HAIgG were then prepared by spectrophotometry and dilution with HBSS-Hepes as above for ultracentrifuged preparations.

2. 2. 5. Stock solutions of chelating agents, divalent cations and TMB-8

Stock solutions of EDTA, EGTA and disodium hydrogen citrate were prepared at a concentration of 200 mM in double distilled water and the pH adjusted to 7.2 with NaOH.

Stock solutions of $CaCl_2$, $MgCl_2$ and $MgSO_4$ were prepared at a concentration of 200 mM in double distilled water.

Stock solutions of TMB-8 were prepared fresh for each experiment as a 5 mM solution in HBSS-Hepes.

2. 2. 6. Preparation and handling of cell suspensions

Cells, reagents and glassware were maintained at 37 °C throughout all assays of cell-shape changes by placing in a room maintained at this temperature. Test media containing stimuli and or inhibitors were prepared in 4 ml siliconised glass test tubes. These tubes were prepared by rinsing briefly with the siliconising solution ("Coatasil"), drained and dried in a fume hood, then finally rinsed 3 times in filtered double distilled water to remove HCl formed during siliconisation and dried.

Each incubation of cells was commenced by addition of one part 10^7 cells/ml stock to nine parts test medium, with the cells being maintained in suspension by gentle agitation once every five minutes. At desired time points, an aliquot of cell

suspension was removed from the incubation tube and added to an equal volume of 2.5% glutaraldehyde in 0.2 M phosphate buffer, pH 7.2, in a 4 ml polycarbonate test tube. For test media containing plasma or serum, it was found necessary to fix aliquots of cell suspension with a volume of fixative approximately five times that of the aliquot in order to prevent precipitation of plasma proteins.

With regard to the length of incubation period chosen, time course studies were generally performed by fixating aliquots of cells at 0.5, 1, 5, 15, 30, 45 and 60 minutes. However, since responses to most stimuli examined were generally maximal by 30 minutes, this time was used as a standard incubation period in subsequent experiments with each stimulus.

2. 2. 7. Assessment of neutrophil shape by visual classification

Cells were prepared for visual classification by suspension in 50 µl of 1:1 glycerol/PBS and mounted between slide and coverslip. Mounted cells were examined with an Olympus BH Vanox microscope equipped with Nomarski optics (differential interference microscopy, DIC) attachments using a x40 objective lens. Three hundred cells were examined per treatment and classified into morphological subtypes according to the following criteria established during initial experiments with FMLP (see section 3. 2. 1. 2.).

Spherical cells were defined as those with a circular outline and no obvious extensions of the cytoplasm. Both 'smooth' spherical (Fig. 2. 1. a.) and 'rough' spherical (Fig. 2. 1. b.) cells as described by Roos et al. (1987) were observed, but were not classified separately because both types were regularly observed in unstimulated preparations of cells (HBSS-Hepes alone).

When the proportion of cells displaying cytoplasmic extensions (i.e. non-spherical cells) exceeded 5% of the total number of cells examined, these cells were usually further classified into four morphological subtypes; types 1, 2, 3 and 4.

Type 1 cells were defined as those cells which displayed more than two extensions of the cytoplasm projecting from random locations on surface of the cell. In some instances, these extensions were restricted to localised points on the cell-surface (Fig. 2. 1. c.), but on other occasions type 1 cells displayed extensions over their entire surface (Fig. 2. 1. d.). The type 1 morphological subtype corresponds to the 'non-polarised' (Keller, 1983; Keller et al., 1983) 'poorly polarised' (Shields and Haston, 1985) and 'short polarised' (Bignold and Ferrante, 1988) shapes of neutrophils documented by other workers.

Type 2 cells (Fig. 2. 1. e.) were defined as those cells which a displayed a polarised pseudopod projecting from the surface of an almost spherical cell-body. This subtype corresponds to the 'spherical smooth cells with unifocal projections' as reported previously (Roos et al, 1987; Zimmermann et al., 1988a; 1988b).

Type 3 cells (Fig. 2. 1. f.) were defined as those cells which displayed a polarised pseudopod projecting from an elongated cell-body with rounded end (i.e. no uropodal tail). This subtype resembles the 'long polarised' cells observed by Bignold and Ferrante (1988), but does not directly correspond to any category of neutrophil-shape previously used in polarisation assays.

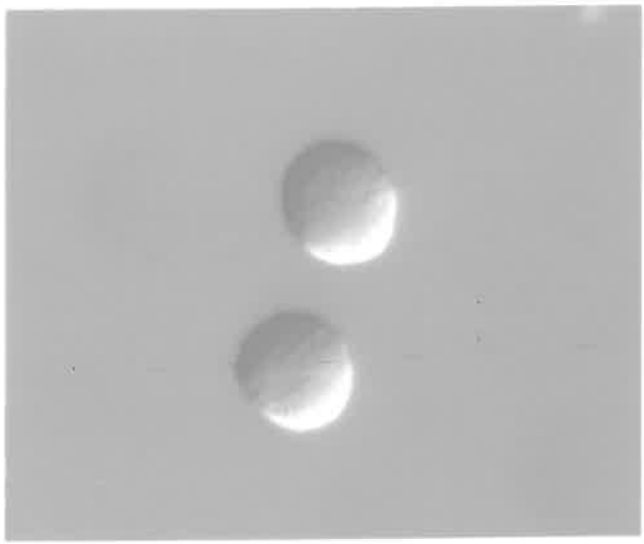
Type 4 cells were defined as those cells which displayed a bi-polar morphology comprised of a flattened pseudopodal extension projecting from one side of the cell and a bulbous uropodal extension at the other. The bodies of type 4 cells were usually elongated (Fig. 2. 1. g.), but in some instances were approximately round (Fig. 2. 1. h.). The type 4 subtype corresponds to the 'polar with tail' category of cells used by Keller (1983) and Keller et al. (1983).

Cells with two pseudopodial extensions were often observed. These cells were classified according to the general shape of their body (spherical or elongated) and the presence or absence of a uropod, into their appropriate subtype (type 2, Fig. 2. 1. i.; type 3, Fig. 2. 1. j.; type 4, Fig. 2. 1. k.).

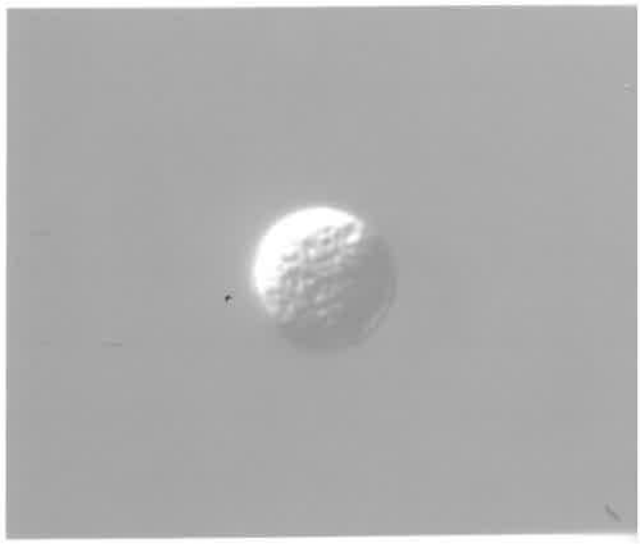
Zeiotic or blebbed cells (Fig. 2. 1. l.) as described by Bessis and de Boisfleury (1976) and Bignold and Ferrante (1988) were occasionally observed throughout

Fig. 2. 1. Subtypes of neutrophil shape identified by visual classification (Nomarski optics; approximately 2000 X).

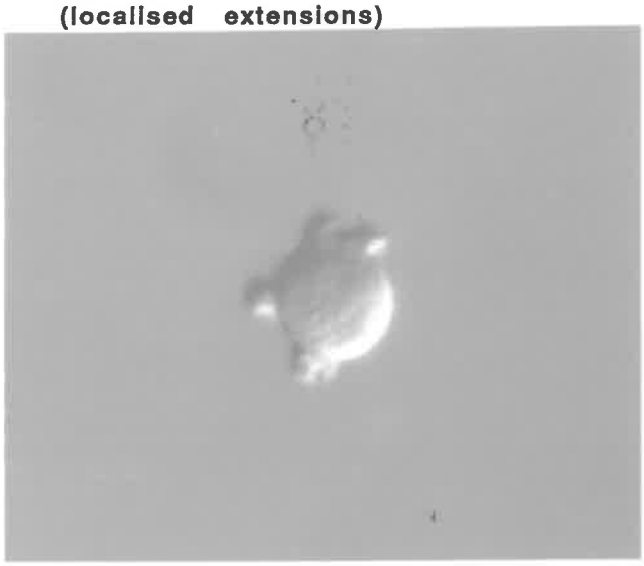
(a) Smooth spherical



(b) Rough spherical

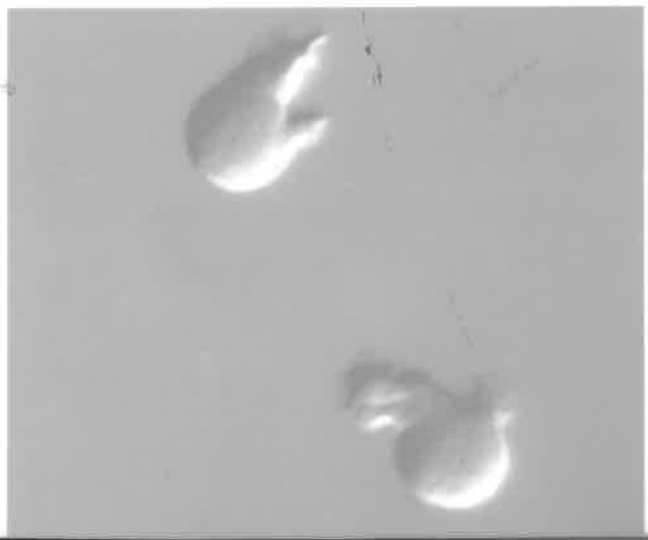


(c) Type 1
(localised extensions)

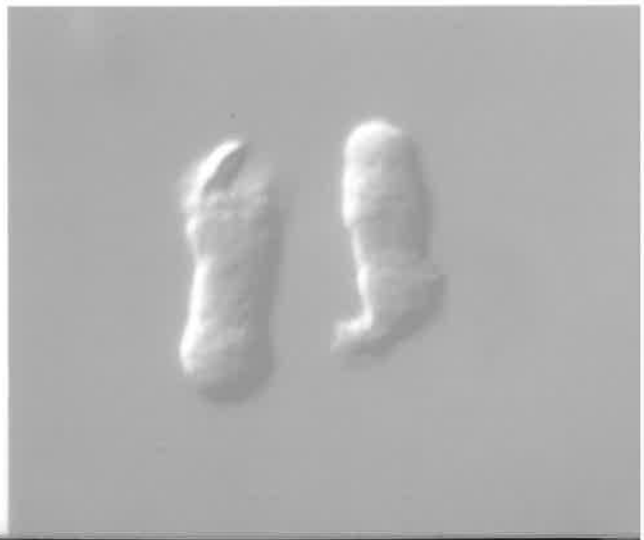


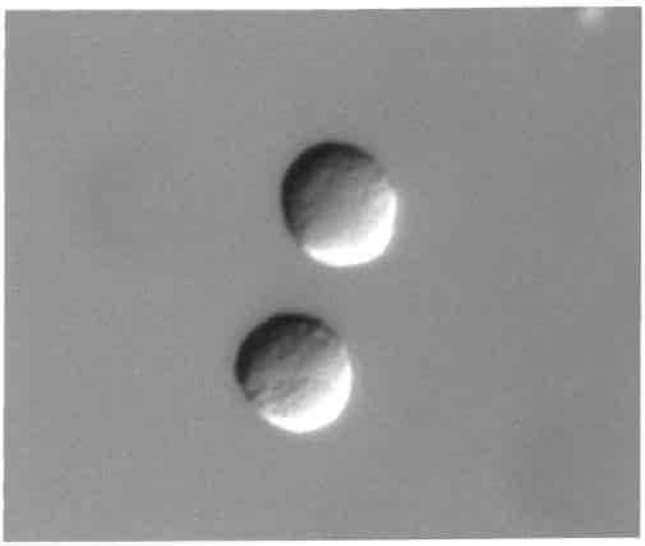
(d) Type 1
(generalised extensions)

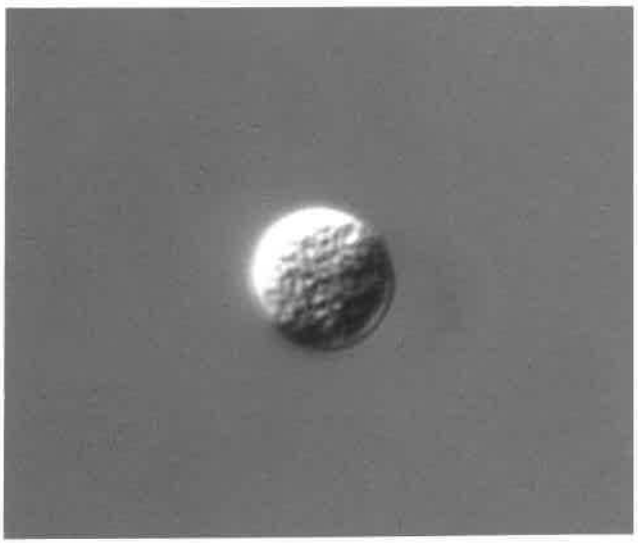
(e) Type 2

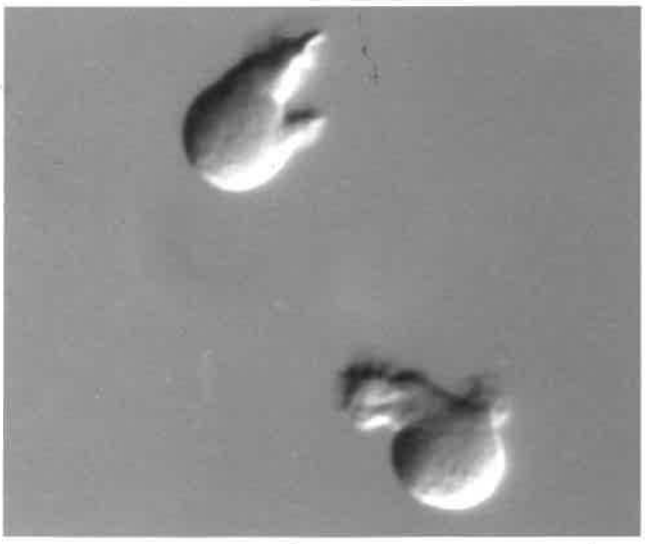


(f) Type 3









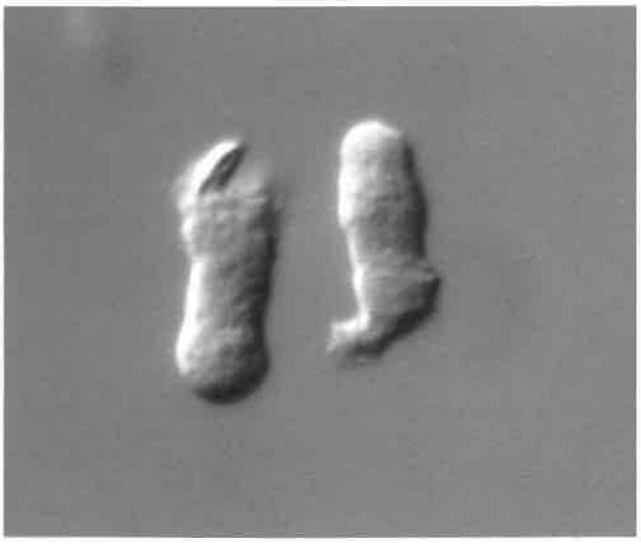
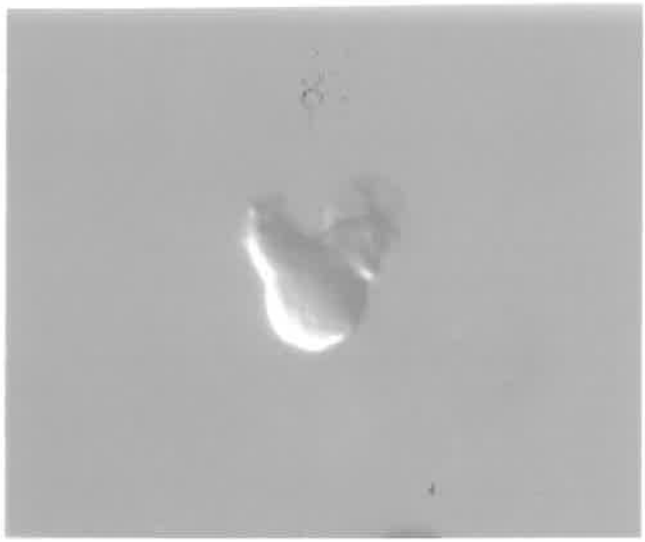


Fig. 2. 1. (continued)

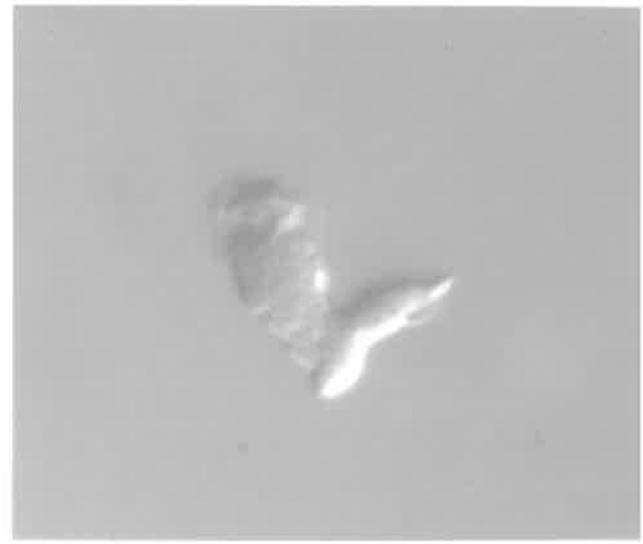
(g) Type 4 (elongated)

(h) Type 4 ("round")

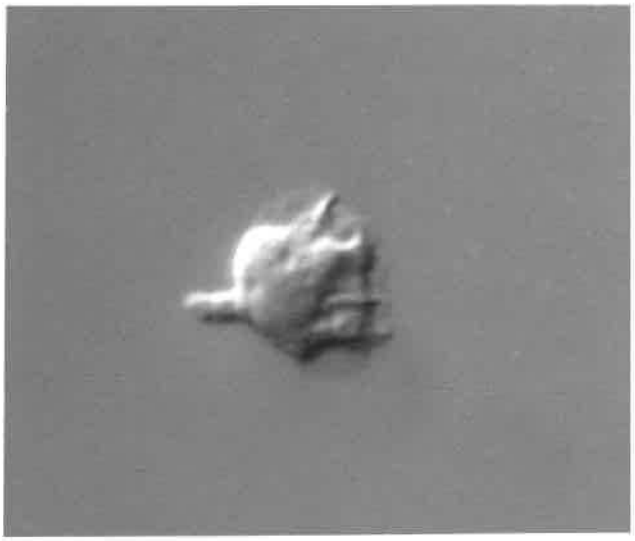
(i) Type 2 (two pseudopodia)

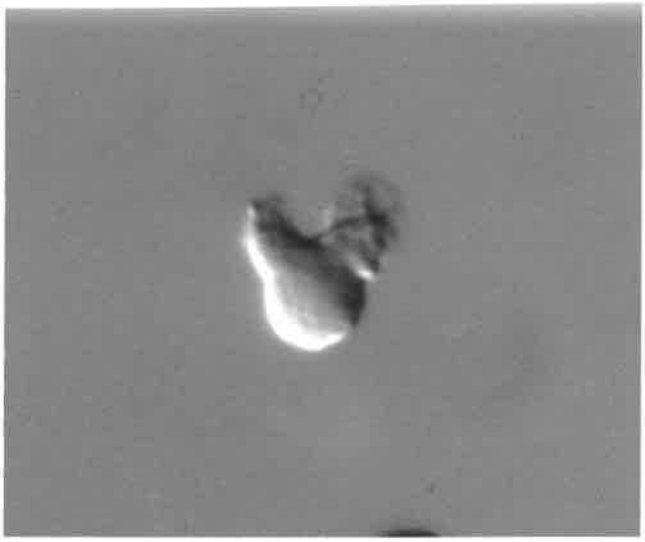


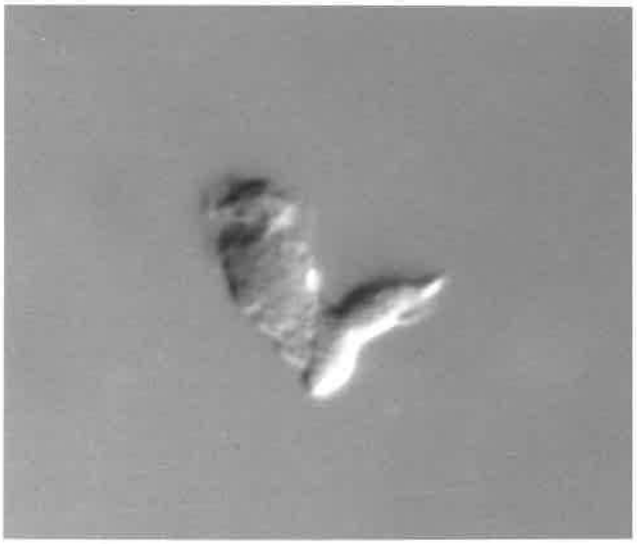
(j) Type 3 (two pseudopodia)



(k) Type 4 (two pseudopodia)







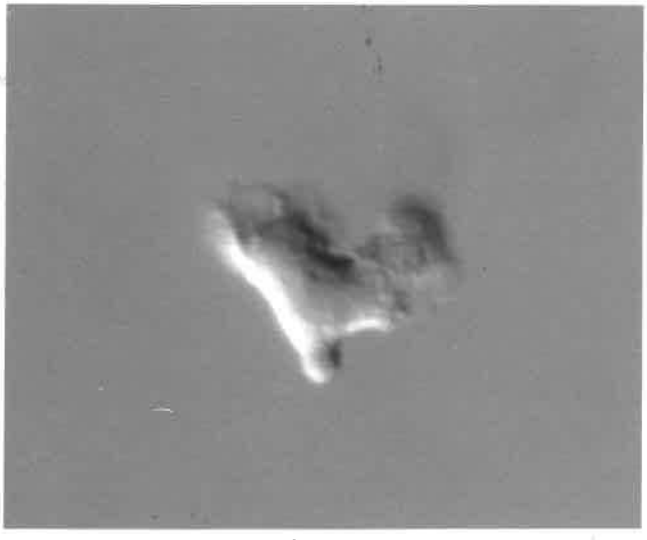
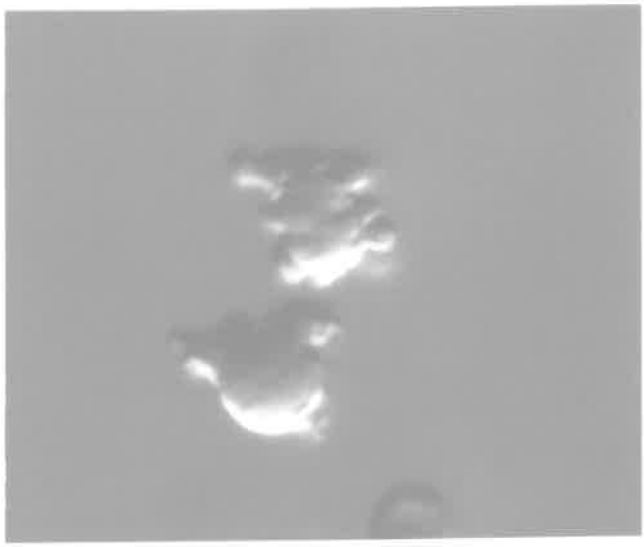
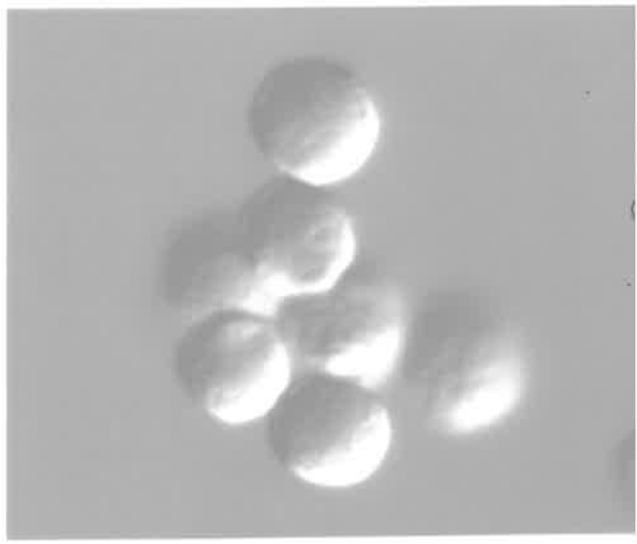


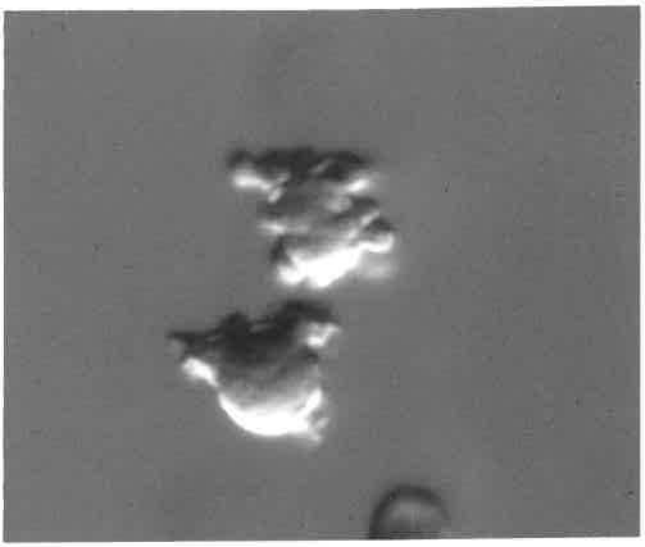
Fig. 2. 1. (continued)

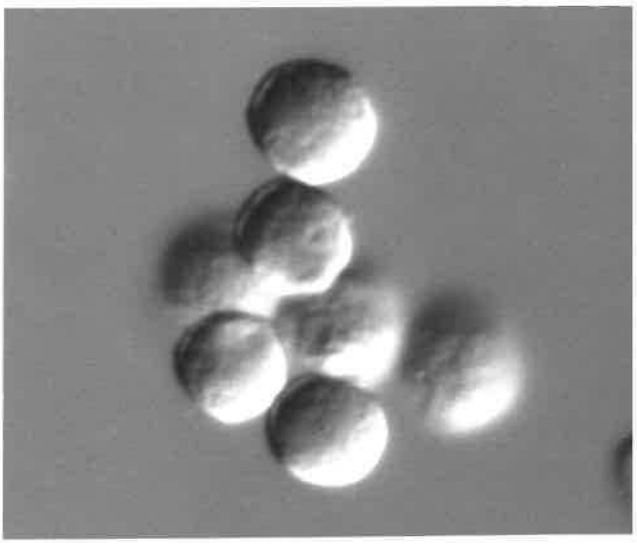
(l) Zeiotic cell (top)



(m) Clump of cells







preparations of both treated and untreated cells, but never exceeded 1% of the total number of cells examined and so were excluded from analysis.

Clumps of cells (Fig. 2. 1. m.) were rarely seen in mounted preparations of fixed cells, but when observed were excluded from analysis on the basis that this contact between cells may have taken place prior to fixation and thus influenced the shape of the cells while in suspension.

Optimal responses of cells to stimuli were defined as those which produced the highest proportion of type 4 cells because these cells displayed the greatest change in shape from spherical (assessed morphometrically, see sections 2. 2. 8. and 3. 2. 3.) and took more time to develop than the other subtypes of shape.

Controls throughout all experiments were performed by suspending cells in HBSS-Hepes. Experiments were discarded whenever the proportion of cells with extensions in HBSS-Hepes exceeded 5% of the total number of cells counted.

2. 2. 8. Assessment of neutrophil shape by computerised morphometry (Fig. 2. 2.)

Computer aided morphometric analysis of neutrophils was carried out on cells viewed by Nomarski optics. While looking through the eyepieces of the microscope the image of cells was superimposed on the image of a digitising tablet by placement of a camera lucida device between the objective and eyepiece lenses. These images of neutrophils were digitised by tracing the outline of the cell with a 'mouse' attached to the digitising tablet. This information was then transmitted via cable to a "Macintosh Plus" computer and analysed using the AREA setting of the "Macmeasure" software application. On this setting the area (A) and perimeter length (P) of the cell's outline were automatically calculated and converted to scale by prior calibration with a graticule microscope slide. In addition, a third value known as the 'shape factor' (F) was calculated which provides a measure of ellipticity. This F value is calculated according to the following equation where 'A' represents area and 'P' represents the perimeter length of the cell:

$$F = \frac{4 \Pi x A}{P^2}$$

The value for F lies between 0 and 1. As the shape of a cell approaches that of a perfect circle the F value approaches 1. Alternatively, as the shape of a cell approaches that of a straight line the F value approaches 0.

The area, perimeter and F value were calculated for 100 cells in each treatment.

2. 2. 9. Assessment of neutrophil shape by FACS analysis (Fig. 2. 3.).

Aliquots of cell suspension were fixed with an equal volume of either 2.5% glutaraldehyde or 8% formalin in PBS then washed and resuspended in 200 µl PBS.

Suspensions of washed, fixed cells were then fed into a fluorescence activated cell sorter (Becton Dickinson, FACScan) and the forward scatter (FSC) of individual cells recorded. The mean and per cent coefficient of variance (i.e. [standard deviation ÷ mean] x 100; % CV) for FSC were then automatically calculated and displayed along with a dot-plot and histogram of the distribution of FSC values within each sample of cells.

Contaminating erythrocytes and other white blood were largely excluded from analysis by creating a 'granulocyte gate' (Jackson and Warner, 1986). This process involved selecting a range of forwards scatter (FSC) and side scatter (SSC) co-ordinates for analysis which corresponded to this population of cells. For time course studies of neutrophil polarisation the granulocyte gate created at time 0 (i.e. cells suspended in HBSS-Hepes) was retained for analysis of samples taken from time points after stimulation. Care was taken to make the FSC axis of granulocyte gate big enough so as to not exclude from analysis cells with large FSC values at later time intervals.

Fig. 2. 2. Assessment of neutrophil shape by computerised morphometry

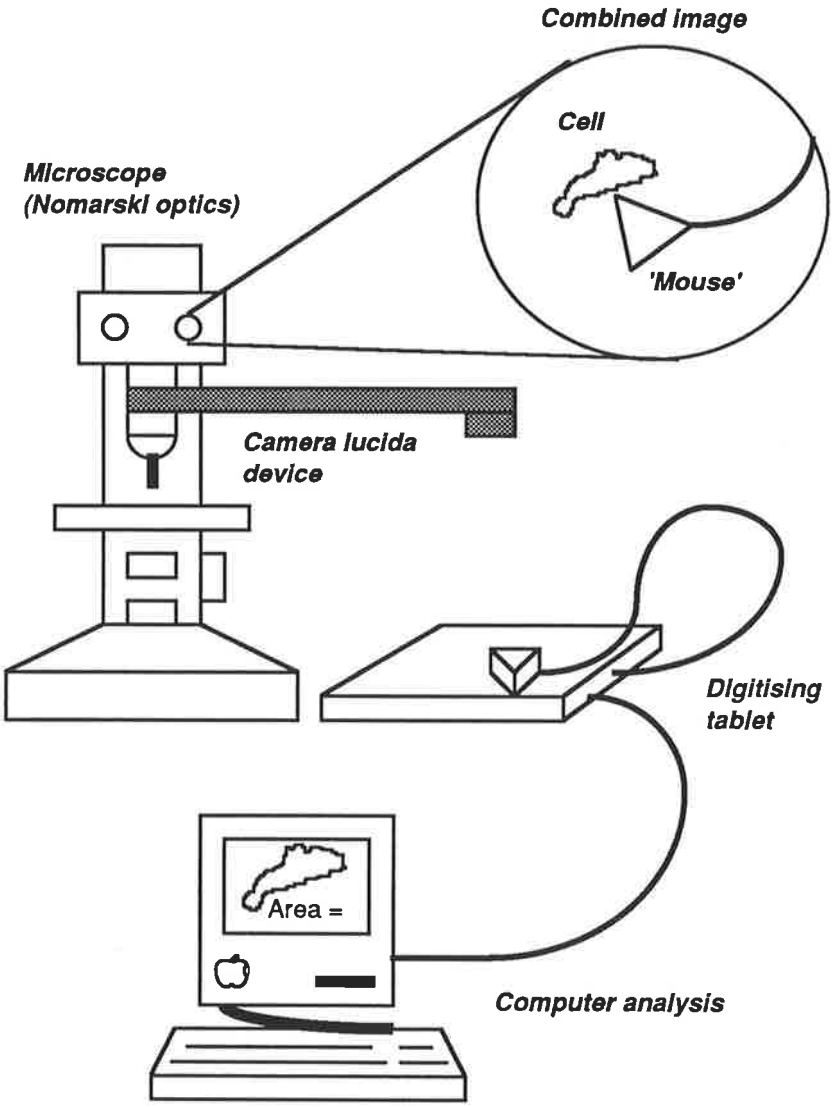
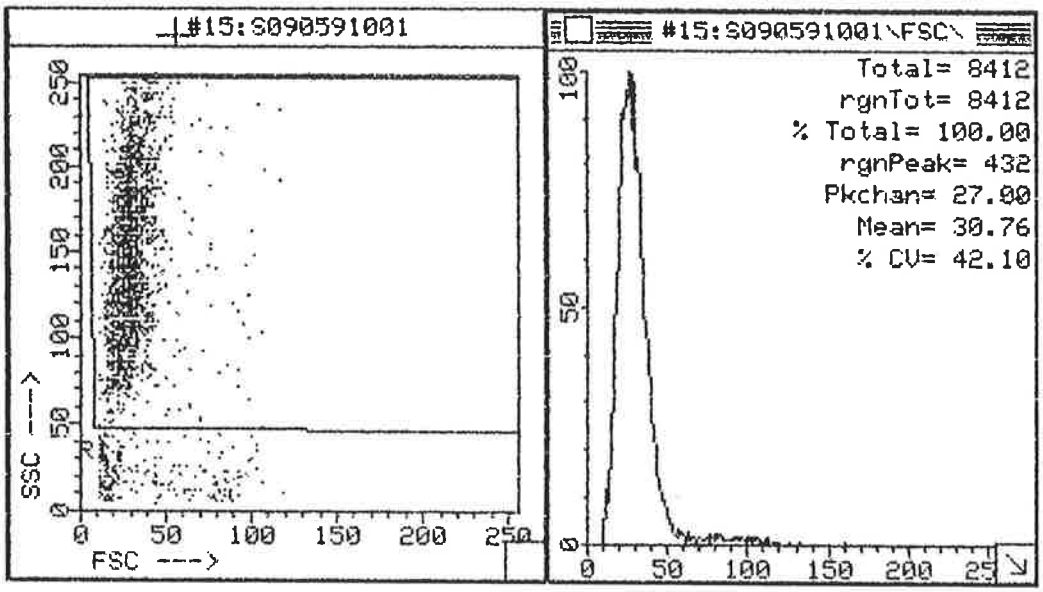
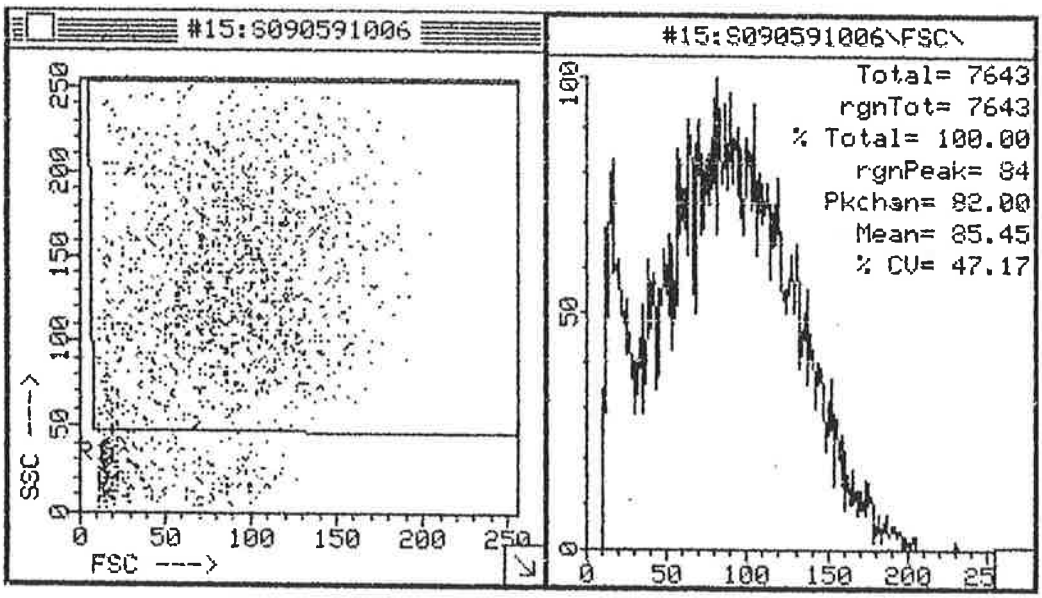


Fig. 2. 3. Assessment of neutrophil shape by FACS analysis

(a) FACS analysis profile for neutrophils suspended in HBSS-Hepes.



(b) FACS analysis profile for neutrophils suspended in 10 nM FMLP for 30 min.



2. 2. 10. Staining of cytoskeletal F-actin with rhodamine phalloidin

2. 2. 10. 1. Fixation

In preliminary experiments cells were fixed with an equal volume of 2.5% glutaraldehyde which resulted in a high background fluorescence of the cells. An attempt to reduce this background fluorescence by using 0.2% glutaraldehyde followed by incubation in HBSS-Hepes with 0.05 M glycine at 4 °C for 15 minutes to "quench" free aldehyde groups (Haston, 1987) proved unsucessful. Similarly, incubation of cells with three changes of 5 mg/ml sodium borohydride (Harlow and Lane, 1988), prior to staining, did not appear to reduce background fluorescence caused by glutaraldehyde. In subsequent studies cells were incubated in the presence of 4% human serum albumin (HSA) and fixed by addition of an equal volume of 8% formalin in PBS. Inclusion of 4% HSA was found to reduce lysis of polarised cells caused by formalin fixation (Haston and Shields, 1984; Haston, 1987) but did not influence the responses to stimuli.

2. 2. 10. 2. Staining

Fixed cells were stained according to an adaptation of a method reported by Roos et al., (1987). A staining solution was prepared by drying down 50 µl of rhodamine phalloidin stock (3.3 µM in methanol; 100 units/ml) in a 4 ml polycarbonate test tube by placing in a fume hood, and adding to 1 ml 0.1 M Tris buffered saline (TBS) pH 7.8 containing 0.05% HSA and 100 µg lysophosphatidyl palmitoyl. Cells were washed with TBS and resuspended in staining solution at a concentration of 10^6 cells/ml. After incubation in the dark for 30 minutes at 37 °C an equal volume of TBS containing 4% HSA was added and the cells washed with TBS. Washed, stained cells were resuspended in a 1:1 mixture of glycerol and PBS and

examined using a Nikon (Microphot-FXA) fluorescence microscope with rhodamine filter block.

2. 2. 11. Molecular sieve chromatography of plasma proteins

Sephacryl 300 molecular separation beads (Pharmacia, Uppsala, Sweden) were equilibriated in TBS pH 8, degassed under vacuum and packed into a glass column (Pharmacia, Uppsala, Sweden) to produce a bed with final dimensions 1.5 cm by 80 cm. The V_0 and V_T of the column were approximated from the elution profile of a 1 ml solution of blue dextran with phenol red in TBS pH 8.

One ml samples of fresh heparinised plasma or fresh serum were prepared for chromatography by centrifugation at 10,000 x g for 5 minutes in an Eppendorf microfuge to remove any contaminating cells and then eluted from the S300 column in TBS pH 8 at a flow rate of approximately 2 ml/cm2/h using a peristaltic pump. One ml fractions were collected and analysed for protein content by absorbance at 280 nm. Fractions containing protein were pooled, dialysed against Milli-Q filtered double distilled water and freeze dried. Freeze dried fractions were prepared for analysis by dissolving in 1 ml HBSS-Hepes and centrifuged at 10,000 x g for 5 minutes to remove insoluble material.

Membrane dialysis tubing (Selby Anax, Adelaide, South Australia) was prepared by boiling in 10% sodium carbonate for 10 minutes to remove impurities and then washed in double distilled water.

2. 2. 12. Preparation of Fc and Fab fragments from IgG

Fc and Fab fragments of human IgG were prepared according to the method of Gorevic et al. (1985). Human purified IgG was dissolved at a concentration of 1% (w/v) in PBS containing 0.01 M cysteine HCl, 0.002 M EDTA and 0.1 mg/ml papain and digested for 18 hours at 37 °C. The digestion solution was then dialysed for 24

hours at 4 °C against TBS pH 8.0 and separated on a Sephacryl 300 column to remove remaining undigested IgG (see section 2. 2. 14). Digested fragments of IgG were separated into an Fab fraction and an Fc fraction by ion exchange chromatography on CM-cellulose. Briefly, lyophilised fragments of IgG were dissolved in 1 ml 0.01 M phosphate buffer, pH 7.6 and applied to a 7 ml column of CM-cellulose equilibriated in the same buffer. One ml fractions were collected under gravity and analysed for protein content by their absorbance at 280 nm in a spectrophotometer. A primary elution peak containing mostly Fc fragments (Gorevic et al., 1985) was collected using a 0.01 M phosphate elution buffer, pH 7.6. A secondary elution peak containing mostly Fab fragments (Gorevic et al., 1985) was then collected by switching to a 0.4 M phosphate elution buffer, pH 7.6. In one experiment the Fc fraction was further purified by ion exchange chromatography on DEAE-cellulose using a gradient elution from 0.01 M to 0.3 M phosphate buffer, pH 8.0. Final fractions of Fc or Fab fragments were dialysed against Milli-Q filtered deionised water and freeze-dried.

2. 2. 13. Digestion of phosphoinositol-linked structures on the surface of neutrophils with phosphoinositol specific phospholipase C (PIPLC)

Cells were incubated for 1 hour at 37 °C in HBSS-Hepes containing 0.05% bovine serum albumin and 0.1 units/ml of the enzyme phosphoinositol specific phospholipase C (PIPLC). Digested cells were then washed in HBSS-Hepes. Remaining contaminating red blood cells were removed during preparation of cells by hypotonic lysis, to improve the availability of enzyme to neutrophils (see section 2. 2. 1.). The level of digestion was assessed by measuring the decrease in binding of a monoclonal antibodies to Fc gamma III receptors and control structures on the neutrophil surface (see section 5. 2. 6.) by flow cytometry (as follows).

2. 2. 14. Detection of cell surface antigens using fluorescein-conjugated monoclonal antibodies

Neutrophils were labelled with fluorescein-conjugated monoclonal antibodies according to the method of Gadd and Ashman (1983). Briefly, cells (10^6) were washed and resuspended in 50 µl PBS containing 0.1% bovine serum albumin with 0.1% sodium azide and incubated for 30 minutes on ice with 20 µg/ml of mouse monoclonal antibody to the cell surface antigen of interest (see section, 5. 2. 6.). Excess antibody was then removed by washing cells three times with PBS. Washed cells were then incubated for 1 hour on ice, in 50 µl of a 1/100 dilution of fluorescein-conjugated, affinity-purified, sheep polyclonal antibody to mouse IgG and IgM (heavy and light chains). Fluorescein labelled cells were then washed twice with 1 ml PBS and resuspended in 1 ml of 1% formalin in PBS. The level of expressed surface antigen was then measured using the fluorescence setting on a fluorescence activated cell sorter (Becton Dickinson, FACScan) as the mean fluorescence (mean FL1) for each population of cells.

2. 2. 15. Statistical analyses

2. 2. 15. 1. Chi-square "goodness-of-fit" test

This test was used exclusively for comparing the proportions of morphological subtypes displayed by neutrophils following two different treatments. The method used for this statistical analysis follows that outlined by Weiss and Hassett, (1987) with the null hypothesis (H_o) being, "that the proportions of morphological subtypes displayed after each treatment are the same", and the alternative hypothesis (H_a) being "that the proportions displayed after each treatment are different". To test the H_o, the mean proportions from one treatment were designated as 'expected frequencies' and those from the second treatment were designated as 'observed

frequencies'. For comparison of data obtained from experiments performed in the presence or absence of a specific agent or condition, the proportions of morphological subtypes displayed in the absence of the agent or condition were designated as 'expected frequencies'.

The squared value for the difference between expected and observed frequencies, divided by the expected frequency was then calculated for each morphological subtype [i.e. $(O-E)^2/E$]. The test statistic (X^2) was then calculated by summating the $(O-E)^2/E$ values obtained for each morphological subtype. The value for the test statistic was then compared with it's critical value ($X^2_{0.05} = 9.488$) obtained from a Chi-square distribution table for four degrees of freedom where '0.05' is the significance level. The number of degrees of freedom was calculated from the total number of different morphological subtypes (five, including 'spherical') minus 1. If the value obtained for X^2 was ≥ 9.488, the null hypothesis was rejected and a significant difference was recorded.

In order to perform this analysis it was necessary for all 'expected frequencies' to be greater than 0.

2. 2. 15. 2. One-way analysis of variance

This test was perfomed exclusively for comparing the morphometric parameters of the different subtypes of cell shape identified by visual classification and was performed using the statistics software package, 'Statview 512+' (Abacus Concepts Inc., published by Brain Power Inc., California). Significant differences between treatments were determined using the Scheffe F-test value for this statistic at a significance level of 95%.

CHAPTER 3.

THE MORPHOLOGICAL RESPONSE OF NEUTROPHILS IN SUSPENSION TO N-FORMYL-METHIONYL-LEUCYL-PHENYLALANINE ASSESSED BY VISUAL CLASSIFICATION, MORPHOMETRY AND FACS ANALYSIS

3. 1. Introduction

In this chapter, visual classification, morphometry and FACS analysis are compared as techniques for assessing polarisation of neutrophils in suspension induced by the synthetic chemotactic peptide, FMLP. Furthermore, the sensitivity of FACS analysis to fixation technique and erythrocyte lysis are examined. In addition, the subtypes of cell-shape identified by visual classification are compared morphometrically and the effect of FMLP concentration on the relative proportions of these subtypes is investigated with respect to time. The distribution of actin microfilaments (F-actin) within the cytoplasm of each morphological subtype is also examined.

3. 2. Results

3. 2. 1. Time course of the morphological response of neutrophils in suspension to FMLP assessed by visual classification, morphometry and FACS analysis

3. 2. 1. 1. Design of experiments

The following experiments were designed to compare visual classification with morphometric and FACS analysis techniques, as methods for studying the time course of neutrophil polarisation induced by FMLP. Neutrophils were suspended in HBSS-Hepes at 37 °C and aliquots of this suspension were fixed immediately before (0 minutes) and 0.5, 1, 5, 15, 30, 45 and 60 minutes after the addition of 10 nM FMLP. Cells fixed at each time point were divided into two samples. Cells in the first sample were prepared for Nomarski optics and their morphology analysed by both visual classification (see section 2. 2. 8.) and computerised morphometry (see section 2. 2. 9.), and cells in the second sample were examined by FACS analysis (see section 2. 2. 10). This experiment was performed three times using cells from a different donor on each occasion. Data obtained by visual classification is expressed as the mean ± standard deviation for all three experiments. Data obtained by computerised morphometry and FACS analysis is expressed graphically as the mean ± standard deviation for individual experiments.

3. 2. 1. 2. Visual classification (Fig. 3. 1. a.)

Neutrophils suspended in HBSS-Hepes were almost entirely spherical (>95% of total number of cells examined) and retained this morphology throughout the experiment. From 0.5 to 60 minutes after the addition of FMLP less than 5% of cells displayed a spherical morphology as the majority of cells displayed cytoplasmic

extensions. These extensions enabled the cells to be classified into four morphological subtypes: type 1 cells, characterised by non-polarised extensions of the cytoplasm; type 2 cells, characterised by a round body with polarised extensions; type 3 cells, characterised by an elongated body with polarised extensions; and type 4 cells, characterised by an elongated body with polarised extensions and uropod. The relative proportions for each morphological subtype varied according to time. The proportion of type 1 cells gradually declined from a maximum of 62.3 ± 6.4(SD)% at 0.5 minutes to <5% by 30 minutes onwards. The proportion of type 2 cells rose from 28.3 ± 7.5% at 0.5 minutes to a maximum of 55.3 ± 1.5% at 1 minute, then gradually declined to <5% by 30 minutes onwards. The proportion of type 3 cells gradually rose from a minimum of 5.3 ± 2.1% at 0.5 minutes to a maximum of 30.3 ± 3.8% by 5 minutes, then gradually decreased to 8.3 ± 3.5% by 45 minutes before rising to 10.3 ± 2.1% at 60 minutes. The proportion of type 4 cells gradually rose from a minimum of 1.0 ± 0.0% at 0.5 minutes to a maximum of 82.3 ± 3.8% by 45 minutes, then declined to 78.0 ± 4.0% at 60 minutes.

3. 2. 1. 3. Computerised morphometry (Fig. 3. 1. b.)

Neutrophils suspended in HBSS-Hepes displayed gradual increases in their mean area and mean perimeter and a gradual decrease in their mean F value over the 60 minutes incubation following the addition of 10 nM M FMLP. The increases in mean area and perimeter were maximal by 45 to 60 minutes after addition of the peptide and were approximately twice the values for unstimulated cells. The decrease in mean F value was also maximal by 45 to 60 minutes and was approximately six tenths the value for unstimulated cells.

Fig. 3. 1. a. Time course of the morphological response of neutrophils in suspension to 10 nM FMLP analysed by visual classification

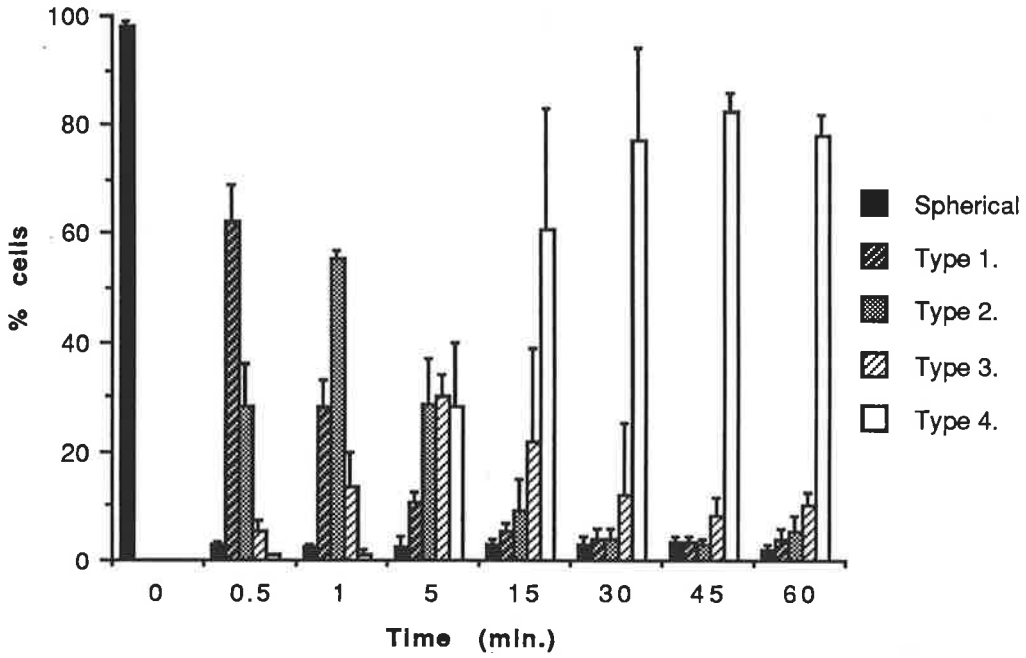
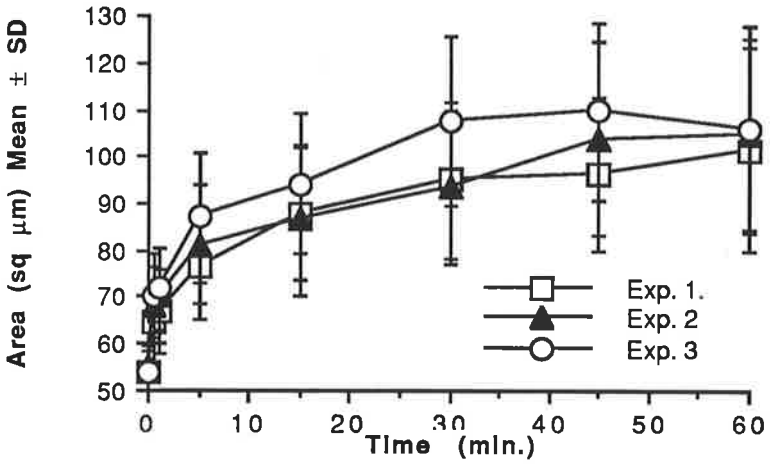
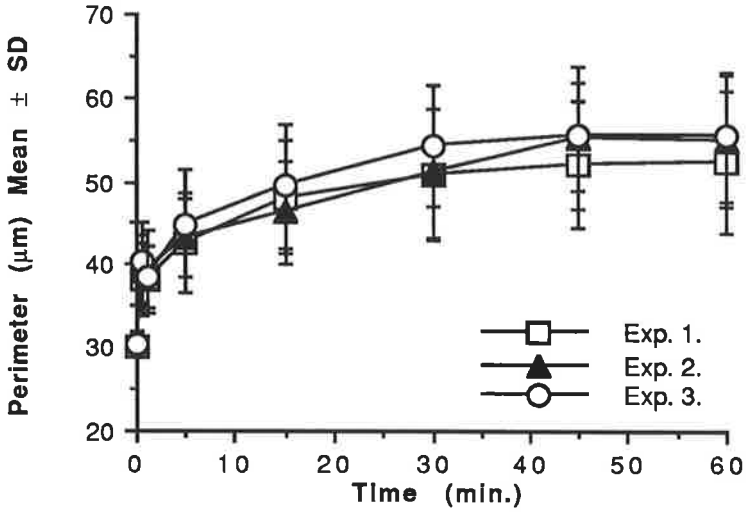


Fig. 3. 1. b. Time course of the morphological response of neutrophils in suspension to 10 nM FMLP analysed by computerised morphometry

(I) Analysis by area



(ii) Analysis by perimeter



(iii) Analysis by Shape Factor

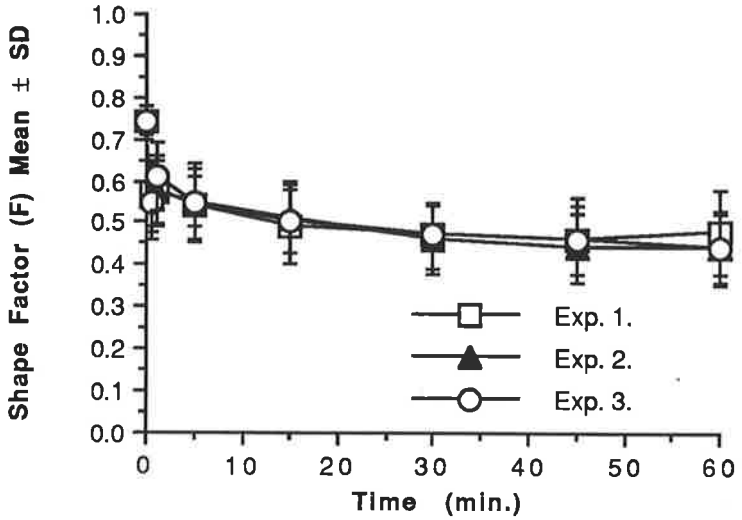
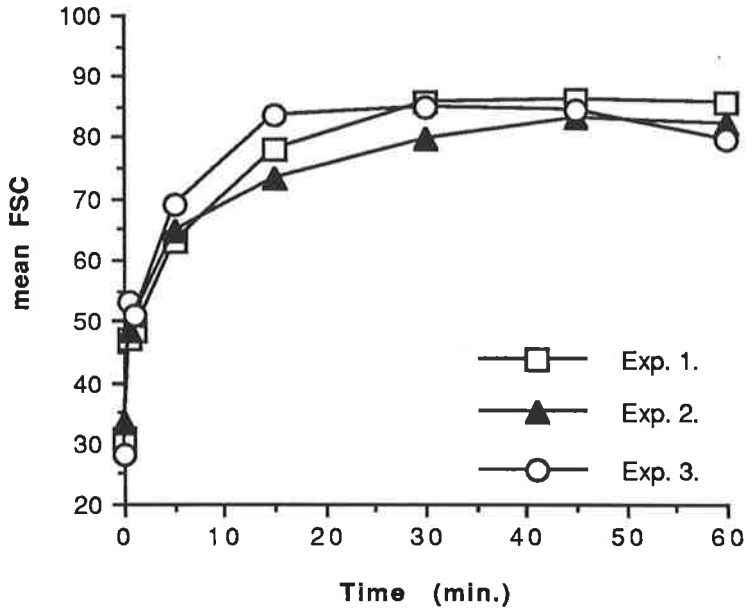
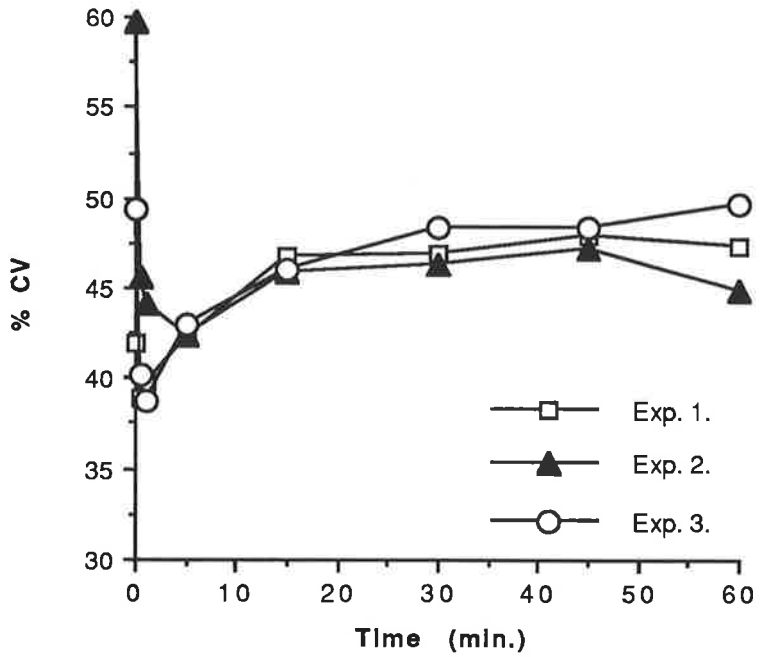


Fig. 3. 1. c. Time course of the morphological response of neutrophils in suspension to 10 nM FMLP measured by FACS analysis

(i) Analysis by forward scatter (FSC)



(II) Analysis by % CV



3. 2. 1. 4. FACS analysis (Fig 3. 1. c.)

Neutrophils suspended in HBSS-Hepes displayed a gradual increase in their mean forward scatter (FSC) following the addition of 10 nM FMLP which was apparent after 0.5 minutes and reached a maximum value of approximately 2.7 times the FSC of unstimulated cells, at 45 minutes after stimulation. Little change in mean FSC was observed between 0.5 minutes and 1 minute, and in one experiment the mean FSC at 1 minute was lower than that at 0.5 minutes, but these findings were not analysed in more detail. The percentage coefficient of variation (% CV) for mean FSC, declined initially, but in general was not considerably altered during the response to FMLP.

3. 2. 2. Effects of formalin versus glutaraldehyde fixation, and erythrocyte lysis, on FACS analysis of the morphological response of neutrophils in suspension to FMLP

3. 2. 2. 1. Design of experiments

Since FMLP increased the mean FSC of neutrophils in the above experiments, when glutaraldehyde fixation was used prior to FACS analysis, but has been reported to decrease the mean FSC of neutrophils when formalin fixation is used prior to FACS analysis (Donabedian et al., 1987), the purpose of this experiment was to investigate whether or not these conflicting observations may result from the different fixation procedures used. This study was performed by dividing a population of cells from one donation of blood into two suspensions of 10 nM FMLP. Cells in the first suspension were fixed at 0, 0.5, 1, 5, 15, 30, 45 and 60 minutes with an equal volume of 2.5% glutaraldehyde, and those in the second suspension were fixed at each time point with an equal volume of 8% formalin.

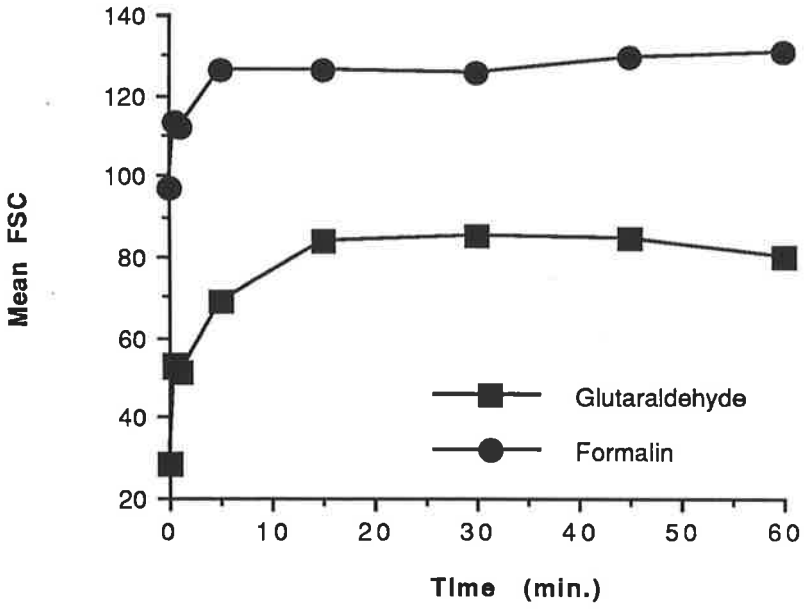
Since erythrocyte lysis is often used to further purify preparations of neutrophils for FACS analysis (Jackson and Warner, 1986) the effect of this technique in combination with the different fixation methods examined above was examined in a second experiment. Neutrophils isolated from one donation of blood were again divided into two samples. Cells in the first sample were further purified by erythrocyte lysis and then suspended in 10 nM FMLP. After 30 minutes incubation two aliquots of cell suspension were removed. The first aliquot was fixed with an equal volume of 2.5% glutaraldehyde and the second aliquot was fixed with an equal volume of 8% formalin. Cells in the second sample were treated in a similar manner to the first, but were not exposed to erythrocyte lysis during their preparation. Controls were performed for both samples of cells by suspending cells for 30 minutes in HBSS-Hepes.

3. 2. 2. 2. Effect of formalin versus glutaraldehyde fixation (Fig 3. 2. a.)

Neutrophils exposed to FMLP displayed increases in mean FSC regardless of the fixation procedure used prior to FACS analysis, but the increase observed for glutaraldehyde fixed cells was approximately twice that recorded for formalin fixed cells. Furthermore, the mean FSC values recorded for formalin fixed cells were consistently greater than those for cells fixed with glutaraldehyde at corresponding time points.

An initial decrease in the % CV values for mean FSC was recorded for glutaraldehyde fixed cells which returned to starting levels after approximately 30 minutes. In contrast, an initial increase in % CV was recorded for formalin fixed cells which returned to approximately basal levels by 15 to 30 minutes. Furthermore, the % CV values for formalin fixed cells were consistently 50-60% lower than those recorded for glutaraldehyde fixed cells at corresponding time points.

Fig 3. 2. a. Effect of formalin fixation versus glutaraldehyde fixation on changes in the FSC of neutrophils induced by 10 nM FMLP



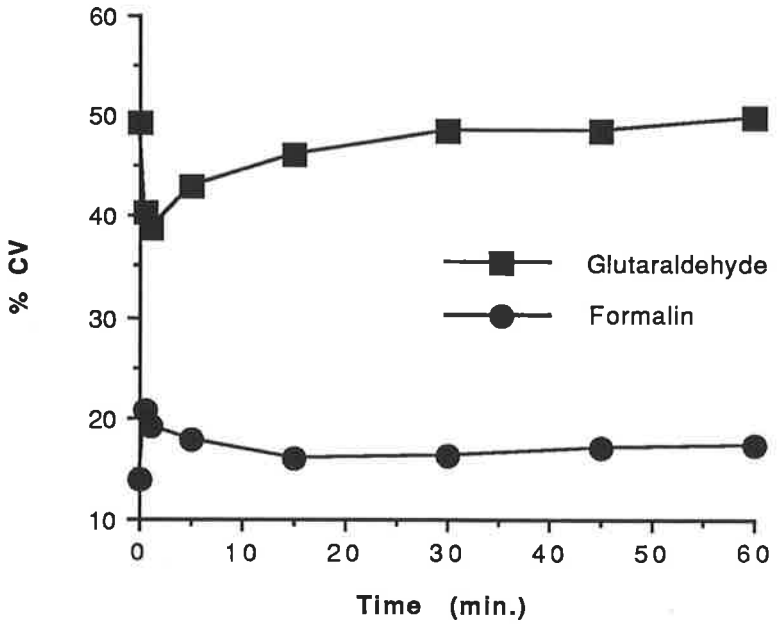
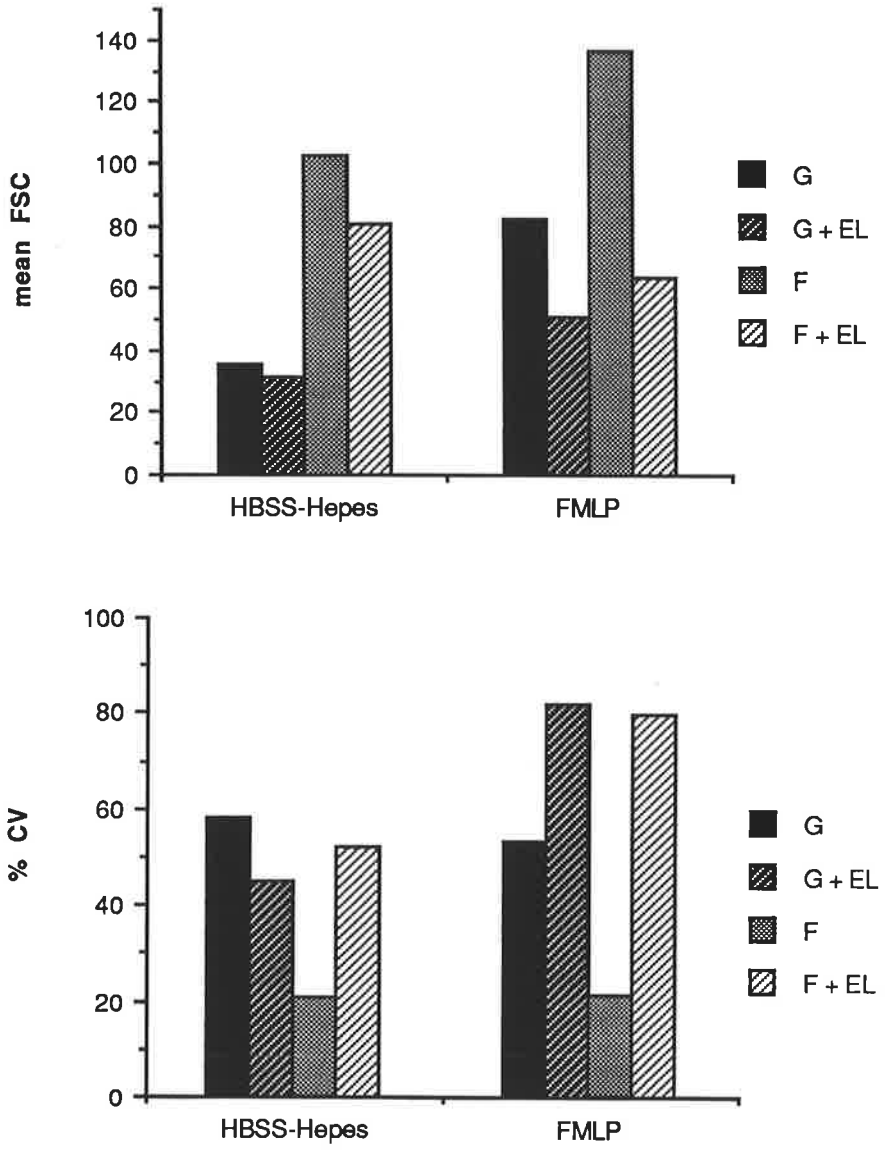


Fig. 3. 2. b. Effect of erythrocyte lysis (EL) on changes in neutrophil FSC induced in suspension by 10 nM FMLP when either glutaraldehyde fixation (G) or formalin fixation (F) is used prior to FACS analysis



3. 2. 2. 3. Effect of erythrocyte lysis (Fig 3. 2. b.)

Neutrophils prepared without exposure to erythrocyte lysis displayed an increased mean FSC after 30 minutes treatment with FMLP. Similar increases were observed regardless of the fixation procedure used prior to FACS analysis. In contrast, neutrophils exposed to erythrocyte lysis during their preparation, displayed different responses to FMLP depending on the fixation procedure employed. When glutaraldehyde fixation was used prior to FACS analysis a higher mean FSC was recorded for neutrophils following treatment with FMLP, compared with untreated cells (HBSS-Hepes). This increase was approximately half that observed for cells which had not been exposed to erythrocyte lysis. When formalin fixation, however, was used prior to FACS analysis, the mean FSC recorded for cells treated with FMLP was approximately 20% lower than that recorded for cells in HBSS-Hepes.

For cells exposed to erythrocyte lysis, the % CV values for FMLP treated cells were 60-100% higher than those recorded for untreated cells (HBSS-Hepes). In comparison, for cells not exposed to erythrocyte lysis, the % CV values for FMLP treated cells were similar to those recorded for untreated cells. These final observations were similar for each fixation procedure used.

3. 2. 3. Morphometric comparison of the subtypes of cell-shape identified by visual classification (Fig. 3. 3.)

3. 2. 3. 1. Design of experiments

The aim of this experiment was to determine whether or not the morphological subtypes of neutrophils identified by visual analysis, can be differentiated from each other on the basis of their morphometric parameters. To investigate this hypothesis, one hundred cells of each morphological subtype were identified at random from

preparations used in the previous set of experiments and their area, perimeter and shape factor calculated by morphometric analysis. The mean (± SD) area, perimeter and shape-factor (F; ellipticity) for each morphological subtype were then calculated and compared using a one-way analysis of variance (see section, 2. 2. 14. 2.).

3. 2. 3. 2. Area (Fig. 3. 3. a.)

The mean area for spherical cells (55.1 ± 4.6 μm^2) was significantly lower than those for cell types 1, 2, 3, and 4. The mean area for type 1 cells (69.0 ± 7.2 μm^2) was less than those for types 2, 3, and 4 cells, but was only significantly different to those of types 3 and 4. The mean area for type 2 cells (69.1 ± 10.1 μm^2) was significantly lower than those for types 3 and 4 cells and the mean area for type 3 cells (78.9 ± 10.3 μm^2) was significantly lower than that for type 4 cells (102.2 ± 20.1 μm^2).

3. 2. 3. 3. Perimeter (Fig. 3. 3. b.)

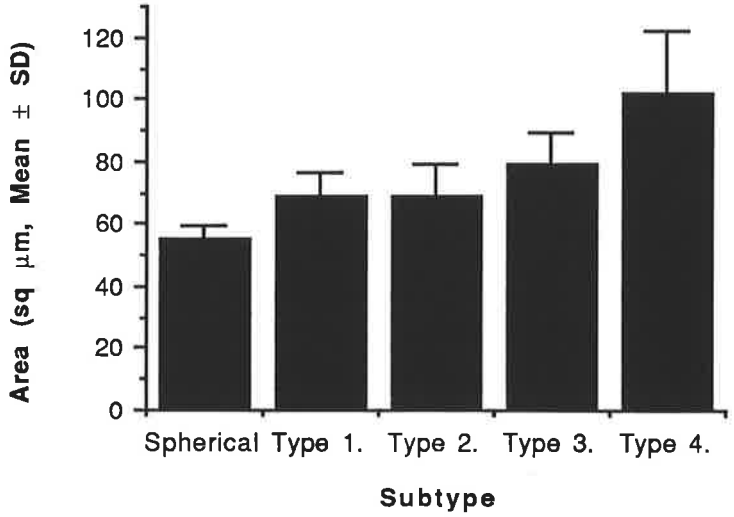
The mean perimeter for spherical cells (30.6 ± 1.3 μm) was significantly less than those for cell types 1, 2, 3, and 4. The mean perimeter for type 1 cells (39.7 ± 3.4 μm) was significantly greater than that for type 2 cells and significantly less than those for cell types 3 and 4. The mean perimeter for type 2 cells (37.4 ± 3.4 μm) was significantly less than those for types 3 and 4 cells and the mean perimeter for type 3 cells (42.0 ± 3.3 μm) was significantly less than that for type 4 cells (53.1 ± 8.3 μm).

3. 2. 3. 4. Shape factor (Fig. 3. 3. c.)

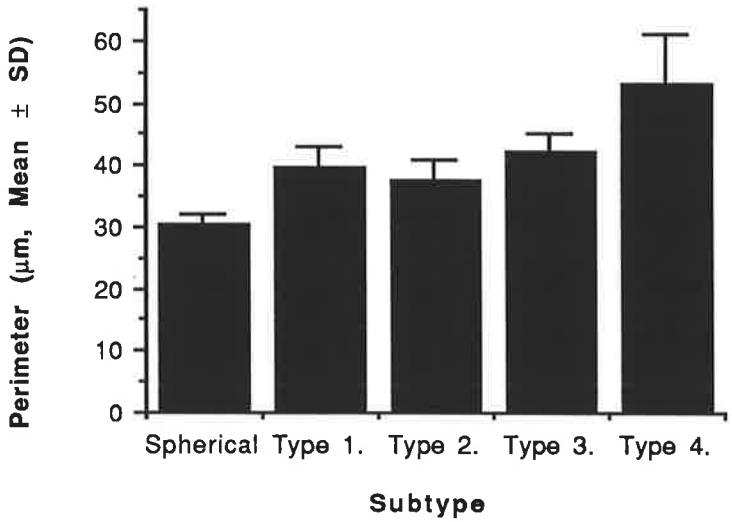
The mean F value for spherical cells (0.74 ± 0.04 shape factor units) was significantly greater than those for cell types 1, 2, 3, and 4. The mean F value for

Fig 3. 3. Morphometric comparison of the subtypes of neutrophil shape identified by visual classification

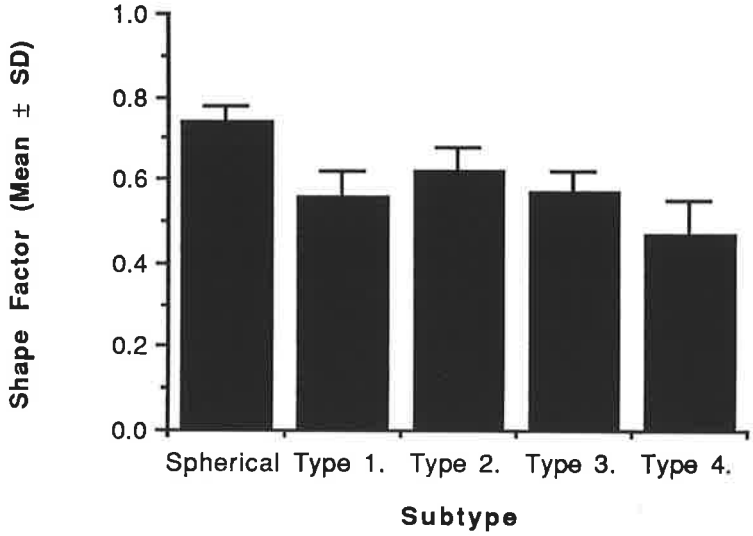
(a) Comparison of areas



(b) Comparison of perimeters



(c) Comparison of Shape factors



type 1 cells (0.56 ± 0.06) was less than those for cell types 2 and 3 and greater than that for type 4 cells, but was only significantly different to those for cell types 2 and 4. The mean F value for type 2 cells (0.62 ± 0.06) was significantly greater than those for types 3 and 4 cells and the mean F value for type 3 cells (0.57 ± 0.05) was significantly greater than that for type 4 cells (0.47 ± 0.08).

3. 2. 4. Effect of FMLP concentration on the proportions of morphological subtypes displayed by neutrophils with time

3. 2. 4. 1. Design of experiments

The aim of this set of experiments was to examine the time course of reported differences between the morphological responses of neutrophils to various concentrations of FMLP (Keller, 1983; Keller et al., 1983; Bignold and Ferrante, 1988). To achieve this, neutrophils were suspended in 1000 nM, 100 nM, 10 nM, 1 nM and 0.1 M FMLP for 0.5, 1, 5, 15, 30, 45 and 60 minutes and their shape assessed by the proportions of morphological subtypes displayed. This experiment was repeated five times using cells from a different donor on each occasion.

3. 2. 4. 3. Effect of FMLP concentration on proportion of cells remaining spherical (Fig. 3. 4. a.)

Addition of 1000, 100 or 10 nM FMLP to neutrophils in suspension decreased the percentage of spherical cells from >95% before stimulation to approximately 5% by 0.5 minutes and maintained similar values for the rest of the hour incubation. Addition of 1 nM FMLP reduced the number of spherical cells from >95% to 60.8 ± 17.5% by 0.5 minutes and maintained levels between 50% and 80% for the remainder of the experiment. No morphological response was measured following the

addition of 0.1 nM FMLP as the percentage of spherical cells remained >95% throughout the hour incubation.

3. 2. 4. 3. Effect of FMLP concentration on the proportion of type 1 cells produced (Fig. 3. 4. b.)

The proportion of type 1 cells observed at each time point after the addition of FMLP was directly proportional to the concentration of peptide. In addition the number of type 1 cells observed was maximal at 0.5 minutes and declined with time for each concentration of FMLP. The highest percentage of type 1 cells was observed at 0.5 minutes after the addition of 1000 nM FMLP (89.7 ± 3.2%).

3. 2. 4. 4. Effect of FMLP concentration on the proportion of type 2 cells produced (Fig. 3. 4. c.)

The concentration of FMLP producing the largest number of type 2 cells increased with time from 1 nM at 0.5 minutes to 10 nM at 1 minute, to 100 nM at 5 minutes and finally to 1000 nM at 15 and subsequent time intervals. The highest percentage of type 2 cells overall was at 1 minute after addition of 10 nM FMLP (62.8 ±14.1%).

3. 2. 4. 5. Effect of FMLP concentration on the proportion of type 3 cells produced (Fig. 3. 4. d.)

Between 0.5 minutes and 15 minutes after the addition of FMLP, 10 nM produced the highest number of type 3 cells, but 1000 nM produced the highest number at 30 minutes and 100 nM FMLP produced the highest number at both 45 and 60 minutes. The highest number of type 3 cells produced overall was at 5 minutes after the addition of 10 nM FMLP (55.4 ± 5.2%).

Fig. 3. 4. Effect of FMLP concentration on the proportions of morphological subtypes displayed by neutrophils with time

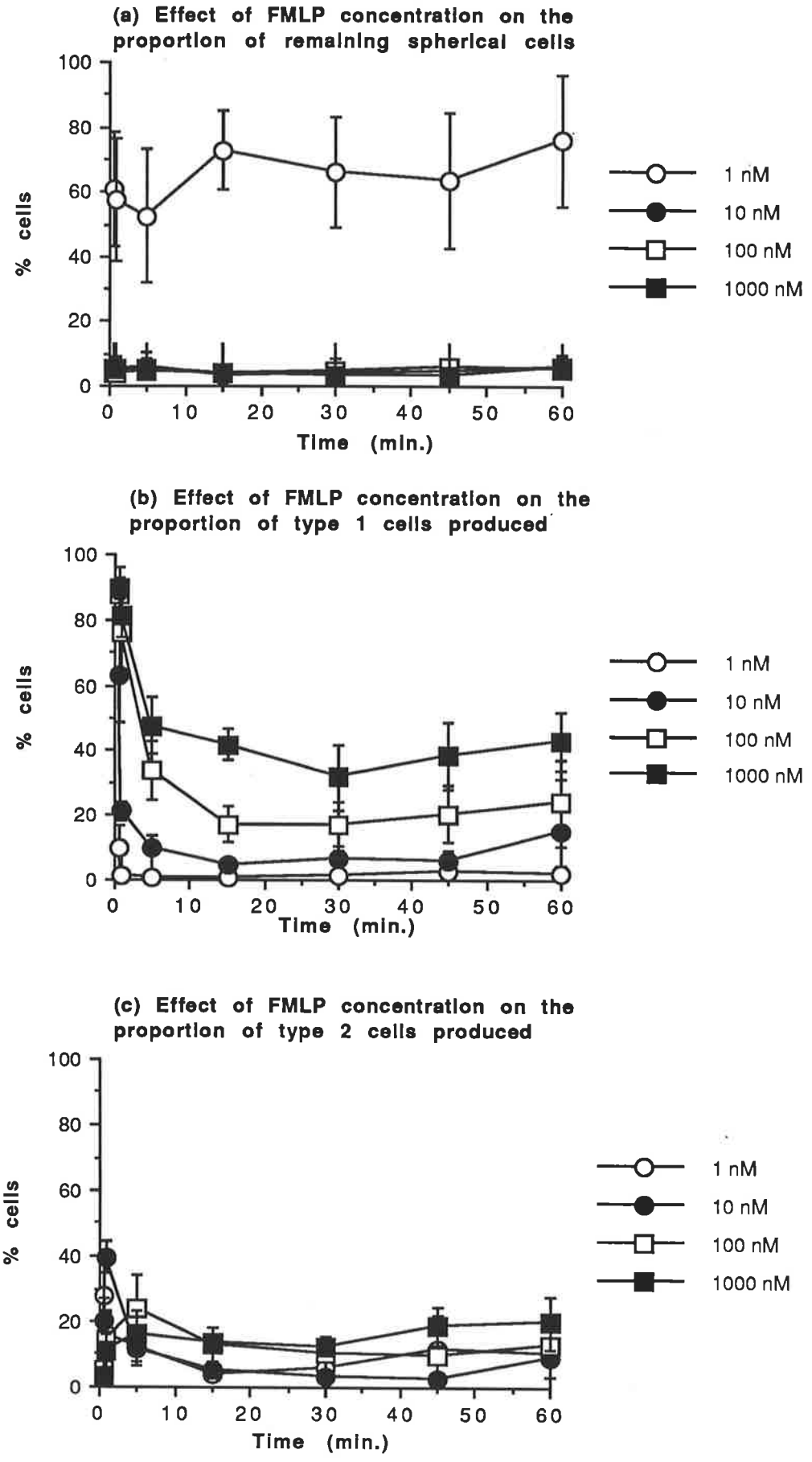
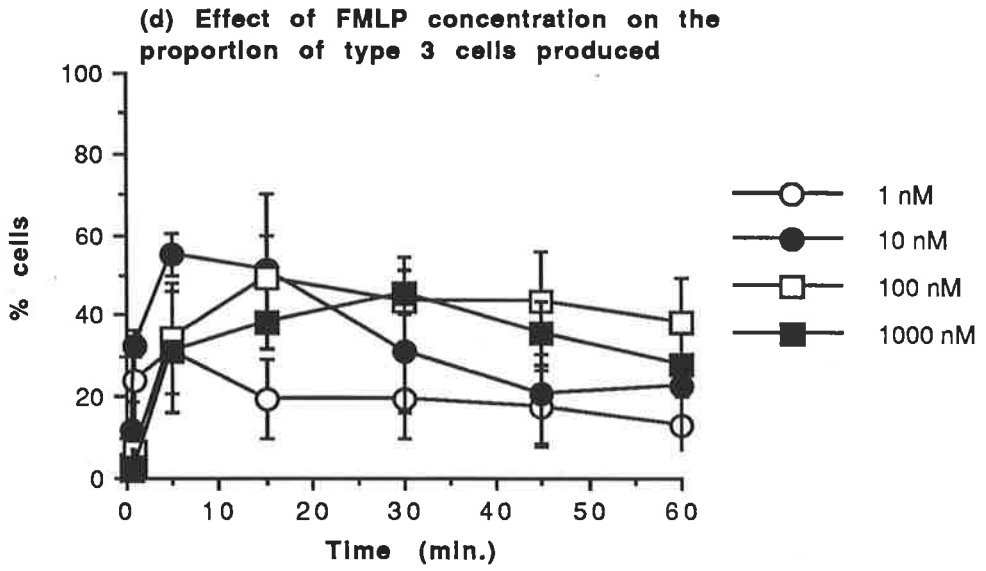
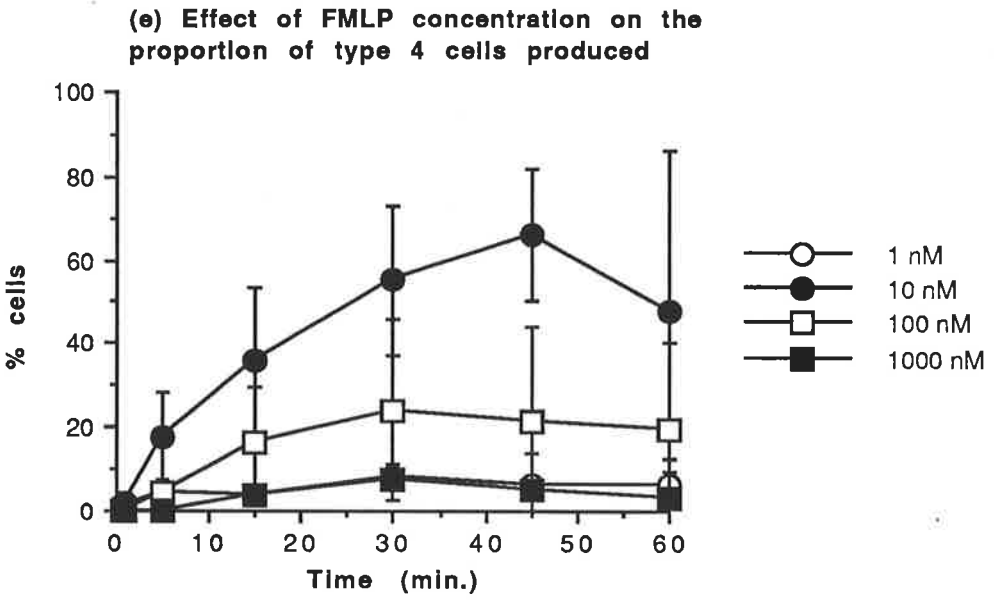


Fig. 3. 4. (continued)





3. 2. 4. 6. Effect of FMLP concentration on the proportion of type 4 cells produced (Fig. 3. 4. e.)

Cell suspensions treated with 10 nM FMLP displayed the highest number of type 4 cells with a maximal value at 45 minutes (66.0 ± 16.0%). The mean number of type 4 cells induced by 100 nM FMLP remained below 25% and those induced by 1000 nM and 1nM FMLP remained below 10%.

3. 2. 5. Comparison of F-actin distributions for morphological subtypes

3. 2. 5. 1. Design of experiments

The aim of this experiment was to establish which aspects of the neutrophil's morphological response to FMLP are associated with the accumulation of F-actin. To determine this, neutrophils were suspended in either HBSS-Hepes/4% human serum albumin or 10 nM FMLP/4% human serum albumin for 1, 15 and 30 minutes before being fixed with 8% formalin and stained with the fluorescent F-actin probe, rhodamine phalloidin. The distribution of staining for F-actin within the cytoplasm of cells with each morphological subtype was then examined by fluorescence microscopy.

3. 2. 5. 2. F-actin distribution for spherical (unstimulated) cells (Fig. 3. 5. a.)

Contrasting regions of moderate and poor staining for F-actin were noted within spherical cells. The regions of moderate staining completely outlined the periphery of each cell and extended into the their centre. The regions of poor staining were usually located towards the centre of each cell and had a lobular appearance.

3. 2. 5. 3. F-actin distribution for Type 1 cells (Fig. 3. 5. b.)

Intense staining for F-actin was observed within peripheral extensions of type 1 cells and weak staining was noted throughout the body of these cells.

3. 2. 5. 4. F-actin distribution for Type 2 cells (Fig. 3. 5. c.)

Intense staining for F-actin was observed within the polarised extensions of type 2 cells. In contrast, the round bodies of type 2 cells displayed weak to moderate staining which was greatest towards the periphery of the cell.

3. 2. 5. 5. F-actin distribution for Type 3 cells (Fig. 3. 5. d.)

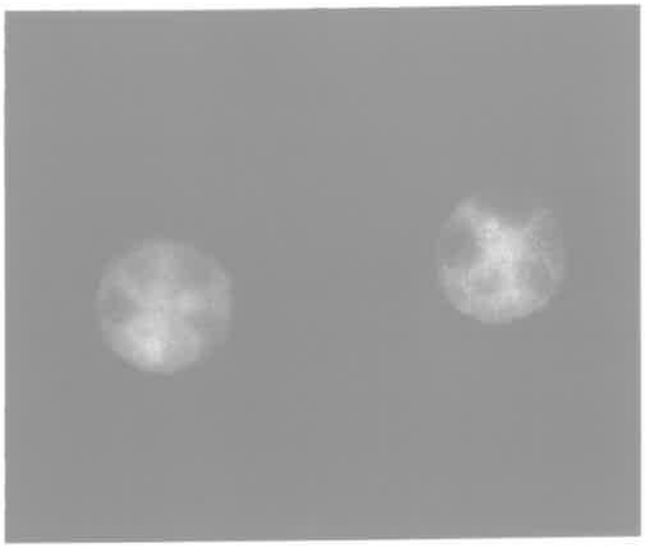
Intense staining for F-actin was observed within the pseudopodia of type 3 cells, but the elongated bodies of these cells displayed similar weak staining to that noted in the round bodies of type 2 cells.

3. 2. 5. 6. F-actin distribution for Type 4 cells (Fig. 3. 5. e. and f.)

While the pseudopod of type 4 cells consistently displayed intense staining for F-actin, the uropod of these cells usually stained weakly (Fig. 3. 5. e.). However, in some instances moderate staining for F-actin was observed within the uropod of type 4 cells (Fig. 3. 5. f.). The body of type 4 cells displayed weak staining similar to that noted within the bodies of types 2 and 3 cells.

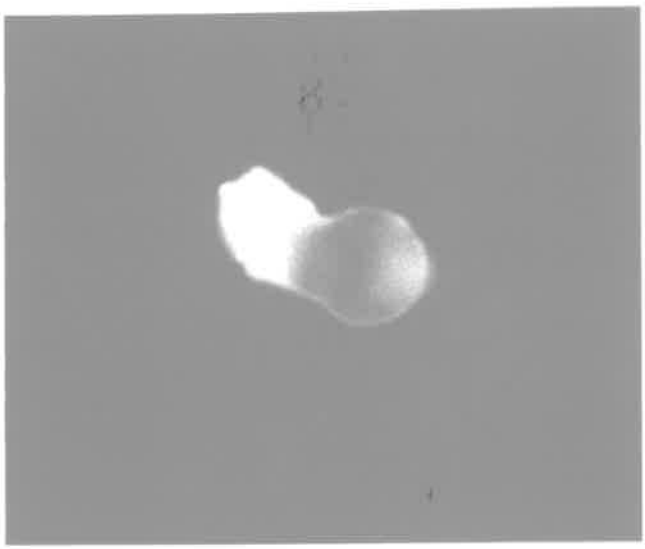
Fig. 3. 5. F-actin distributions for subtypes of neutrophil shape identified by visual classification (fluorescence microscopy; approximately 2000 X).

(a) Spherical

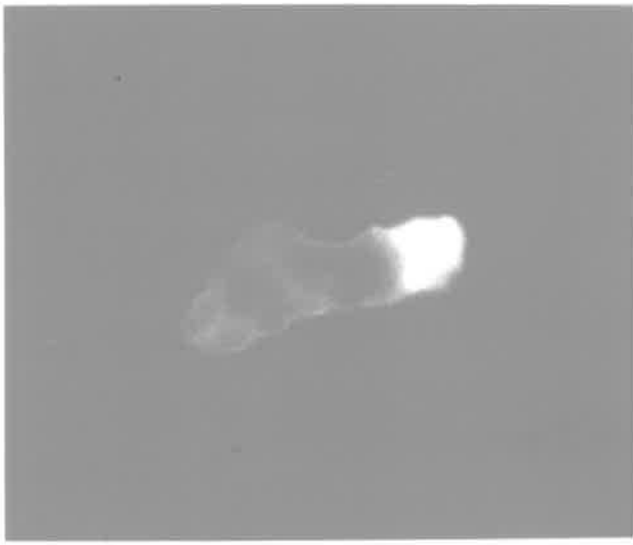


(b) Type 1

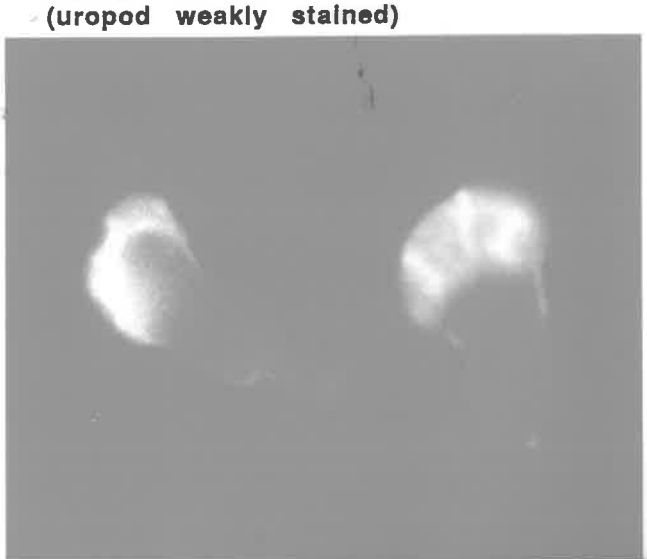
(d) Type 2



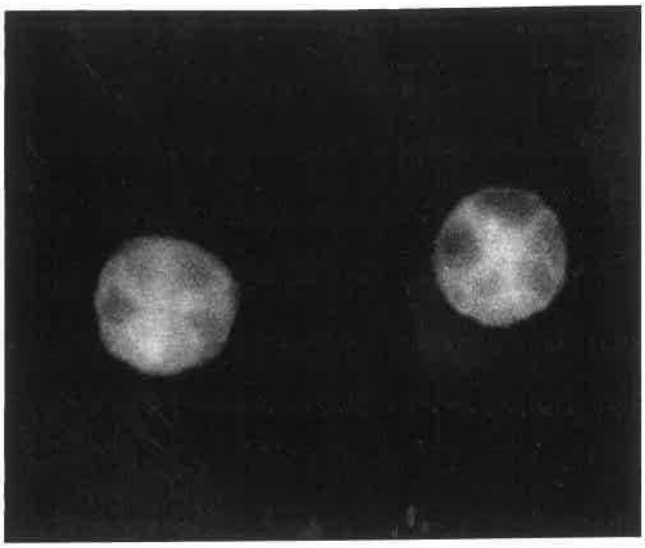
(d) Type 3

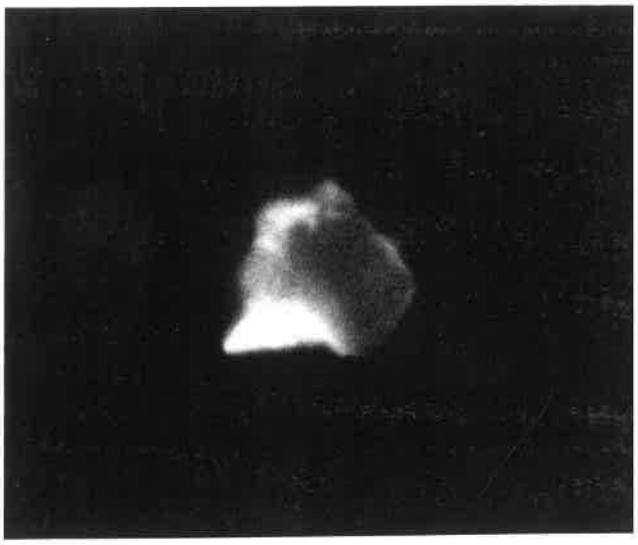


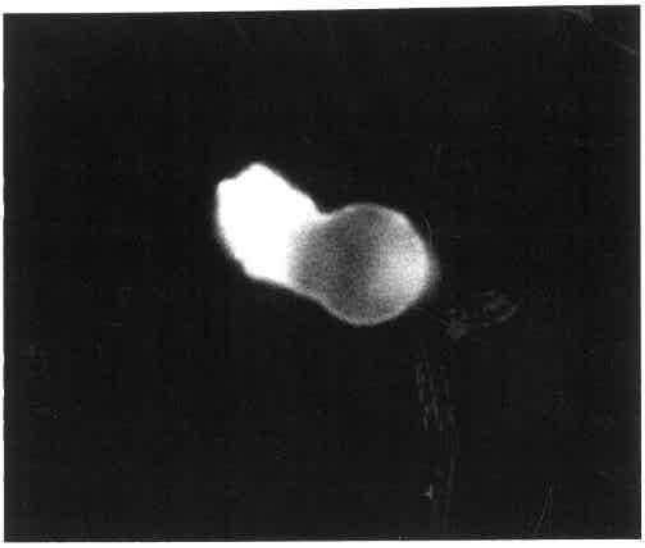
(e) Type 4
(uropod weakly stained)

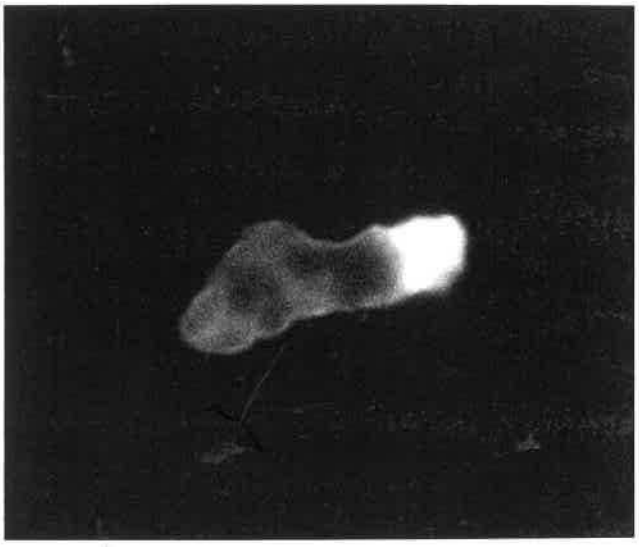


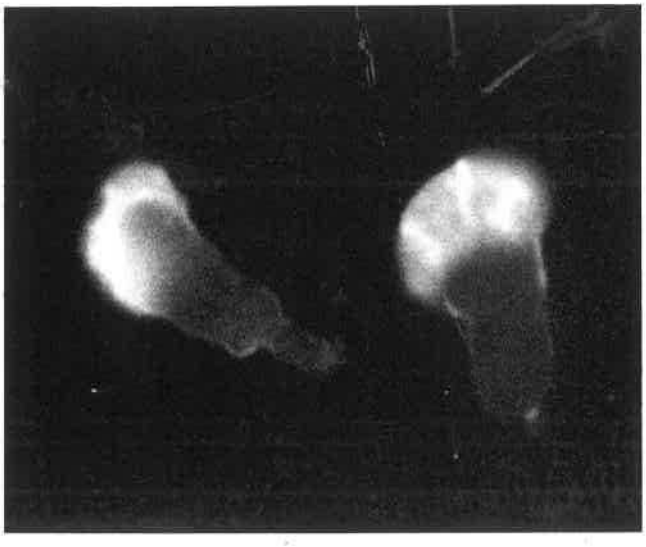
(f) Type 4
(uropod moderately stained)

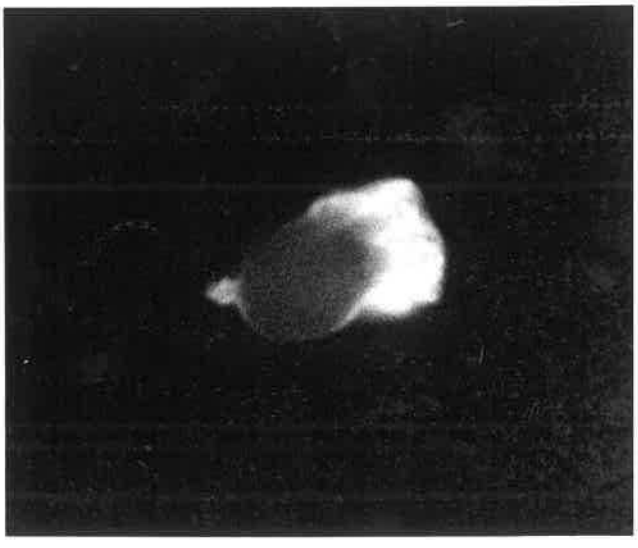












3. 3. Summary

Neutrophils suspended in HBSS-Hepes at 37 °C remained spherical (>95%) for the period of the experiments (60 min). In contrast, neutrophils exposed to 10 nM FMLP developed extensions of their cytoplasm within 0.5 minutes which remained for at least 1 hour. The distribution of these cytoplasmic extensions and the elongation of the cell body, allowed neutrophils to be visually classified into four morphological subtypes which altered in relative proportions according to the following time course. Type 1 cells were characterised by non-polarised extensions of the cytoplasm and occurred in highest numbers after 0.5 minutes incubation. Type 2 cells were round with polarised extensions and occurred in highest numbers after 1 minute incubation. Type 3 cells were elongated with polarised extensions and occurred in highest numbers after 5 minutes. Type 4 cells were elongated with polarised extensions and a uropod or tail, and were observed in maximal numbers after 45 minutes incubation.

Morphometric analysis of the same preparations of FMLP-treated cells as used for visual classification showed gradual increases in the mean area and mean perimeter of neutrophils and a gradual decrease in their mean shape factor. These changes in morphometric parameters were greatest by approximately 45 minutes after the addition of FMLP. However, morphometry did not consistently distinguish between non-polarised (type 1 cells) and certain subtypes of polarised cells (types 2 and 3).

FACS analysis of samples obtained from the same preparations of FMLP-treated cells as used for visual and morphometric analyses showed an inconsistent change in mean FSC for neutrophils during the first minute followed by a consistent and gradual rise in mean FSC which peaked at 30 to 45 minutes. This rise was observed regardless of whether glutaraldehyde or formalin fixation was used prior to FACS analysis. In a separate study of the importance of fixation techniques, a decrease in mean FSC in response to FMLP was recorded for cells exposed to erythrocyte lysis

during their preparation, when formalin but not glutaraldehyde fixation was used prior to FACS analysis. However, FACS analysis did not distinguish between preparations of neutrophils treated with FMLP for 0.5 minutes containing mostly non-polarised cells (type 1 cells) and those treated with FMLP for 1 minute which contained mostly polarised cells (type 2).

Morphometric comparisons of the subtypes of neutrophils revealed significant differences between their mean area, mean perimeter and mean shape factor value, with the exceptions of similar mean areas for subtypes 1 and 2 and similar mean shape factors for subtypes 1 and 3.

In concentration-effect studies of FMLP, the highest proportion of type 4 cells was observed with 10 nM compared to higher and lower concentrations. Supra-optimal concentrations of FMLP (100 nM and 1000 nM) increased the proportions of type 1 cells observed throughout the 60 minutes incubation, but the relative proportion of cells with this shape still declined from highest values at 0.5 minutes.

Microfilaments (F-actin) accumulated within cytoplasmic extensions of cells with each morphological subtype and were sparsely distributed within both round (type 2) and elongated (type 3 and 4) bodies of these cells.

CHAPTER 4.

THE MORPHOLOGICAL RESPONSES OF NEUTROPHILS IN SUSPENSION TO WHOLE AND FRACTIONATED PLASMA

4. 1. Introduction

In this chapter, polarisation of neutrophils in plasma is measured by using the visual classification method established in the preceding chapter. Possible variations in this morphological response of neutrophils to plasma are examined with respect to time and plasma concentration. Furthermore, the roles of anticoagulants, extracellular divalent cations, intracellular Ca^{2+} ions, plasma proteases and F-actin distribution during neutrophil polarisation in plasma are assessed. The morphological responses of neutrophils in suspension to commercial and chromatographically separated fractions of plasma proteins are also assessed.

4. 2. Results

4. 2. 1. Time courses of the morphological responses of neutrophils to various concentrations of plasma

4. 2. 1. 1. Design of experiments

The aim of these initial experiments was to characterise polarisation of neutrophils suspended in plasma with respect to time, plasma concentration and the morphological subtypes displayed. Briefly, a cell shape assay was perfomed by suspending neutrophils for 0.5, 1, 5, 15, 30, 45 and 60 minutes in 90%, 50%, 10% and 1% heparinised (12.5 I.U./ml) plasma diluted with HBSS-Hepes (pH 7. 2, 37 °C). Cells fixed at each time point were then prepared for Nomarski optics and analysed by visual classification of morphological subtypes. Results are expressed as the mean ± standard deviation for the proportions of morphological subtypes obtained from 8 experiments using blood from a different donor on each occasion.

4. 2. 1. 2. Response to 90% plasma (Fig. 4. 1. a.)

Neutrophils suspended in 90% plasma displayed an immediate morphological response with only 12.3 ± 7.6% of cells retaining a spherical shape at 0.5 minutes after commencing incubation. The number of spherical cells further declined to a minimum of 7.0 ± 4.5% at 5 minutes then gradually rose to a maximum of 65.3 ± 15.3% by 60 minutes. The number of type 1 cells produced declined from 16.7 ± 3.1% at 0.5 minutes to <5% for the remainder of the experiment. The number of type 2 cells produced gradually declined from a maximum of 67.7 ± 4.7% at 0.5 minutes to 26.1 ± 10.5% by 5 minutes, then rose to 29.9 ± 7.3% at 15 minutes before gradually declining to a minimum value of 17.3 ± 6.1% by 60 minutes. The

number of type 3 cells produced gradually rose from a minimum of 2.7 ± 4.6% at 0.5 minutes to a maximum of 64.8 ± 10.5% by 5 minutes and then gradually declined to 14.0 ± 9.2% by 60 minutes. The number of type 4 cells produced was always <5% and ranged from a minimum of 0.0 ± 0.0% at 0.5 minutes to a maximum of 2.7 ± 3.8% at 45 minutes.

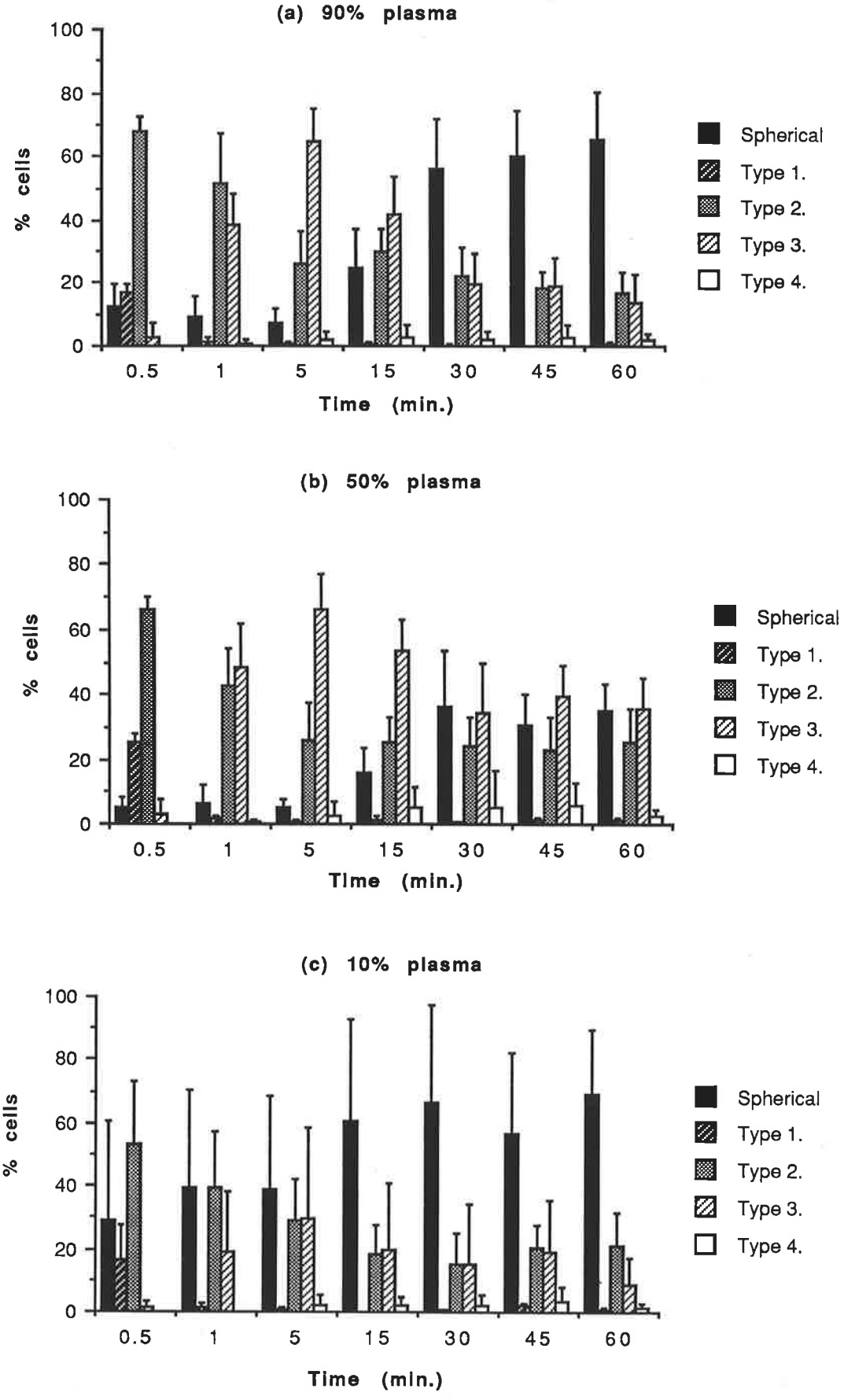
4. 2. 1. 3. Response to 50% plasma (Fig. 4. 1. b.)

Neutrophils suspended in 50% plasma displayed an immediate morphological response with only 5.3 ± 3.2% of cells retaining a spherical shape at 0.5 minutes after commencing incubation. The number of spherical cells remained close to 5% until 5 minutes then gradually rose to a maximum of 36.1 ± 17.4% by 30 minutes, declined to 30.7 ± 9.2% at 45 minutes and rose to 35.0 ± 8.5% at 60 minutes. The number of type 1 cells produced declined from a maximum of 25.3 ± 2.5% at 0.5 minutes to <5% for the remainder of the experiment. The number of type 2 cells produced gradually declined from a maximum of 66.3 ± 3.5% at 0.5 minutes to a minimum of 23.0 ± 10.4% by 45 minutes and then rose to 25.7 ± 9.9% at 60 minutes. The number of type 3 cells produced gradually rose from a minimum of 3.0 ± 4.4% at 0.5 minutes to a maximum of 66.1 ± 10.7% by 5 minutes then gradually declined to 34.3 ± 15.1% by 30 minutes, rose to 39.3 ± 9.9% at 45 minutes and declined to 35.7 ± 9.5% at 60 minutes. The number of type 4 cells produced was always ≤ 6% and ranged from a minimum of 0.0 ± 0.0% at 0.5 minutes to a maximum of 6.0 ± 7.0% at 45 minutes.

4. 2. 1. 4. Response to 10% plasma (Fig. 4. 1. c.)

Neutrophils suspended in 10% plasma displayed an immediate morphological response with only 29.0 ± 31.2% of cells retaining a spherical shape at 0.5 minutes after commencing incubation. The number of spherical cells rose to 39.6 ± 30.7% at

Fig 4. 1. Time courses of the morphological responses of neutrophils in suspension to various concentrations of heparinised plasma



1 minute, declined to 38.7 ± 30.0% at 5 minutes and then gradually rose to a maximum of 69.3 ± 20.0% by 60 minutes. The number of type 1 cells produced declined from a maximum of 16.3 ± 11.2% at 0.5 minutes to <5% of cells for the remainder of the experiment. The number of type 2 cells produced gradually declined from a maximum of 53.3 ± 19.7% at 0.5 minutes to a minimum of 15.4 ± 9.6% by 30 minutes and then gradually rose to 21.0 ± 10.8% by 60 minutes. The number of type 3 cells produced gradually rose from a minimum of 1.3 ± 2.3% at 0.5 minutes to a maximum of 29.7 ± 29.1% by 5 minutes then gradually declined to 14.9 ± 19.4% by 30 minutes, rose to 19.0 ± 16.8% at 45 minutes and declined to 8.3 ± 8.5% at 60 minutes. The number of type 4 cells produced was always <5% and ranged from a minimum of 0.0 ± 0.0% at 0.5 minutes to a maximum of 3.3 ± 4.9% at 45 minutes.

4. 2. 1. 5. Response to 1% plasma

Neutrophils did not display any noticeable change in morphology when suspended in 1% plasma as the percentage of spherical cells remained >95% throughout the hour incubation (data not shown graphically).

4. 2. 2. Effects of heparin on the morphological responses of neutrophils to plasma and FMLP

4. 2. 2. 1. Design of experiments

Since heparin has been reported to reduce the motility of neutrophils in plasma (Platten, 1973) and was used in the preceding studies to prevent coagulation, the purpose of this experiment was to determine the effect of this anticoagulant on the ability of plasma to induce polarisation of neutrophils in suspension. This study was made by suspending neutrophils for 30 minutes at 37 °C in 50% buffered plasma

from blood anticoagulated with either 5, 20 or 50 I.U./ml heparin. The final concentrations of heparin in suspension media containing plasma were therefore 2.5, 10 and 25 I.U./ml. Each plasma was examined visually prior to use for evidence of coagulation as indicated by the presence or absence of a fibrin clot. Controls were performed by suspending cells in 10 nM FMLP containing 0, 5, 20, 50, or 100 units/ml heparin, and HBSS-Hepes containing 0 or 100 units/ml heparin. Results are expressed as the mean ± standard deviation from experiments with three donors. Significant differences between treatments were assessed using a Chi-square 'goodness-of-fit test (see section, 2. 2. 15. 1).

4. 2. 2. 2. Effect of heparin on response to plasma (Fig. 4. 2. a.)

The proportions of morphological subtypes displayed by neutrophils after suspension in 50% plasma containing 2.5 I.U./ml heparin were: 34.3 ± 43.5% spherical, 2.0 ± 2.0% type 1, 13.3 ± 11.7% type 2, 22.3 ± 25.8% type 3, and 28.3 ± 45.7% type 4.

The proportions of morphological subtypes displayed by neutrophils after suspension in 50% plasma containing 10 I.U./ml heparin were: 51.3 ± 36.3% spherical, 0.3 ± 0.6% type 1, 26.7 ± 7.6% type 2, 18.3 ± 26.6% type 3, and 3.3 ± 4.9% type 4. These proportions were significantly different to those observed in the presence of 2.5 I.U./ml heparin (X^2= 46.2).

The proportions of morphological subtypes displayed by neutrophils after suspension in 50% plasma containing 25 I.U./ml heparin were: 66.3 ± 9.1% spherical, 0.0 ± 0.0% type 1, 29.7 ± 10.5% type 2, 3.0 ± 1.7% type 3, and 0.0 ± 0.0% type 4. These proportions of morphological subtypes were significantly different to those obtained in the presence of 2.5 I.U./ml heparin (X^2= 97.05) and were also significantly different to those obtained in the presence of 10 I.U./ml heparin (X^2= 21.10).

Clotting was not was not apparent in any of the preparations of plasma used.

4. 2. 2. 3. Effect of heparin on responses to FMLP (Fig. 4. 2. b.)

The proportions of morphological subtypes displayed by neutrophils after suspension in 10 nM FMLP, without heparin were: 4.7 ± 2.9% spherical, 7.0 ± 1.7% type 1, 4.7 ± 1.2% type 2, 3.7 ± 0.6% type 3, and 80.3 ± 2.5% type 4.

The proportions of morphological subtypes displayed by neutrophils after suspension in 10 nM FMLP, with 5 I.U./ml heparin were: 5.0 ± 3.0% spherical, 7.7 ± 2.9% type 1, 5.3 ± 1.2% type 2, 4.7 ± 0.6% type 3, and 77.0 ± 5.3% type 4. These proportions of morphological subtypes were not significantly different to those observed in 10 nM FMLP alone (X^2= 0.58).

The proportions of morphological subtypes displayed by neutrophils after suspension in 10 nM FMLP, with 20 I.U./ml heparin were: 3.7 ± 0.6% spherical, 5.0 ± 0.0% type 1, 2.7 ± 1.2% type 2, 4.7 ± 0.6% type 3, and 84.0 ± 0.0% type 4. These proportions of morphological subtypes were not significantly different to those observed in 10 nM FMLP alone (X^2= 2.07).

The proportions of morphological subtypes displayed by neutrophils after suspension in 10 nM FMLP, with 50 I.U./ml heparin were: 3.3 ± 0.6% spherical, 7.3 ± 3.1% type 1, 6.7 ± 2.1% type 2, 4.0 ± 1.0% type 3, and 79.3 ± 3.8% type 4. These proportions of morphological subtypes were not significantly different to those observed in 10 nM FMLP alone (X^2= 1.31).

The proportions of morphological subtypes displayed by neutrophils after suspension in 10 nM FMLP, with 100 I.U./ml heparin were: 3.0 ± 1.7% spherical, 6.0 ± 3.5% type 1, 4.0 ± 1.0% type 2, 4.7 ± 2.1% type 3, and 83.0 ± 4.4% type 4. These proportions of morphological subtypes were not significantly different to those observed in 10 nM FMLP alone (X^2= 1.21).

Cells suspended in HBSS-Hepes remained >95% spherical in the presence of 100 units/ml heparin.

Fig 4. 2. a. Effect of heparin concentration on the morphological response of neutrophils to 50% plasma

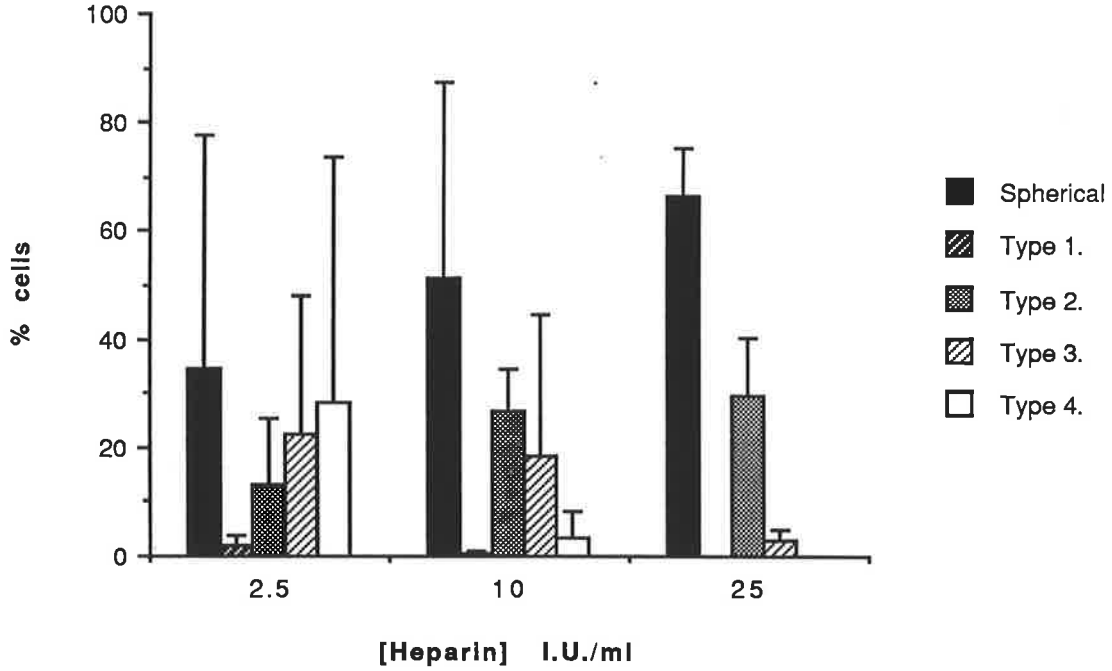
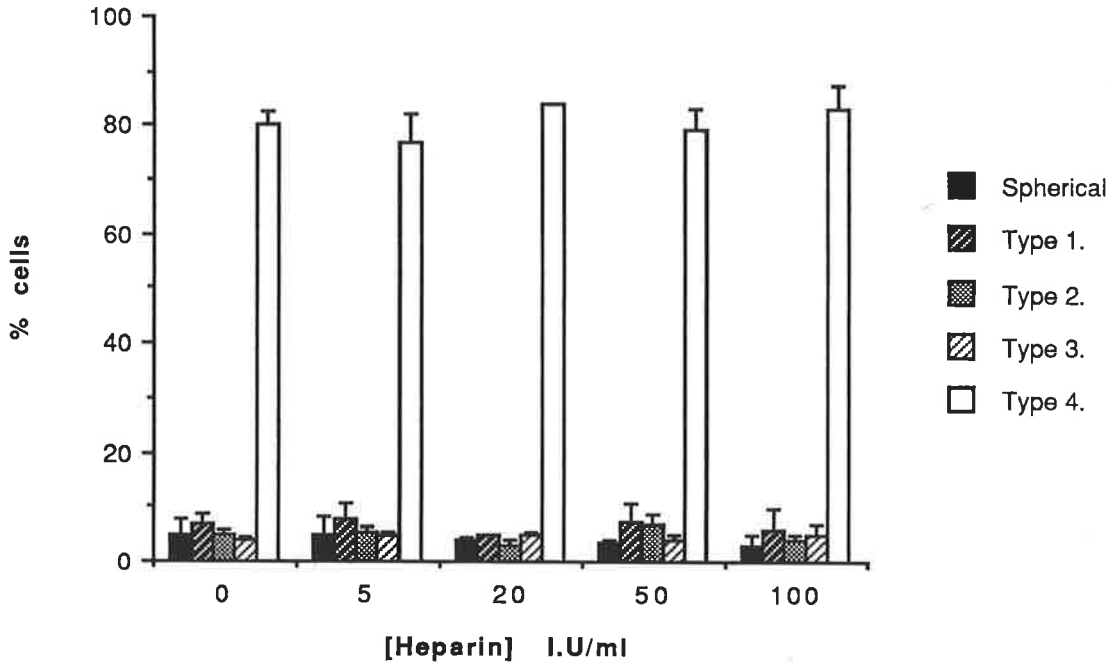


Fig 4. 2. b. Effect of heparin concentration on the morphological response of neutrophils to 10 nM FMLP



4. 2. 3. Comparison of the morphological responses of neutrophils to plasma and serum

4. 2. 3. 1. Design of experiments

The purpose of this study was to compare the morphological response of neutrophils in plasma to that observed in serum (i.e. plasma following coagulation when have activation of the coagulation system). This comparison was achieved by preparing neutrophils, plasma and serum from the one donation of blood and the following procedure perfomed. Neutrophils were suspended for 30 minutes at 37 °C in either 50% serum or 50% plasma. This experiment was performed twice using blood from a different donor on each occasion.

4. 2. 3. 2. Comparison of responses to plasma and serum (Fig. 4. 3.)

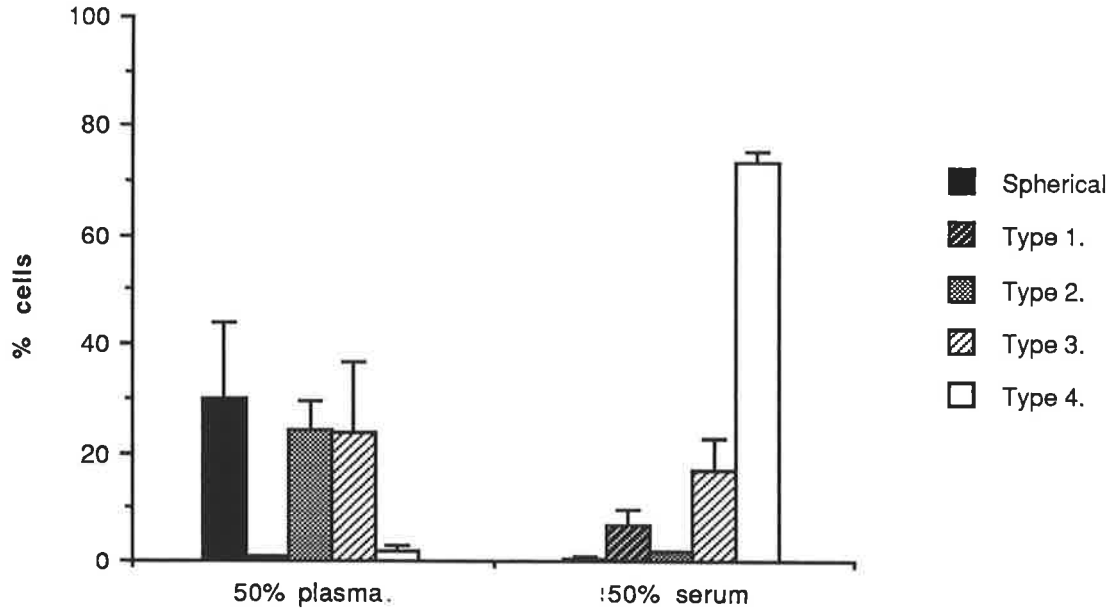
The proportions of morphological subtypes displayed by neutrophils after suspension for 30 minutes in 50% plasma were: 30.0 ± 14.1% spherical, 1.0 ± 0.0% type 1, 24.0 ± 5.7% type 2, 23.5 ± 13.4% type 3, and 2.0 ± 1.0% type 4.

The proportions of morphological subtypes displayed by neutrophils after suspension for 30 minutes in 50% serum were: 0.5 ± 0.7% spherical, 7.0 ± 2.8% type 1, 2.0 ± 0.0% type 2, 17.0 ± 5.7% type 3, and 73.5 ± 2.1% type 4. These proportions of morphological subtypes were significantly different (X^2= 2643.10) to those displayed by cells suspended in 50% plasma .

4. 2. 4. Effects of cation chelating anticoagulants on the morphological response of neutrophils to plasma

4. 2. 4. 1. Design of experiments

Fig 4. 3. Comparison of morphological responses of neutrophils in suspension to plasma and serum



The purpose of these experiments was to examine the effect of anticoagulants, other than heparin, on the morphological response. This study was perfomed by suspending neutrophils for 30 minutes at 37 °C in 50% plasma obtained from blood anticoagulated with either 5 mM EDTA, 5 mM EGTA or 4 mg/ml disodium hydrogen citrate. All responses were assessed in three experiments, using blood from a different donor on each occasion.

4. 2. 4. 2. Responses to plasma anticoagulated with EDTA, EGTA or citrate (Fig. 4. 4.)

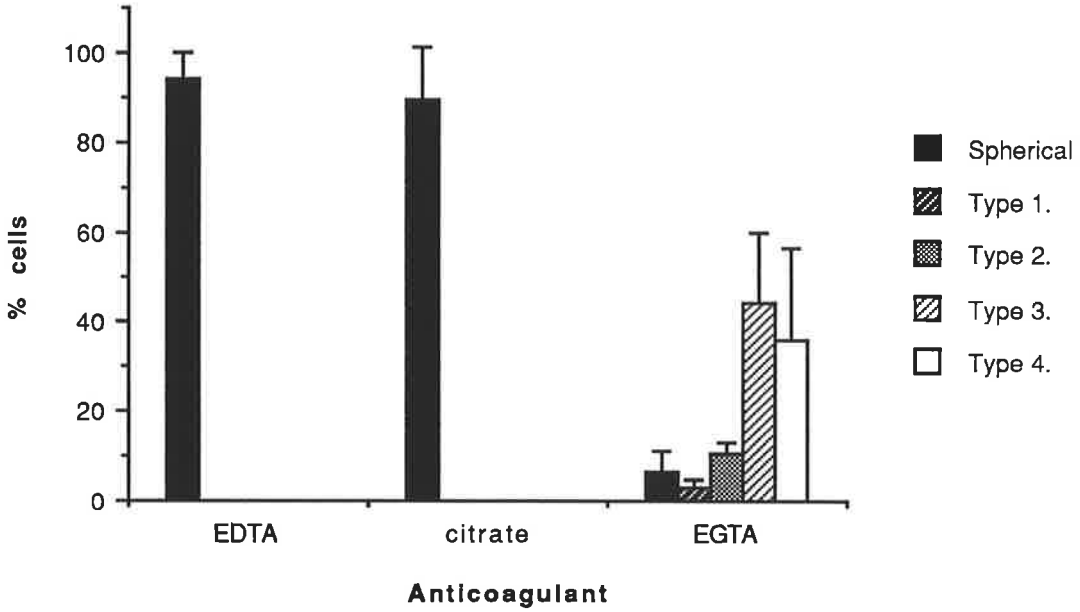
Only 5.7 ± 5.7% of neutrophils displayed extensions of cytoplasm after suspension for 30 minutes in EDTA anticoagulated plasma and only 10.7 ± 11.9% displayed extensions after suspension for 30 minutes in citrate anticoagulated plasma. The morphological subtypes 1, 2, 3 and 4 were therefore not consistently observed in sufficient quantities to be individually quantified in these treatments. In contrast, 93.7 ± 5.1% of neutrophils displayed extensions of the cytoplasm after suspension for 30 minutes in EGTA anticoagulated plasma. The proportions of morphological subtypes displayed by neutrophils following this treatment were: 6.3 ± 5.1% spherical, 3.0 ± 2.0% type 1, 10.7 ± 2.5% type 2, 44.3 ± 15.9% type 3, and 35.7 ± 20.8% type 4.

4. 2. 5. Effects of cation chelating agents on the morphological responses of neutrophils to FMLP and heparinised plasma

4. 2. 5. 1. Design of experiments

Since extracellular Ca^{2+} and Mg^{2+} ions have been reported to be necessary for optimal motile responses of neutrophils (Marasco et al., 1980), and these cations are chelated by EDTA and EGTA, it was considered that the effects of these

Fig 4. 4. Morphological responses of neutrophils to plasmas (50%) obtained from blood anticoagulated with either EDTA (5 mM), citrate (4 mg/ml) or EGTA (5 mM)



anticoagulants in the previous set of experiments might be related to a requirement for divalent cations during polarisation. To test this hypothesis, neutrophils were suspended for 30 minutes at 37 °C in 85%, 50% and 10% heparinised plasma (v/v) in HBSS-Hepes containing either 5 mM EDTA, 5 mM EGTA or no chelating agent. A comparative study for these experiments was perfomed by suspending neutrophils for 30 minutes at 37 °C in 100, 10 and 1 nM FMLP in HBSS-Hepes containing either 5 mM EDTA, 5 mM EGTA or no chelating agent. Both experiments were repeated at least three times using blood from a different donor on each occasion.

4. 2. 5. 2. Effects of chelating agents on response to plasma (Fig. 4. 5. a.)

4. 2. 5. 2. 1. Effects on response to 85% plasma

The proportions of morphological subtypes displayed by neutrophils after suspension in 85% plasma, in the absence of chelating agents, were: 53.3 ± 18.9% spherical, 0.3 ± 0.5% type 1, 28.5 ± 8.6% type 2, 17.3 ± 11.5% type 3, and 1.0 ± 0.8% type 4.

The proportions of morphological subtypes displayed by neutrophils after suspension in 85% plasma, in the presence of 5 mM EDTA, were: 80.8 ± 10.8% spherical, 0.0 ± 0.0% type 1, 15.5 ± 8.4% type 2, 3.5 ± 2.1% type 3, and 0.0 ± 0.0% type 4. These proportions of morphological subtypes were significantly different to those observed in 85% plasma alone (X^2=32.43).

The proportions of morphological subtypes displayed by neutrophils after suspension in 85% plasma, in the presence of 5 mM EGTA, were: 58.5 ± 19.0% spherical, 0.0 ± 0.0% type 1, 28.8 ± 9.9% type 2, 11.8 ± 8.0% type 3, and 1.3 ± 1.5% type 4. These proportions of morphological subtypes were not significantly different to those observed in 85% plasma alone (X^2=2.38).

4. 2. 5. 2. 2. Effects on response to 50% plasma

The proportions of morphological subtypes displayed by neutrophils after suspension in 50% plasma, in the absence of chelating agents, were: 19.5 ± 12.4% spherical, 1.0 ± 1.2% type 1, 25.3 ± 7.6% type 2, 47.3 ± 13.5% type 3, and 8.0 ± 3.4% type 4.

The proportions of morphological subtypes displayed by neutrophils after suspension in 50% plasma, in the presence of 5 mM EDTA, were: 53.5 ± 20.6% spherical, 0.5 ± 0.6% type 1, 24.5 ± 5.2% type 2, 18.3 ± 12.0% type 3, and 3.3 ± 3.2% type 4. These proportions of morphological subtypes were significantly different to those observed in 50% plasma alone (X^2=80.10).

The proportions of morphological subtypes displayed by neutrophils after suspension in 50% plasma, in the presence of 5 mM EGTA, were: 35.0 ± 17.8% spherical, 0.3 ± 0.5% type 1, 25.0 ± 5.0% a type 2, 34.0 ± 18.4% type 3, and 5.8 ± 3.6% type 4. These proportions of morphological subtypes were significantly different to those observed in 50% plasma alone (X^2=17.16).

4. 2. 5. 2. 3. Effects on response to 10% plasma

The proportions of morphological subtypes displayed by neutrophils after suspension in 10% plasma, in the absence of chelating agents, were: 74.5 ± 18.4% spherical, 0.3 ± 0.5% type 1, 16.0 ± 9.6% type 2, 8.8 ± 8.7% type 3, and 0.3 ± 0.5% type 4.

Neutrophils suspended in 10% plasma in the presence of 5 mM EDTA were 96.5 ± 1.7% spherical. Other morphological subtypes were therefore not present in sufficient numbers to be individually quantified.

The proportions of morphological subtypes displayed by neutrophils after suspension in 10% plasma, in the presence of 5 mM EGTA, were: 86.5 ± 7.9% spherical, 0.0 ± 0.0% type 1, 9.0 ± 4.2% type 2, 4.0 ± 3.2% type 3, and 0.5 ±

0.6% type 4. These proportions of morphological subtypes were not significantly different to those observed in 10% plasma alone (X^2= 8.04).

4. 2. 5. 3. Effect of chelating agents on response to FMLP (Fig 4. 5. b.)

4. 2. 5. 3. 1. Effects on response to 100 nM FMLP

The proportions of morphological subtypes displayed by neutrophils after suspension in 100 nM FMLP, in the absence of chelating agents, were: 5.7 ± 2.1% spherical, 14.7 ± 3.2% type 1, 17.3 ± 10.0% type 2, 32.0 ± 13.0% type 3, and 30.7 ± 28.1% type 4 morphology.

The proportions of morphological subtypes displayed by neutrophils after suspension in 100 nM FMLP, in the presence of 5 mM EDTA, were: 6.0 ± 2.6% spherical, 6.3 ± 3.2% type 1, 8.3 ± 5.0% type 2, 21.0 ± 7.2% type 3, and 59.0 ± 14.7% type 4. These proportions of morphological subtypes were significantly different to those observed in 100 nM FMLP alone (X^2= 39.37).

The proportions of morphological subtypes displayed by neutrophils after suspension in 100 nM FMLP, in the presence of 5 mM EGTA, were: 6.3 ± 0.6% spherical, 5.0 ± 3.0% type 1, 14.3 ± 8.7% type 2, 26.3 ± 15.0% type 3, and 48.0 ± 23.8% type 4. These proportions of morphological subtypes were significantly different to those observed in 100 nM FMLP alone (X^2= 17.75).

4. 2. 5. 3. 2. Effects on response to 10 nM FMLP

The proportions of morphological subtypes displayed by neutrophils after suspension in 10 nM FMLP, in the absence of chelating agents, were: 6.3 ± 4.2% spherical, 5.0 ± 1.0% type 1, 7.3 ± 3.1% type 2, 22.0 ± 4.4% type 3, and 60.3 ± 3.8% type 4.

The proportions of morphological subtypes displayed by neutrophils after suspension in 10 nM FMLP, in the presence of 5 mM EDTA, were: 6.7 ± 1.5% spherical, 6.0 ± 2.0% type 1, 4.7 ± 3.5% type 2, 15.3 ± 7.0% type 3, and 67.7 ± 5.1% type 4. These proportions of morphological subtypes were not significantly different to those observed in 10 nM FMLP alone (X^2= 4.11).

The proportions of morphological subtypes displayed by neutrophils after suspension in 10 nM FMLP, in the presence of 5 mM EGTA, were: 5.7 ± 2.5% spherical, 4.0 ± 2.6% type 1, 5.7 ± 2.3% type 2, 15.0 ± 6.2% type 3, and 70.3 ± 5.0% type 4. These proportions of morphological subtypes were not significantly different to those observed in 10 nM FMLP alone (X^2= 4.50).

4. 2. 5. 3. 3. Effects on response to 1 nM FMLP

The proportions of morphological subtypes displayed by neutrophils after suspension in 1 nM FMLP, in the absence of chelating agents, were: 78.7 ± 16.2% spherical, 0.3 ± 0.6% type 1, 3.0 ± 2.0% type 2, 9.7 ± 7.4% type 3, and 8.0 ± 7.8% type 4.

The proportions of morphological subtypes displayed by neutrophils after suspension in 1 nM FMLP, in the presence of 5 mM EDTA, were: 67.0 ± 24.2% spherical, 0.7 ± 0.6% type 1, 5.0 ± 3.6% type 2, 10.7 ± 8.1% type 3, and 16.3 ± 16.4% type 4. These proportions of morphological subtypes were significantly different to those observed in 1 nM FMLP alone (X^2=12.3).

The proportions of morphological subtypes displayed by neutrophils after suspension in 1 nM FMLP, in the presence of 5 mM EGTA, were: 77.0 ± 23.4% spherical, 0.3 ± 0.6% type 1, 3.3 ± 2.1% type 2, 8.7 ± 9.8% type 3, and 11.0 ± 13.0% type 4 morphology. These proportions of morphological subtypes were not significantly different to those observed in 1 nM FMLP alone (X^2= 1.30).

Fig 4. 5. a. Effects of cation chelating agents (5 mM) on the morphological responses of neutrophils in suspension to various concentrations of heparinised plasma

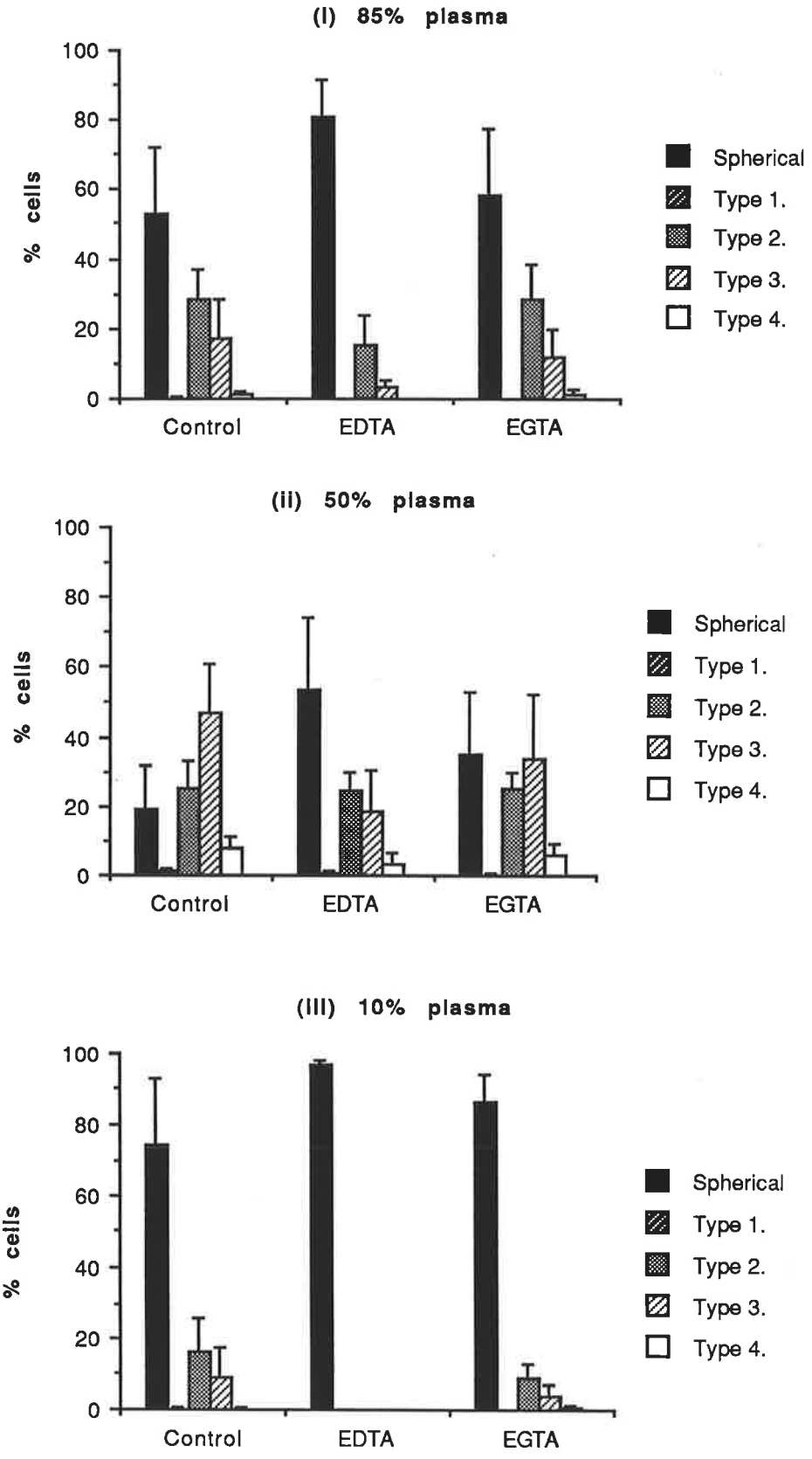
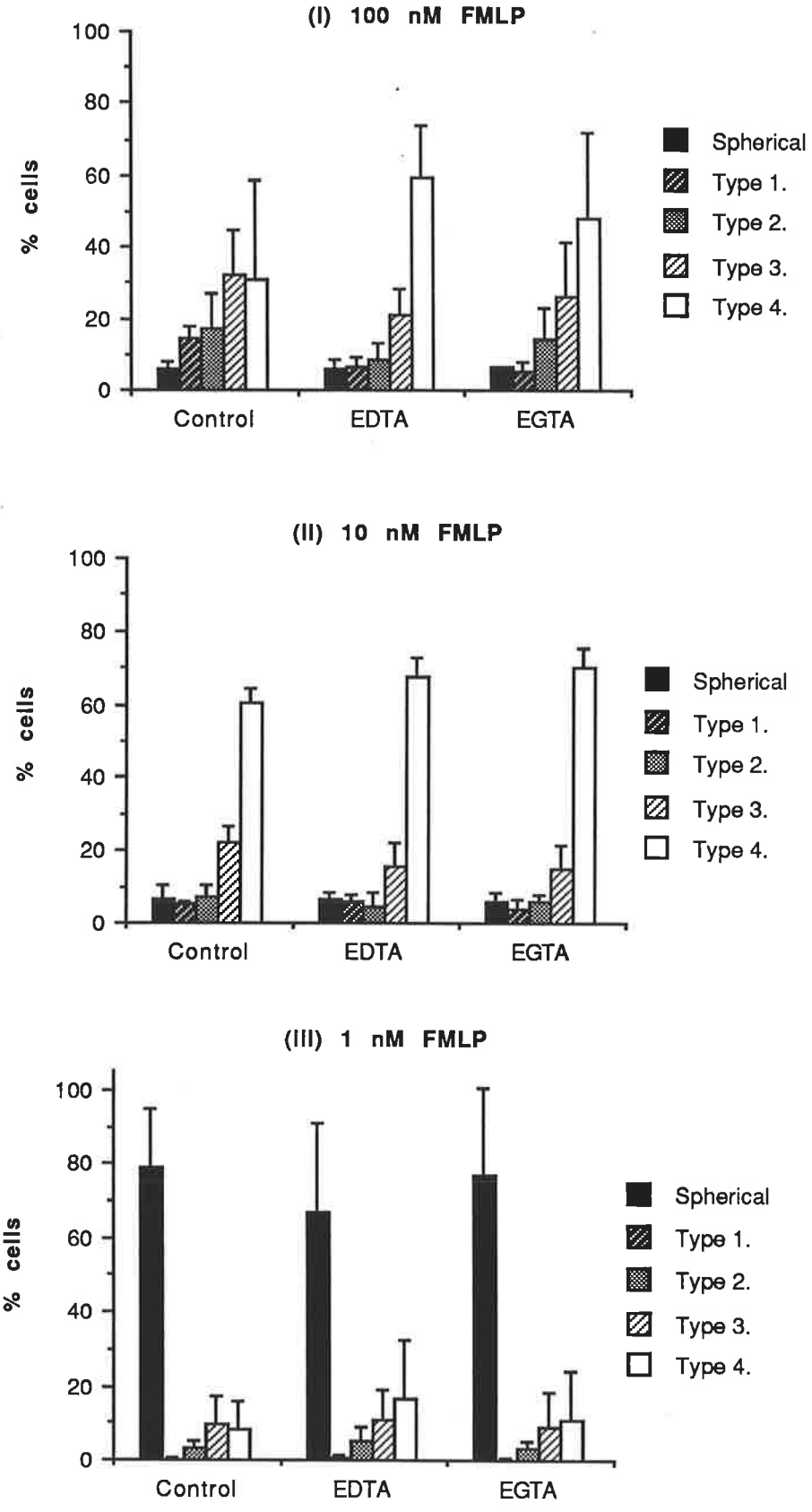


Fig. 4. 5. b. Effects of cation chelating agents (5 mM) on the morphological responses of neutrophils in suspension to various concentrations of FMLP



4. 2. 6. Effect of cation chelating agents on the morphological response of neutrophils to heparinised plasma, in the presence of additional divalent cations (Fig 4. 6.)

4. 2. 6. 1. Design of experiments

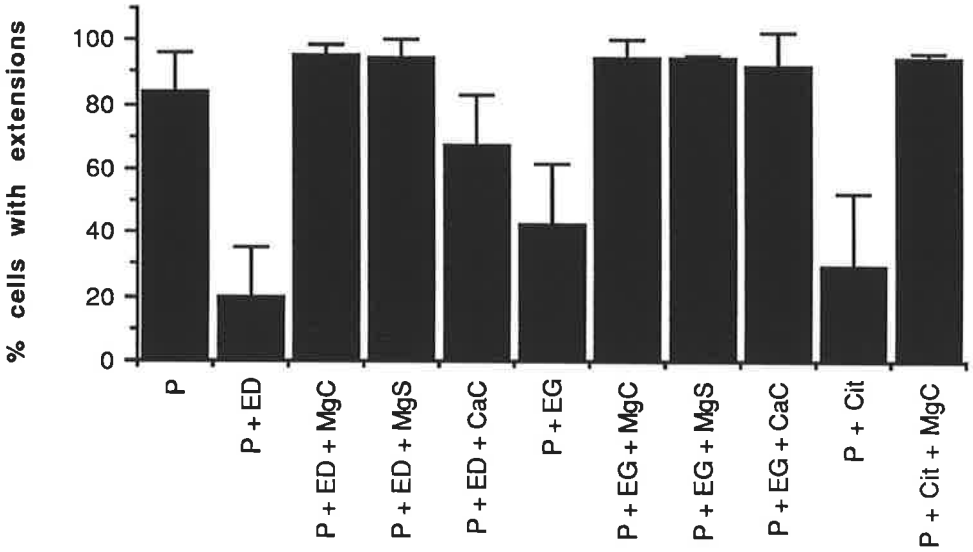
The aim of this experiment was to confirm that the effects of chelating agents on the morphological response of neutrophils to plasma, noted in preceding experiments, are due to their ability to remove divalent cations from the suspension medium. To assess this, neutrophils were suspended in 85% plasma alone, 85% plasma with 5 mM chelating agent (EDTA, EGTA or disodium hydrogen citrate), or 85% plasma with 5 mM chelating agent plus 5 mM additional cations ($CaCl_2$, $MgCl_2$ or $MgSO_4$). Controls were perfomed by suspending cells in HBSS-Hepes in the presence or absence of additional cations. All responses were assessed by visual classification and the final results are expressed as the mean (± SD) proportion of cells displaying extensions of the cytoplasm from three experiments using blood from a different donor on each occasion.

4. 2. 6. 2. Effects of additional cations

After suspending neutrophils for 30 minutes in 85% plasma, in the absence of chelating agents and additional divalent cations, 84.1 ± 11.9% of these cells displayed extensions of the cytoplasm.

After suspending neutrophils for 30 minutes in 85% plasma with 5 mM EDTA, in the absence of additional cations, 20.0 ± 15.6% of these cells displayed extensions of the cytoplasm. In the presence of EDTA plus 5 mM $MgCl_2$ or $MgSO_4$, 95.2 ± 3.0% and 94.4 ± 5.4% of cells displayed extensions of the cytoplasm, respectively. In the presence of EDTA plus 5 mM $CaCl_2$, 67.6 ± 15.2% of cells displayed extensions of the cytoplasm.

Fig. 4. 6. Effects of cation chelating agents (EDTA, EGTA or citrate; 5 mM) on the morphological response of neutrophils to heparinised plasma (P; 85%) in the presence of additional Mg2+ (MgCl2 or MgSO4) or Ca2+ (CaCl2) ions (5 mM)



After suspending neutrophils for 30 minutes in 85% plasma with 5 mM EGTA, in the absence of additional cations, 42.5 ± 19.3% of these cells displayed extensions of the cytoplasm. In the presence of EGTA plus 5 mM $MgCl_2$ or $MgSO_4$, 94.2 ± 5.9% and 94.0 ± 1.4% of cells displayed extensions of the cytoplasm, respectively. In the presence of EGTA plus 5 mM $CaCl_2$, 92.0 ± 11.0% of cells displayed extensions of the cytoplasm.

After suspending neutrophils for 30 minutes in 85% plasma with 5 mM disodium hydrogen citrate, in the absence of additional cations, 29.4 ± 22.9% of these cells displayed extensions of the cytoplasm. In the presence of citrate plus 5 mM $MgCl_2$, 94.0 ± 2.1% of cells displayed extensions of the cytoplasm. Addition of $CaCl_2$ to citrate treated plasma resulted in formation of a precipitate which precluded analysis of cell shape.

Neutrophils suspended in HBSS-Hepes remained >95% spherical following addition of either 5 mM $MgCl_2$, 5 mM $MgSO_4$ or 5 mM $CaCl_2$.

4. 2. 7. Effect of additional magnesium ions on the morphological response of neutrophils in suspension to plasma (Fig 4. 7.)

4. 2. 7. 1. Design of experiments

Since the proportion of cells displaying pseudopodia in plasma was higher in the preceding experiments when additional Mg^{2+} ions were present, the aim of this experiment was to examine this response in more detail by studying the proportions of morphological subtypes displayed in plasma when additional Mg^{2+} ions are supplied. To assess this, neutrophils were suspended for 30 minutes at 37 °C in 50% plasma with either 0 mM, 0.5 mM, 1 mM, 2 mM or 5 mM of additional Mg^{2+} ions ($MgCl_2$). This experiment was performed three times using cells from different individuals.

4. 2. 7. 2. Effect of magnesium concentration

The proportions of morphological subtypes displayed by neutrophils after suspension in 50% plasma, in the absence of additional Mg^{2+} ions, were: 34.0 ± 14.9% spherical, 0.3 ± 0.6% type 1, 29.0 ± 3.6% type 2, 32.7 ± 18.7% type 3, and 4.3 ± 0.6% 4 morphology.

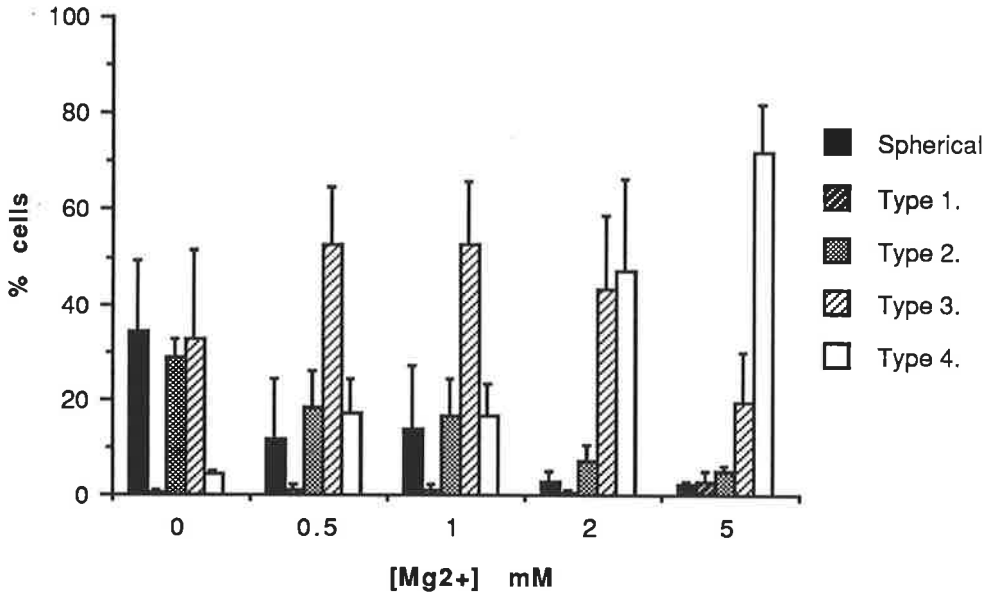
The proportions of morphological subtypes displayed by neutrophils after suspension in 50% plasma, in the presence of 0.5 mM additional Mg^{2+} ions, were: 11.7 ± 12.5% spherical, 1.0 ± 1.0% type 1, 18.3 ± 7.6% type 2, 52.3 ± 12.4% type 3, and 17.0 ± 7.5% type 4. These proportions of morphological subtypes were significantly different to those observed in plasma alone (X^2= 69.47).

The proportions of morphological subtypes displayed by neutrophils after suspension in 50% plasma, in the presence of 1 mM additional Mg^{2+} ions, were: 13.7 ± 13.3% spherical, 1.0 ± 1.0% type 1, 16.7 ± 7.6% type 2, 52.3 ± 13.6% type 3, and 16.3 ± 6.7% type 4. These proportions of morphological subtypes were significantly different to those observed in plasma alone (X^2= 64. 21), but were not significantly different to those observed in plasma containing 0.5 mM additional Mg^{2+} ions (X^2= 0.53).

The proportions of morphological subtypes displayed by neutrophils after suspension in 50% plasma, in the presence of 2 mM additional Mg^{2+} ions, were: 2.7 ± 2.1% spherical, 0.7 ± 0.6% type 1, 7.3 ± 3.1% type 2, 43.0 ± 15.4% type 3, and 46.7 ± 19.8% type 4. These proportions of morphological subtypes were significantly different to those observed in plasma alone (X^2= 466.90), and were also significantly different to those observed in plasma containing 1 mM additional Mg^{2+} ions (X^2= 72.56).

The proportions of morphological subtypes displayed by neutrophils after suspension in 50% plasma, in the presence of 5 mM additional Mg^{2+} ions, were: 2.0 ± 1.0% spherical, 2.7 ± 2.1% type 1, 4.7 ± 1.2% type 2, 19.3 ± 10.3% type 3, and 71.7 ± 10.0% type 4. These proportions of morphological subtypes were

Fig. 4. 7. Effect of additional Mg2+ ion concentration on the morphological response of neutrophils to 50% plasma



significantly different to those observed in plasma alone (X^2= 1131.63), and were also significantly different to those observed in plasma containing 2 mM additional Mg^{2+} ions (X^2= 33.26).

4. 2. 8. Role of magnesium ions during the morphological response of neutrophils in suspension to plasma

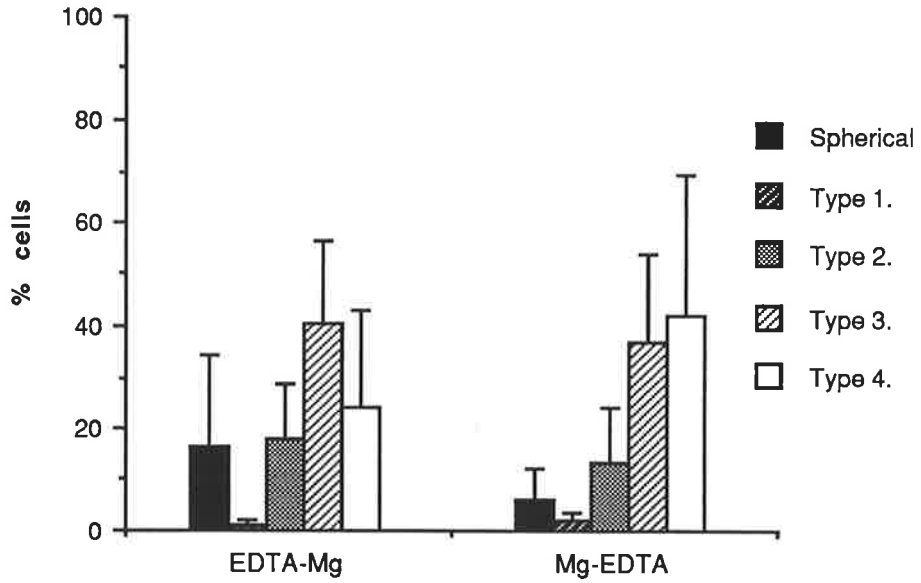
4. 2. 8. 1. Design of experiments

Because additional magnesium ions were found in the preceding experiments to enhance the morphological responses of neutrophils to plasma (i.e. increase the formation of type 4 cells), the aim of this experiment was to investigate whether this effect might be mediated by an interaction between these cations and plasma prior to addition of the cells. If this were to be so, then plasma pre-incubated with Mg^{2+} ions before addition of EDTA, might display a greater activity than plasma treated with EDTA before addition of Mg^{2+}. To test this hypothesis, the proportions of morphological subtypes displayed by neutrophils after suspension for 30 minutes at 37 °C in two different solutions of 50% plasma were compared. The first solution of 50% plasma (EDTA-Mg) was pre-incubated for 10 minutes at 37 °C with 5 mM EDTA, and then 5 mM additional Mg^{2+} ions were added immediately before suspension of cells. The second solution of 50% plasma (Mg-EDTA) was incubated for 10 minutes at 37 °C with 5 mM additional Mg^{2+} ions, and then 5 mM EDTA added immediately before suspension of cells. This experiment was repeated three times.

4. 2. 8. 2. Comparison of responses to EDTA-Mg plasma and Mg-EDTA plasma (Fig. 4. 8.)

The mean (± SD) proportions of morphological subtypes displayed by neutrophils after suspension for 30 minutes at 37 °C in 50% plasma (EDTA-Mg)

Fig. 4. 8. Effect of preincubating plasma with Mg2+ ions before addition of EDTA on the morphological response of neutrophils to 50% plasma. (EDTA-Mg, EDTA added to plasma before Mg2+ ions; Mg-EDTA, Mg2+ ions added to plasma before EDTA).



were: 16.3 ± 17.9% spherical, 1.0 ± 1.0% type 1, 17.7 ± 11.2% type 2, 40.7 ± 15.5% type 3, and 24.3 ± 18.6% type 4.

The proportions of morphological subtypes displayed by neutrophils after suspension for 30 minutes at 37 °C in 50% plasma (Mg-EDTA) were: 6.3 ± 5.9% spherical, 2.0 ± 1.7% type 1, 13.3 ± 11.0% type 2, 36.7 ± 17.4% type 3, and 42.3 ± 26.8% type 4 cells. These proportions of morphological subtypes were significantly different (X^2=21.94) to those displayed by cells suspended in 50% plasma (EDTA-Mg).

4. 2. 9. Effect of soybean trypsin inhibitor (STI) on the morphological response of neutrophils to plasma (Fig. 4. 9.)

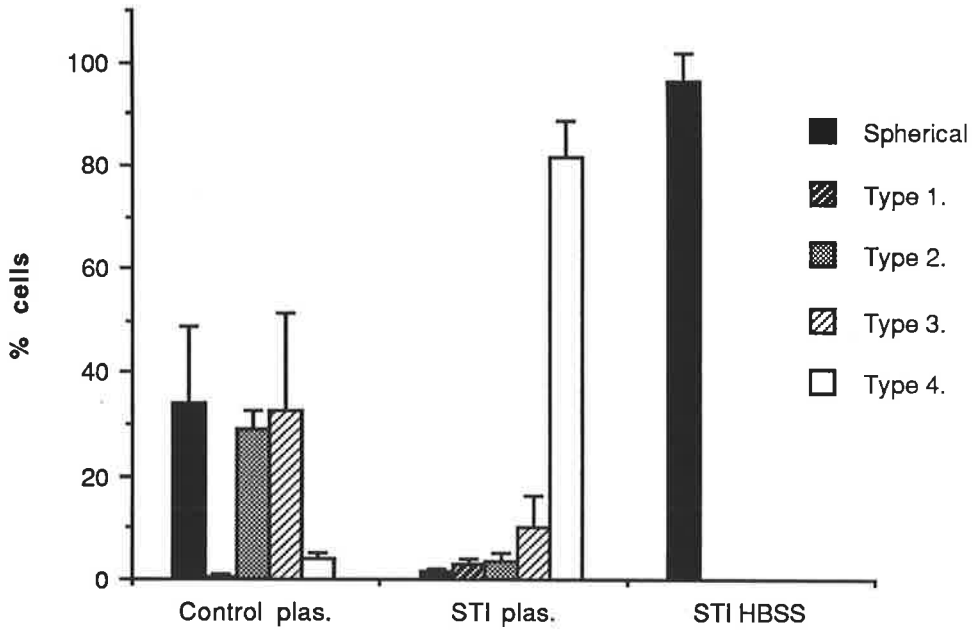
4. 2. 9. 1. Design of experiments

Since kallikrein has been reported to be chemotactic for neutrophils (Kaplan et al., 1972), and this activity is blocked by pretreating this plasma protease with soybean trypsin inhibitor (STI), the aim of this experiment was to indirectly examine the possible role of kallikrein during polarisation of neutrophils in plasma by pretreating plasma with STI. This study was performed (three times) by dividing plasma obtained from heparinised blood into two samples. The first sample was pre-incubated with 0.25 mM STI for 5 minutes at 37 °C, and the second sample was pre-incubated in the absence of STI. Neutrophils prepared from the same donation of blood as plasma were then incubated for 30 minutes at 37 °C in either 50% untreated plasma, 50% STI treated plasma, or HBSS-Hepes containing 0.125 mM STI.

4. 2. 9. 2. Effect of STI pre-treatment on activity of plasma

The mean (± SD) proportions of morphological subtypes displayed by neutrophils after suspension in 50% untreated plasma for 30 minutes were: 34.0 ±

Fig. 4. 9. Effect of soy bean trypsin inhibitor (STI) on the morphological response of neutrophils to heparinised plasma. (Control plas.), 50% plasma; (STI plas.), 50% plasma pre-treated with STI (0.25 mM); (STI HBSS), HBSS-Hepes with 0.125 mM STI



14.9% spherical, 0.3 ± 0.6% type 1, 29.0 ± 3.6% type 2, 32.7 ± 18.7% type 3, and 4.3 ± 0.6% type 4.

The proportions of morphological subtypes displayed by neutrophils after suspension in 50% STI treated plasma were: 1.3 ± 0.6% spherical, 3.3 ± 0.6% type 1, 3.7 ± 1.5% type 2, 10.0 ± 6.1% type 3, and 81.7 ± 6.7% type 4. These proportions of morphological subtypes were significantly different to those observed in untreated plasma (X^2= 1492.48).

4. 2. 9. 3. Effect of STI on morphology of neutrophils suspended in HBSS-Hepes

Cells suspended in HBSS-Hepes displayed no apparent morphological response in the presence of STI as >95% of cells remained spherical.

4. 2. 10. Effect of TMB-8 on the morphological responses of neutrophils to plasma and FMLP

4. 2. 10. 1. Design of experiments

The purpose of this experiment was to investigate the role of intracellular divalent cations during this morphological response by use of the intracellular calcium antagonist, TMB-8. This study was performed by pretreating neutrophils (10^7/ml) for 10 minutes at 37 °C with 5 x 10^{-4} M TMB-8 prior to their suspension (10^6/ml) for 5, 15 and 30 minutes at 37 °C in either 50% plasma with 5 x 10^{-4} M TMB-8 or 10 nM FMLP with 5 x 10^{-4} M TMB-8. Controls were perfomed by suspending untreated cells in 50% plasma and 10 nM FMLP without TMB-8.

Fig. 4. 10. a. Effect of TMB-8 on the morphological response of neutrophils to 50% plasma

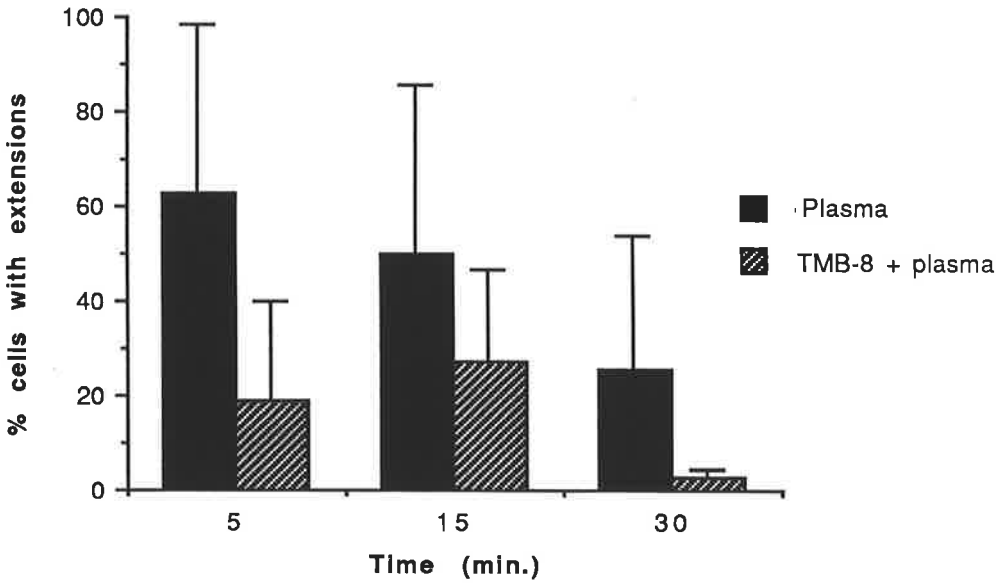
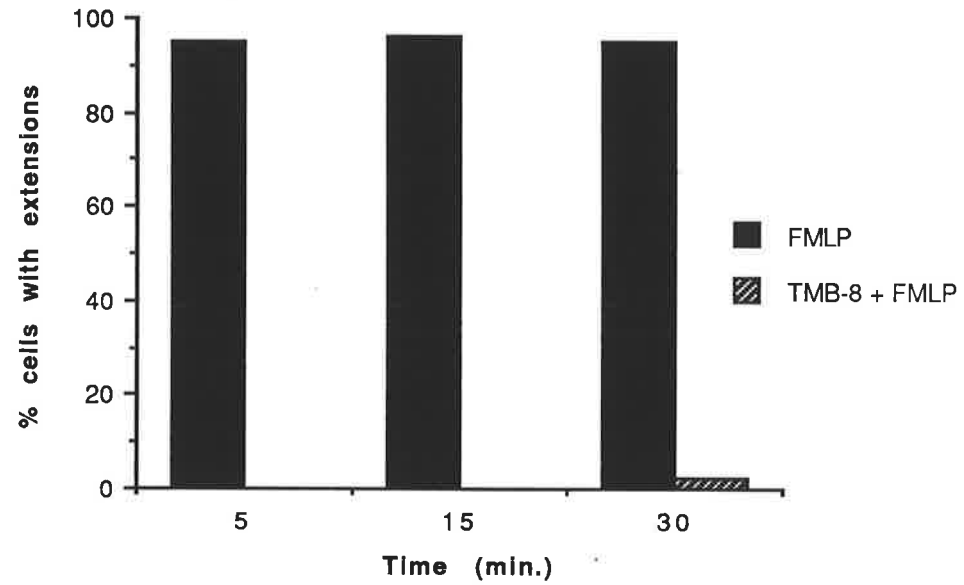


Fig. 4. 10. b. Effect of TMB-8 on the morphological response of neutrophils to 10 nM FMLP



4. 2. 10. 2. Effect of TMB-8 on response to plasma (Fig. 4. 10. a.)

The proportions of neutrophils displaying cytoplasmic extensions after suspension in plasma for 5, 15 and 30 minutes (62.7 ± 35.5%, 50.0 ± 35.6% and 25.3 ± 28.4% respectively) were lower for cells treated with TMB-8 (18.7 ± 21.5%, 27.0 ± 19.5% and 3.0 ± 1.7%).

4. 2. 10. 3. Effect TMB-8 on response to FMLP (Fig. 4. 10. b.)

While >95% of untreated neutrophils displayed cytoplasmic extensions throughout their 30 minutes exposure to FMLP, <5% of TMB-8 treated neutrophils displayed cytoplasmic extensions when stimulated with FMLP.

4. 2. 11. Reversibility of the effect of TMB-8 on the morphological response of neutrophils to FMLP (Fig. 4. 11.)

4. 2. 11. 1. Design of experiments

The purpose of this experiment was to investigate whether the effects of TMB-8 on neutrophil polarisation, documented in the preceding experiment, might be due to a toxic effect on the cells. Neutrophils were suspended (10^6/ml) in HBSS-Hepes with 5 x 10^{-4} M TMB-8 for 10 minutes at 37 °C, washed with HBSS-Hepes and then resuspended (10^6/ml) in HBSS-Hepes containing 10 nM FMLP for 1, 5, 15 and 30 minutes. Controls were cells pre-incubated for 10 minutes in HBSS-Hepes or HBSS-Hepes containing 5 x 10^{-4} M TMB-8, followed by immediate stimulation with 10 nM FMLP for 1, 5, 15 and 30 minutes.

4. 2. 11. 2. Effect of washing on the response of TMB-8 treated neutrophils to FMLP

Cells pretreated with TMB-8 remained >95% spherical when immediately stimulated with FMLP. In contrast, <5% of 'TMB-8 treated and washed' cells remained spherical when stimulated with FMLP. This morphological response of washed cells appeared similar to that of untreated cells towards FMLP, but was not analysed in more detail.

4. 2. 12. Effect of TMB-8 on the morphological response of neutrophils to FMLP in the presence of human serum albumin (Fig. 4. 12.)

4. 2. 12. 1. Design of experiments

Because TMB-8 was found in an earlier experiment to inhibit the morphological response of neutrophils to FMLP to a greater degree than that induced by plasma, it was considered that the effect of TMB-8 on neutrophil polarisation might be reduced in the presence of plasma proteins. To test this hypothesis neutrophils were pretreated with TMB-8 as described above and then suspended for 30 minutes in 10 nM FMLP/5 x 10^{-4} M TMB-8 containing either 0, 0.5, 1, 2 or 4% (w/v) human serum albumin (HSA). Controls were perfomed by suspending untreated cells in HBSS-Hepes, HBSS-Hepes with 4% HSA, 10 nM FMLP and 10 nM FMLP with 4% HSA.

4. 2. 12. 2. Effect of TMB-8 in the presence of human serum albumin

The inhibitory effects of TMB-8 treatment on the morphological response of neutrophils in suspension to FMLP were reduced in a concentration dependent manner by inclusion of human serum albumin in the suspension medium. The highest

Fig. 4. 11. Reversibility of effect of TMB-8 on the morphological response of neutrophils to FMLP

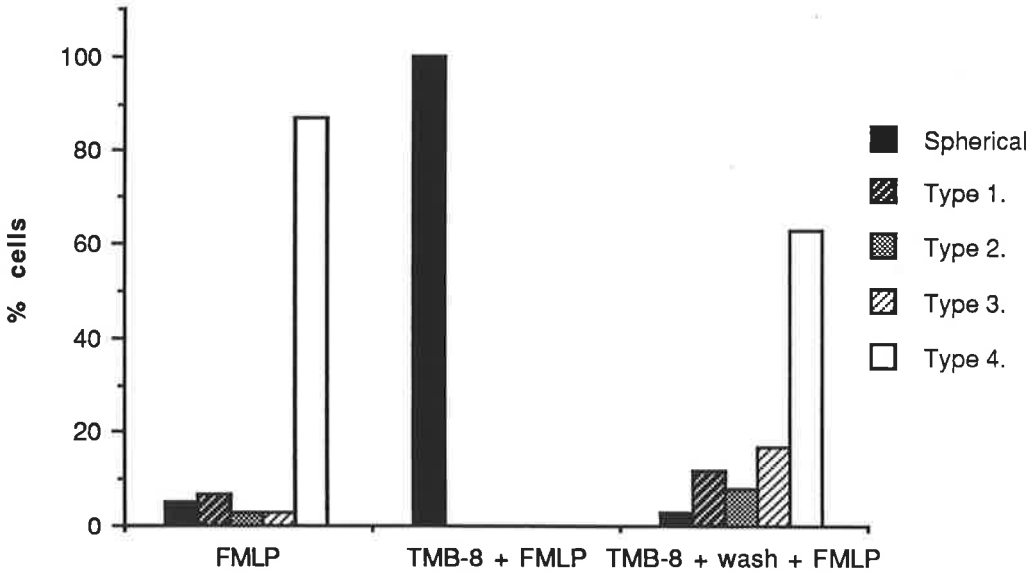
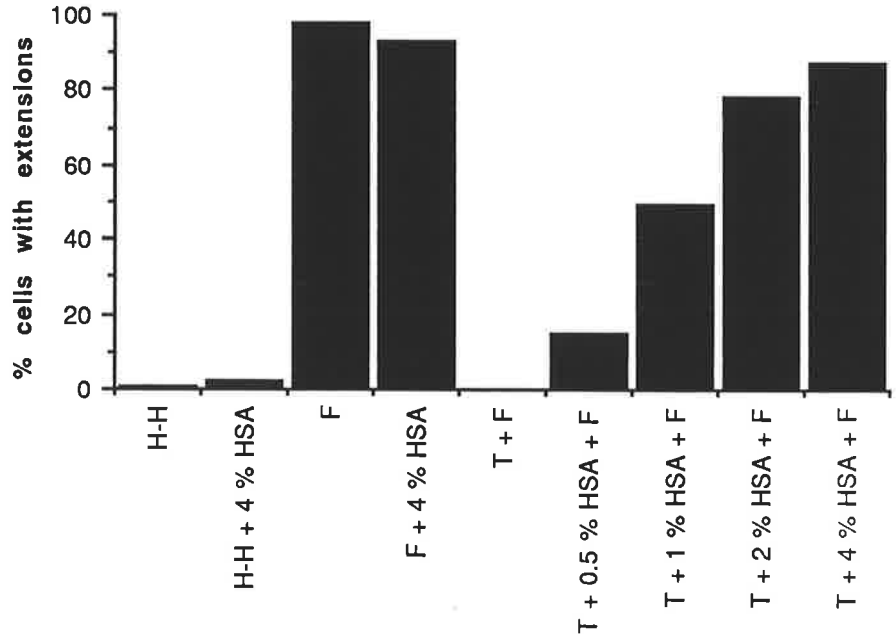


Fig. 4. 12. Effect of TMB-8 (T) on the morphological response of neutrophils suspended in HBSS-Hepes (H-H) to FMLP (F), in the presence of various concentrations of human serum albumin (HSA)



concentration of HSA used (4% w/v) did not affect the morphology of cells suspended in either HBSS-Hepes or 10 nM FMLP.

4. 2. 13. Effects of chromatographically purified fractions of plasma and serum on the morphology of neutrophils in suspension

4. 2. 13. 1. Design of experiments

The purpose of these experiments was to characterise the factor(s) responsible for inducing polarisation of neutrophils in plasma and serum with regard to their molecular weight. This analysis was achieved by separating plasma and serum into several fractions by molecular sieve chromatography on Sephacryl 300 (see section 2. 2. 11.). Morphological responses of neutrophils to plasma and serum fractions were assessed by calculating the proportion of cells displaying cytoplasmic extensions after incubation in HBSS-Hepes at 37 °C for 30 minutes. The effects of additional Mg^{2+} ions ($MgCl_2$) and/or EDTA on responses to active fractions were examined at concentrations of 5 mM for plasma studies and 10 mM for serum studies. Fractions from three donors were tested for both plasma and serum studies. Fractions were usually tested on cells from a different individual, but one experiment was perfomed using autologous cells and fractions for plasma and serum studies.

4. 2. 13. 2. Sephacryl 300 separation of plasma (Fig. 4. 13. a)

Plasma was eluted from Sephacryl 300 as four major protein peaks: (1) a 'pre IgG' peak, (2) an 'IgG' peak, (3) an 'albumin' peak, and (4) a 'low molecular weight' peak (containing proteins of <10 kDa). Fractions pooled from either the 'pre IgG' peak, the 'IgG' peak, or the 'low molecular weight' peak induced morphological responses, but cells suspended in fractions pooled from the 'albumin' peak remained predominantly spherical (>95% spherical). The proportion of cells displaying

Fig. 4. 13. a. Human plasma fractionated on Sephacryl 300

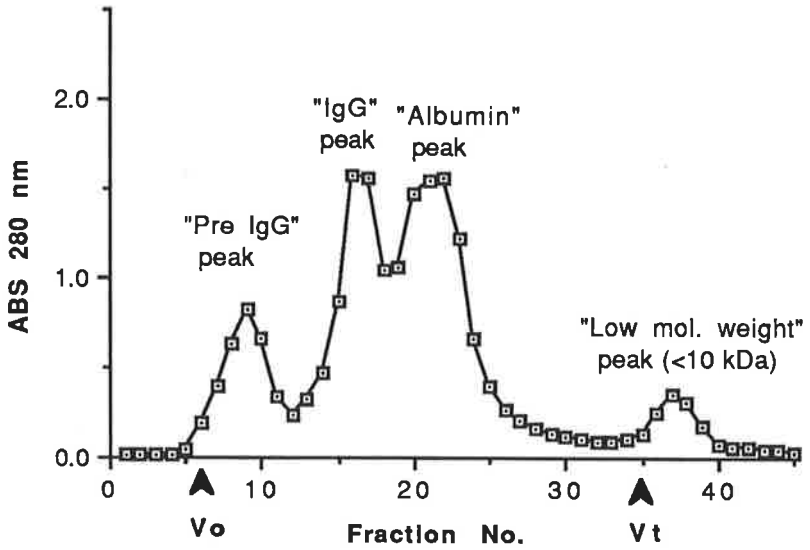
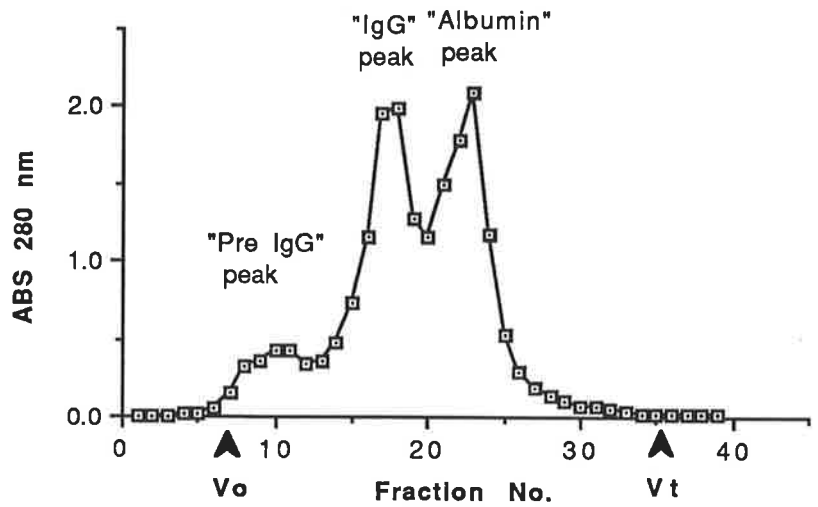


Fig. 4. 13. b. Human serum fractionated on Sephacryl 300



extensions of the cytoplasm was consistently between 50 and 95% for the 'pre IgG' peak, >95% for the 'IgG peak' and between 30% and 90% for the 'low molecular weight' peak. Responses to the 'pre IgG' pooled fractions were reduced by approximately 50% in the presence of EDTA, but only in two out of three experiments. This inhibitory effect of EDTA was not reduced in the presence of additional Mg^{2+} ions. Responses to the 'IgG peak' fraction were consistently unaffected by additional Mg^{2+} ions and/or EDTA. Responses to the 'low molecular weight' peak fraction were consistently reduced by 35% to 85% in the presence of EDTA. This inhibitory effect of EDTA was reduced in two out of three experiments in the presence of additional Mg^{2+} ions.

4. 2. 13. 3. Sephacryl 300 separation of serum (Fig. 4. 13. b.)

Serum was eluted from Sephacryl 300 as three major protein peaks: (1) a 'pre IgG' peak, (2) an 'IgG' peak, and (3) an 'albumin' peak, but a low molecular weight peak similar to that observed for plasma was not observed. Fractions pooled from the 'pre IgG' peak consistently induced neutrophils to develop cytoplasmic extensions, but fractions pooled from the 'IgG' peak induced neutrophils to produce extensions in only one experiment. Cells suspended in fractions pooled from the 'albumin' peak remained predominantly spherical (>95% spherical). Responses to the 'pre IgG' peak fraction were reduced by 40% to 80% in the presence of EDTA. This inhibitory effect of EDTA was enhanced in the presence of additional Mg^{2+} ions. The effects of EDTA and additional Mg^{2+} ions on the activity of the 'IgG' peak fractions observed in one experiment were not examined.

4. 2. 14. Effect of commercial preparations of plasma proteins on the morphology of neutrophils in suspension

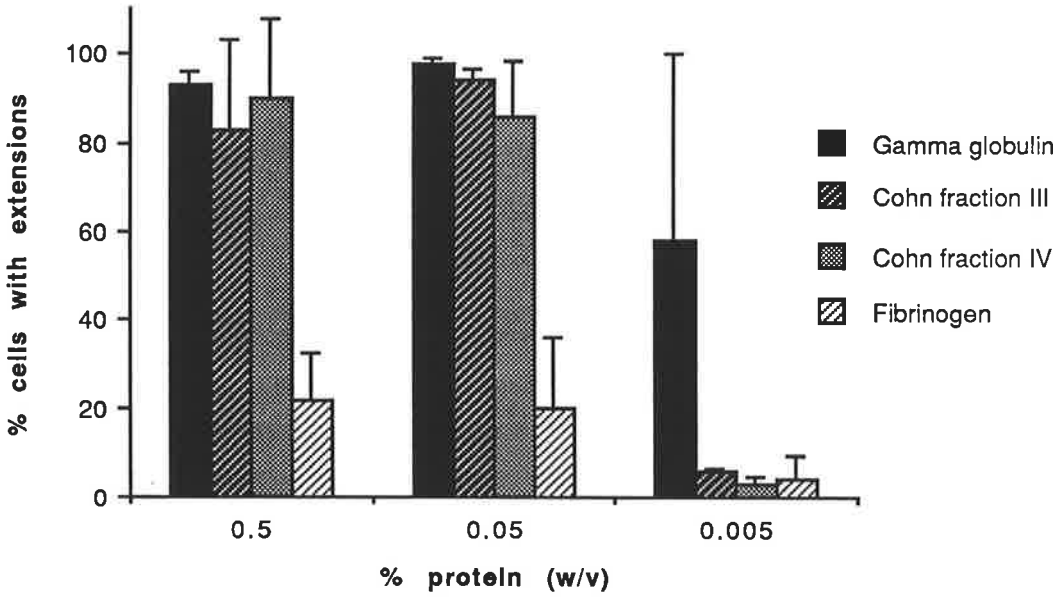
4. 2. 14. 1. Design of experiments

The aim of these experiments was to determine whether or not commercial preparations of various plasma proteins stimulate polarisation of neutrophils in cell suspensions. Neutrophils were suspended for 30 minutes at 37 °C in solutions of commercially-prepared ethanol-precipitated fractions of human plasma proteins dissolved in HBSS-Hepes (pH 7.2). The preparations tested were; gamma globulins (predominantly immunoglobulins; 0.5%, 0.05% and 0.005% w/v), Cohn fractions III and IV (0.5%, 0.05% and 0.005%, w/v), fibrinogen (0.5%, 0.05% and 0.005% w/v), albumin (0.5%, w/v) and fibronectin (0.01%, w/v). All responses were assessed by measuring the proportion of cells displaying cytoplasmic extensions.

4. 2. 14. 2. Responses to commercial preparations of plasma proteins (Fig. 4. 14.)

Large numbers of neutrophils suspended in 0.5% and 0.05% gamma globulin displayed extensions of the cytoplasm (92.8 ± 3.3% and 97.7 ± 1.2% respectively) and moderate numbers were observed in 0.005% gamma globulin (57.7 ± 42.1%). Cohn fractions III and IV induced formation of large numbers of cells with cytoplasmic extensions at 0.5% (83.0 ± 19.8% and 90.0 ± 17.7% respectively) and 0.05% (94.3 ± 2.3% and 85.7 ± 12.7% respectively) but did not alter the morphology of spherical cells at 0.005% (5.7 ± 0.6% and 3.0 ± 2.0% respectively). Small morphological responses were observed for cells suspended in 0.5% and 0.05% fibrinogen (21.7 ± 10.7% and 20.3 ± 15.5% respectively) but not for those suspended in 0.005% fibrinogen (4.0 ± 5.2%).

Fig. 4. 14. Effect of commercial preparations of plasma proteins of the morphology of neutrophils in suspension



Cells suspended in 0.5% human serum albumin or 0.01% fibronectin remained predominantly (>95%) spherical.

4. 2. 15. Effect of plasma on the F-actin distribution of neutrophils in suspension

4. 2. 15. 1. Design of experiments

Neutrophils were suspended in 50% plasma for 1, 15 and 30 minutes before being fixed with 8% formalin and stained with the fluorescent F-actin probe, rhodamine phalloidin. The distribution of staining for F-actin within the cytoplasm of cells with each morphological subtype was then examined by fluorescence microscopy.

4. 2. 15. 2. F-actin distribution of cells

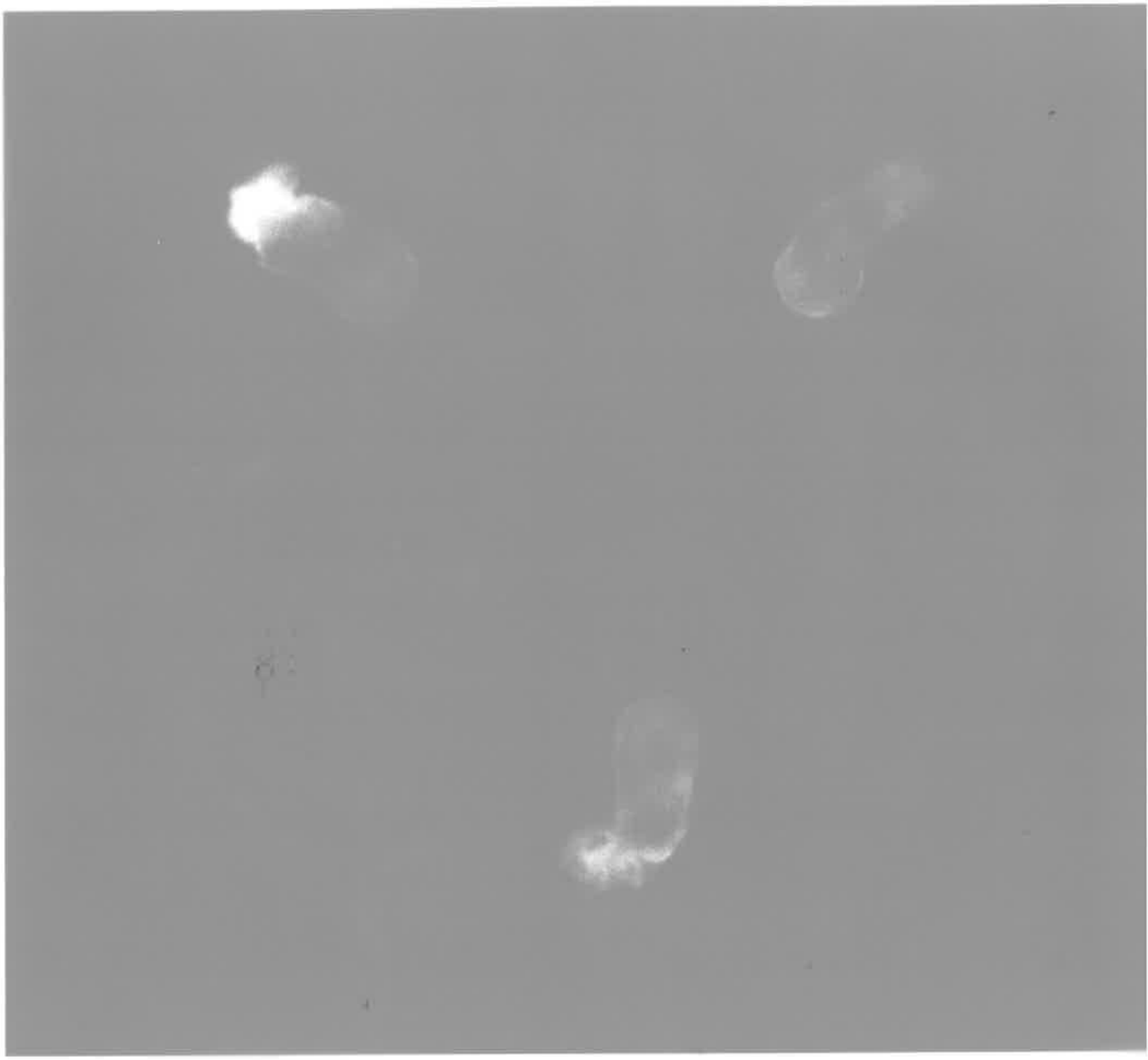
Cells suspended in plasma generally displayed intense staining for F-actin within their extensions and weak staining within their bodies in a similar manner to cells treated with FMLP (see previous chapter, Fig. 3. 5.). However, in a considerable number of cells, moderate to weak staining was observed throughout the entire cell even when marked polarisation was evident (Fig. 4. 15.). These poor staining cells appeared to be more apparent at later time intervals (especially 30 minutes) as the proportion of spherical cells increased.

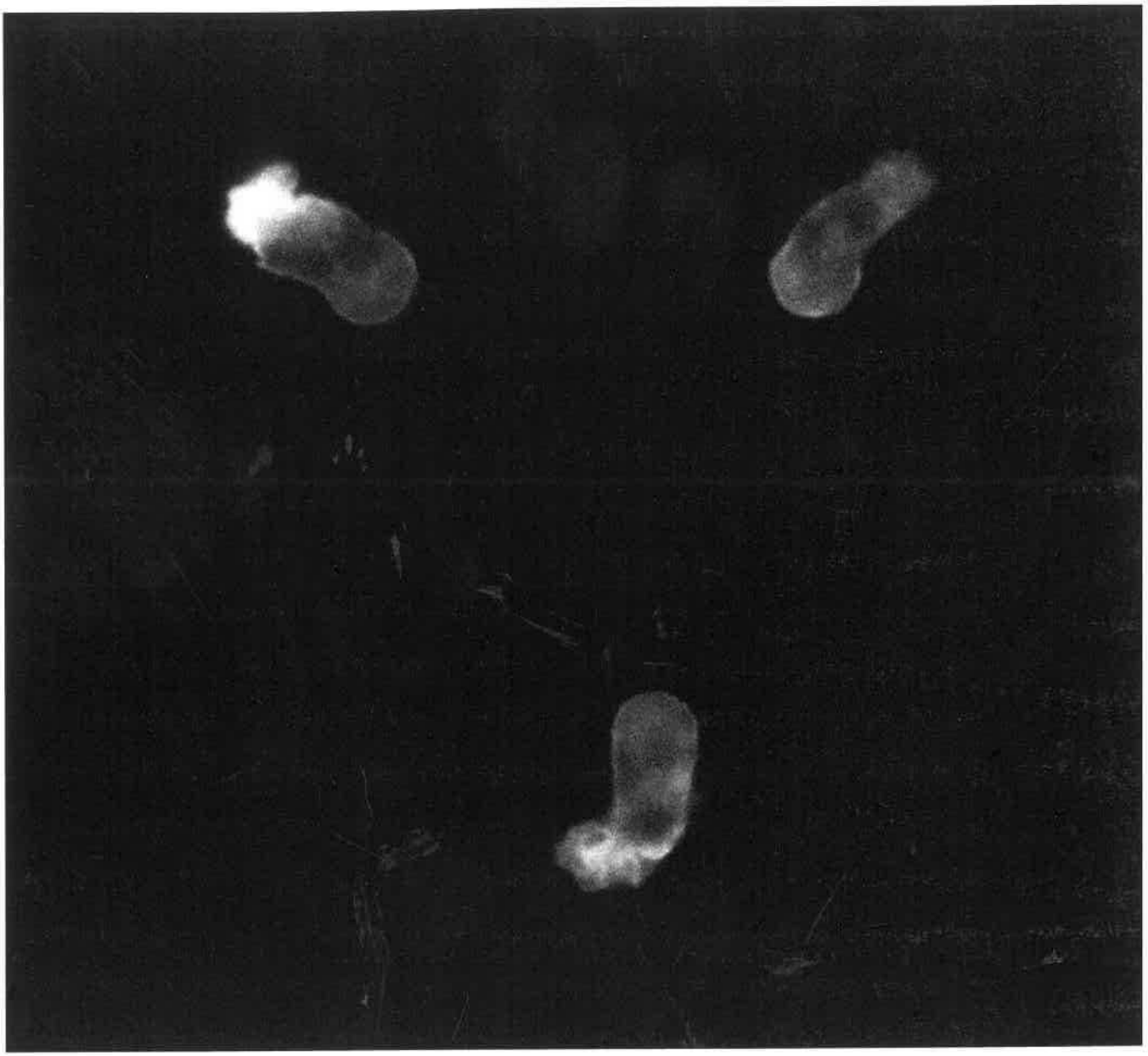
4. 3. Summary

Standard preparations of heparinised (12.5 I.U./ml) plasma (10%, 50% and 90% v/v, but not 1%) induced neutrophils to develop cytoplasmic extensions within 30 seconds which were maintained after 60 minutes, with highest numbers being maintained in 50% plasma. These cytoplasmic extensions enabled visual

Fig. 4. 15. F-actin distributions for neutrophils suspended in plasma.

Top left, polarised cell with intensely staining pseudopod. Top right and bottom, polarised cells with moderate to weak staining throughout cytoplasm. (fluorescence microscopy; approximately 2000 X).





classification of cells into morphological subtypes 1, 2, 3 and 4. These subtypes appeared in a similar order to that displayed during responses to FMLP with type 1 cells appearing early and type 4 cells appearing last, but type 4 cells were rarely observed even in 50% plasma. However, when the concentration of heparin used as anticoagulant was lowered to 5 I.U/ml or serum was used (i.e. no anticoagulant added) neutrophils often developed a type 4 morphology. In contrast, the ability of neutrophils to develop a type 4 morphology in response to FMLP (10 nM) was unaffected by heparin (5-100 I.U/ml).

Plasma (50%) anticoagulated with EGTA (5 mM) also induced many neutrophils to develop a type 4 morphology, but plasma anticoagulated with EDTA (5 mM) or disodium hydrogen citrate (4 mg/ml) induced little or no change in cell shape. However, all three cation chelating agents (EDTA, EGTA and citrate; 5 mM) reduced the morphological response of neutrophils to standard preparations of heparinised plasma with EDTA having the greatest effect. These inhibitory effects of chelating agents were reduced or totally abolished in the presence of additional Ca^{2+} or Mg^{2+} ions. In contrast, the responses of neutrophils to 100 and 1 nM FMLP (but not 10 nM FMLP) were slightly enhanced (i.e. more type 4 cells) in the presence of cation chelating agents.

Addition of extra Mg^{2+} ions (0.5 to 5 mM) to standard preparations of heparinised plasma (50%) enhanced the morphological response of neutrophils to this medium with many type 4 cells being produced in a concentration dependent manner. Furthermore, plasma pre-incubated with additional Mg^{2+} ions (5 mM) followed by addition of EDTA (5 mM) induced greater polarisation of neutrophils (i.e. more type 4 cells) than plasma pre-incubated with EDTA before addition of Mg^{2+} ions.

Pre-treatment of standard plasma with soy bean trypsin inhibitor (0.25 mM; STI) increased the polarisation response of neutrophils to this medium, with a high proportion of type 4 cells being formed. However, STI (0.125 mM) had no effect on the morphology of cells suspended in HBSS-Hepes.

Pre-treatment of neutrophils with TMB-8 (5 x 10^{-4} M) reduced their response to 50% plasma and abolished their response to FMLP (10 nM). The inhibitory effect of TMB-8 on the response to FMLP was reversed by washing cells in fresh HBSS-Hepes and was reduced in a concentration dependent manner by including human serum albumin in the suspension medium.

Neutrophils polarised while suspended in plasma consistently displayed poor staining for F-actin within their cell-bodies, even when elongated. However, the intense staining associated with extensions of the cytoplasm became less apparent after 15 minutes, even in those cells which continued to display marked polarisation.

Chromatographically purified fractions of plasma containing proteins of high molecular weight similar to and greater than that of immunoglobulin type G, induced neutrophils to develop cytoplasmic extensions. Responses to these fractions were not consistently reduced by EDTA. However, a fraction of plasma containing proteins of low molecular weight (≤10 kDa) induced neutrophils to develop cytoplasmic extensions and this response was reduced by chelation of extracellular Mg^{2+} ions. Nevertheless, fractionation of serum revealed activity only in fractions containing of proteins of high molecular weight.

Commercial fractions of plasma proteins induced neutrophils to develop cytoplasmic extensions with fractions containing immunoglobulin having the greatest potency (active at 0.005% w/v). Since IgG is the primary immunoglobulin present in human plasma and is a known stimulus of neutrophils, the next chapter was devoted to a detailed study of the effects of a purified preparation of this protein on the morphology of neutrophils in suspension.

CHAPTER 5.

THE MORPHOLOGICAL RESPONSE OF NEUTROPHILS IN SUSPENSION TO IMMUNOGLOBULIN TYPE G

5. 1. Introduction

In the preceding chapter, fractions of plasma proteins containing immunoglobulin type G (IgG) were found to stimulate changes in the morphology of neutrophils, but the role of IgG in this response was unknown. In this chapter, the effects of purified preparations of IgG on the morphology of neutrophils in suspension are examined with regard to the time course and type of shape changes induced, and the concentration and form of IgG used. Furthermore, the roles of membrane receptors, divalent cations and F-actin during responses to IgG are assessed.

5. 2. Results

5. 2. 1. Preliminary studies of the morphological response of neutrophils in suspensions to purified IgG

5. 2. 1. 1. Design of experiments

The purpose of these preliminary experiments was to establish whether neutrophils display a morphological response to purified IgG in cell suspensions. Furthermore, since only polymeric molecules (i. e. aggregates or immune complexes) of IgG have been reported to bind to normal human neutrophils it was necessary to examine the role of these molecules during any responses observed. This study was performed by suspending neutrophils in 0.005%, 0.0005% and 0.00005% (w/v in HBSS-Hepes) solutions of purified IgG for 0.5, 1, 5, 15 and 30 minutes at 37 °C. The role of polymeric IgG was assessed by comparing responses of cells to equivalent concentrations of normal and ultracentrifuged preparations of IgG. Ultra-centrifugation was used to reduce the quantity of any polymeric IgG present in the suspension medium. The quantities of polymeric IgG present in normal and ultracentrifuged preparations was assessed by molecular sieve chromatography on a Sephacryl 300 column. This experiment was performed only once and all morphological responses were assessed by visual classification of morphological subtypes.

5. 2. 1. 2. Responses to commercial preparations of IgG (Fig. 5. 1. a.)

Neutrophils suspended in 0.005% IgG remained > 95% spherical at 0.5 minutes and 1 minute, but at 5 minutes only 27% of cells remained spherical. Furthermore, < 5% of cells remained spherical at 15 and 30 minutes. Cells

displaying extensions of the cytoplasm were therefore not present in sufficient quantity to be classified into types 1, 2, 3, and 4 at 0.5 minutes and 1 minute but were so at later time intervals. The number of type 1 cells produced rose from a minimum of 3% at 5 minutes to a maximum of 35% at 15 minutes then declined to 16% at 30 minutes. The number of type 2 cells produced declined from a maximum of 67% at 5 minutes to 49% at 15 minutes then rose to 52% at 30 minutes. The number of type 3 cells produced gradually rose from a minimum of 3% at 5 minutes to a maximum of 26% by 30 minutes. The number of type 4 cells produced remained ≤1% for the entire experiment.

Neutrophils suspended in 0.0005% IgG remained > 95% spherical at 0.5 minutes and 1 minute, but at 5 minutes only 86% of cells remained spherical. Furthermore, only 47% of cells remained spherical at 15 and 30 minutes. Cells displaying extensions of the cytoplasm were therefore not present in sufficient quantity to be classified into types 1, 2, 3, and 4 at 0.5 minutes and 1 minute but were so at later time intervals. The number of type 1 cells produced rose from a minimum of 1% at 0.5 minutes to a maximum of 7% at 15 and 30 minutes. The number of type 3 cells produced rose from a minimum of 13% at 0.5 minutes to a maximum of 46% at 15 and 30 minutes. Cell types 3 and 4 were not observed at any time.

Neutrophils suspended in 0.00005% IgG remained >95% spherical throughout the experiment.

5. 2. 1. 3. Responses to ultra-centrifuged commercial preparations of IgG (Fig. 5. 1. b.)

Neutrophils suspended in 0.005% ultracentrifuged IgG remained >95% spherical at 0.5, 1 and 5 minutes, but at 15 minutes only 24% of cells remained spherical. Furthermore, only 5% of cells remained spherical at 30 minutes. Cells displaying extensions of the cytoplasm were therefore not present in sufficient

Fig. 5. 1. a. Time course of the morphological response of neutrophils in suspension to 0.005% (w/v) IgG

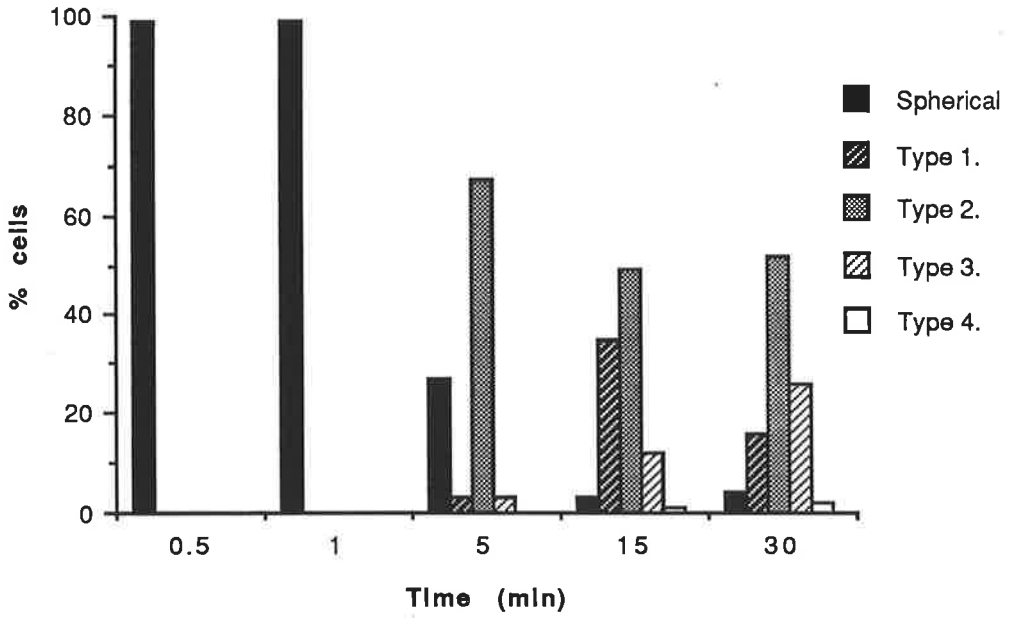


Fig. 5. 1. b. Time course of the morphological response of neutrophils in suspension to 0.005% (w/v) ultra-centrifuged IgG

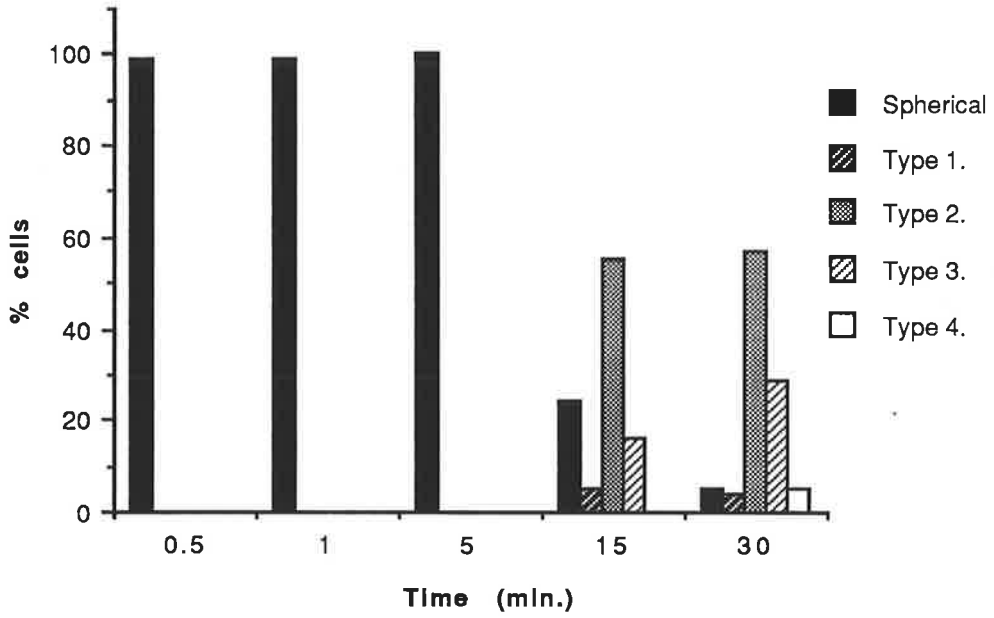


Fig. 5. 2. a. Commercial preparation of IgG fractionated on Sephacryl 300

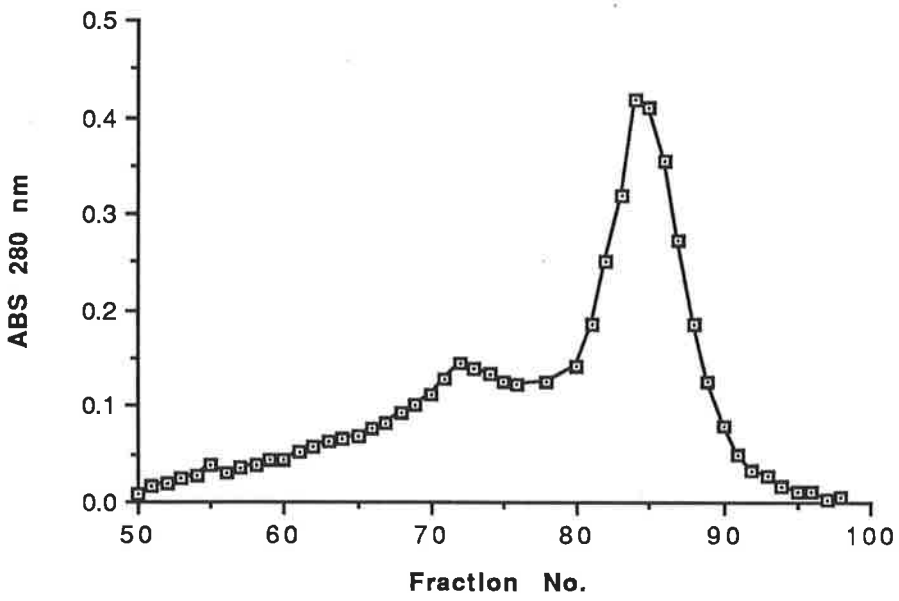
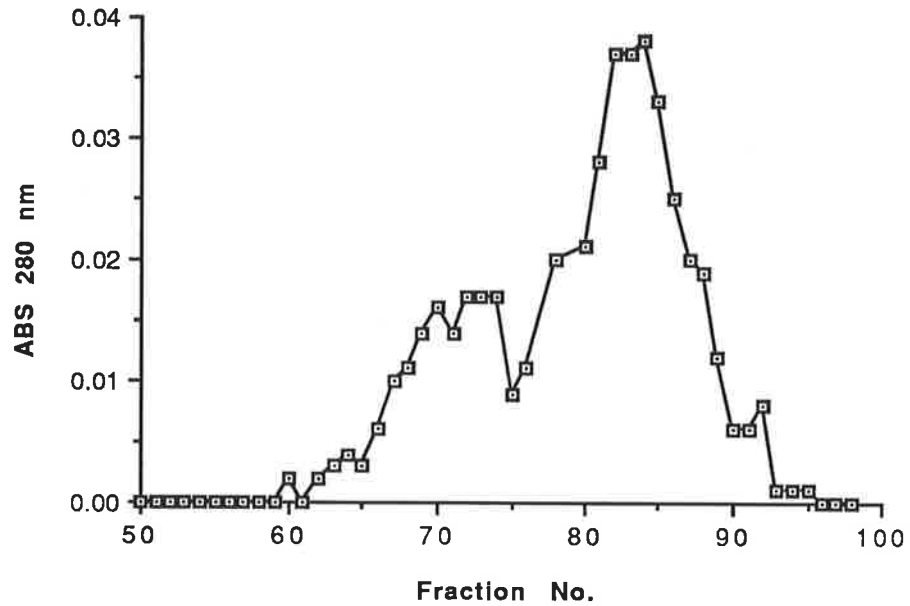


Fig. 5. 2. b. Ultra-centrifuged commercial preparation of IgG fractionated on Sephacryl 300



quantity to be classified into types 1, 2, 3, and 4 at 0.5 ,1 and 5 minutes but were so at 15 and 30 minutes. Over this time, the number of type 1 cells remained ≤5%, the number of type 2 cells rose slightly from 55% and 57%, the number of type 3 cells produced rose from 16% to 29% and the number of type 4 cells produced rose from 0% to 5%.

Neutrophils suspended in 0.0005% (w/v) and 0.00005% (w/v) ultracentrifuged IgG, remained >95% spherical throughout the experiment.

5. 2. 1. 4. Chromatographic analysis of normal and ultracentrifuged preparations of IgG

Chromatographic analysis of a 0.5% solution of normal IgG (Fig. 5. 2. a.) revealed a broad elution peak followed closely by a narrower and higher elution peak of lower molecular weight material. Chromatographic analysis of a 0.1% solution of ultra-centrifuged IgG (Fig. 5. 2. b.) revealed two elution peaks similar to that observed for the normal preparation, but the peak corresponding to material of higher molecular weight was less broadly distributed than before.

5. 2. 2. Morphological responses of neutrophils in suspension to Fab and Fc fragments of IgG

5. 2. 2. 1. Design of experiments

The aim of this experiment was to establish whether sub-components of the IgG molecule are capable of inducing neutrophil polarisation. This was achieved by examining the morphological responses of neutrophils to Fc and Fab fragments of IgG purified from a papain digest of IgG. In the first experiment, Fc and Fab fragments were purified by molecular sieve chromatography on Sephacryl 300 followed by ion exchange chromatography on CM-cellulose. In the second experiment an Fc fraction was further purified by ion exchange chromatography on DEAE-cellulose. The effects

of purified fragments on the morphology of neutrophils in suspension were assessed by incubating neutrophils for 30 minutes at 37 °C in solutions of Fc and Fab fragments prepared in HBSS-Hepes.

5. 2. 2. 2. Response to Fc fragments of IgG

Initial preparations of Fc fragments induced extensions of the cytoplasm on 30.2% of neutrophils after 30 minutes suspension in a 0.05% (w/v). However, when neutrophils were suspended in solutions of Fc fragments (1.0%, 0.1% and 0.01% w/v) which had been further purified by ion exchange on DEAE-cellulose, >95% of cells remained spherical.

5. 2. 2. 3. Response to Fab fragments of IgG

Greater than 95% of neutrophils remained spherical when suspended in a 0.05% (w/v) solution of Fab fragments.

5. 2. 3. Morphological responses of neutrophils to various concentrations of heat aggregated IgG

5. 2. 3. 1. Design of experiments

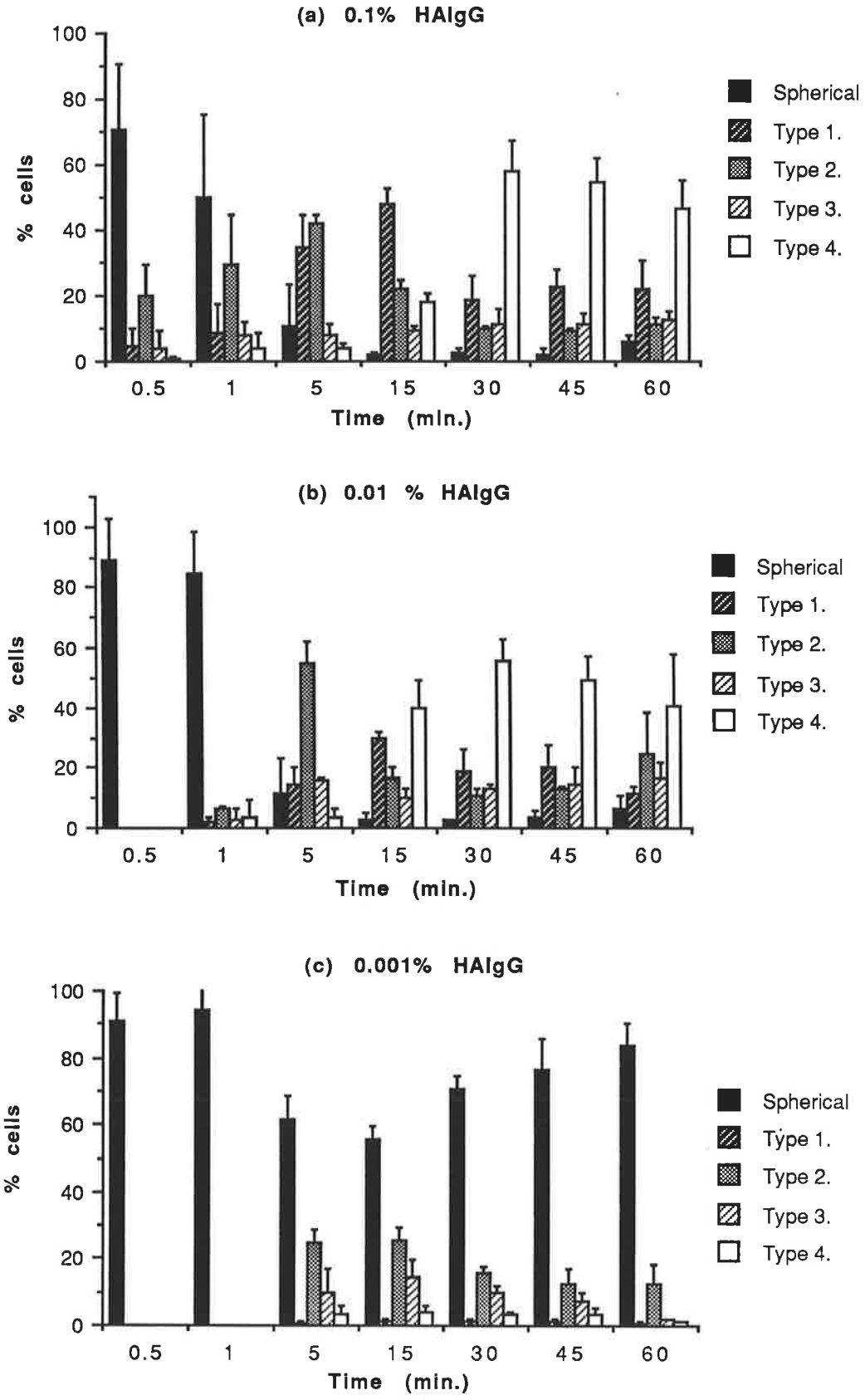
Since heat aggregated IgG (HAIgG) has been routinely used to stimulate activities of neutrophils and the results of the preceding studies suggest that aggregates of IgG are necessary for the morphological response of neutrophils to this immunoglobulin, the aim of these experiments was to examine the morphological response of neutrophils to HAIgG. This study was perfomed by suspending neutrophils for 0.5, 1, 5, 15, 30, 45 and 60 minutes in 0.1%, 0.01%, 0.001%, and 0.0001% (w/v) solutions of HAIgG in HBSS-Hepes at 37 °C. To examine the possibility that

responses to HAIgG might be due to a non-specific effect of heat denatured protein, a control was performed by suspending cells for 30 minutes in 1.0% (w/v) heated and 1.0% (w/v) un-heated human serum albumin (HSA). All morphological responses were assessed by visual classification and the results are expressed as the mean ± standard deviation for the proportions of morphological subtypes obtained from three experiments. Cells from a different donor were used for each experiment.

5. 2. 3. 2. Morphological response to 0.1% HAIgG (Fig. 5. 3. a.)

Neutrophils suspended in 0.1% HAIgG initially displayed a weak morphological response with 70.7 ± 20.0% of cells retaining a spherical shape at 0.5 minutes after commencing incubation. Nevertheless, the number of spherical cells gradually declined with time to below <5% by 15 minutes and remained close to this value for the duration of the experiment. The number of type 1 cells produced gradually rose from a minimum of 4.7 ± 5.0% at 0.5 minutes to a maximum of 48.0 ± 4.6% by 15 minutes then declined to approximately 20% for the remainder of the experiment. The number of type 2 cells produced gradually rose from 20.0 ± 9.6% at 0.5 minutes to a maximum of 42.0 ± 2.6% by 5 minutes then gradually declined to approximately 10% by 30 minutes and remained at this level for the duration of the experiment. The number of type 3 cells produced gradually rose from a minimum of 3.7 ± 5.5% at 0.5 minutes to a maximum of 12.7 ± 2.5% by 60 minutes. The number of type 4 cells produced gradually rose from a minimum of 0.7 ± 0.6% at 0.5 minutes to a maximum of 57.7 ± 9.7% by 30 minutes then gradually declined to 47.0 ± 8.5% by 60 minutes.

Fig. 5. 3. Time courses of the morphological responses of neutrophils in suspension to various concentrations of heat aggregated IgG (HAIgG)



5. 2. 3. 3. Morphological response to 0.01% HAIgG (Fig. 5. 3. b.)

Neutrophils suspended in 0.01% HAIgG initially displayed a weak morphological response with 89.3 ± 13.4% of cells retaining a spherical shape at 0.5 minutes after commencing incubation and 84.7 ± 13.7% at 1 minute. Nevertheless, the number of spherical cells declined to 11.7 ± 11.5% at 5 minutes and remained approximately ≤5% for the remainder of the experiment. Cell types 1, 2, 3 and 4 were not consistently produced in sufficient quantities to be individually quantified after 0.5 minutes, but were so at later time intervals. The number of type 1 cells produced gradually rose from a minimum of 2.0 ± 1.7% at 1 minute to a maximum of 30.0 ± 1.7% by 15 minutes, then eventually declined to 11.7 ± 2.3% by 60 minutes. The number of type 2 cells produced rose from a minimum of 6.3 ± 1.2% at 1 minute to a maximum of 54.7 ± 7.5% at 5 minutes then gradually declined to 10.7 ± 2.1% by 30 minutes before rising to 24.7 ± 13.6% at 60 minutes. The number of type 3 cells produced rose from 2.7 ± 3.8% at 1 minute to 16.0 ± 1.0% at 5 minutes then declined to 10.3 ± 2.9% at 15 minutes before gradually rising to a maximum of 16.7 ± 5.1% by 60 minutes. The number of type 4 cells produced gradually rose from a minimum of 3.3 ± 5.8% at 0.5 minutes to a maximum of 55.7 ± 7.4% by 30 minutes then gradually declined to 40.7 ± 16.9% by 60 minutes.

5. 2. 3. 4. Morphological response to 0.001% HAIgG (Fig. 5. 3. c.)

When neutrophils were suspended in 0.001% HAIgG, the number of spherical cells remained >90% for the first minute, then gradually declined to a minimum of 55.3 ± 4.5% by 15 minutes before gradually rising to 83.7 ± 6.4% by 60 minutes. Cell types 1, 2, 3 and 4 were not consistently produced in sufficient quantities to be individually quantified at 0.5 minutes and 1 minute, but were so at later time intervals. The number of type 1 cells produced remained <2% for the entire

experiment. The number of type 2 cells rose from 25.0 ± 4.0% at 5 minutes to a maximum of 25.3 ± 4.0% at 15 minutes then gradually declined to a minimum of 12.7 ± 5.8% by 60 minutes. The number of type 4 cells remained <5% for the entire experiment.

5. 2. 3. 5. Morphological response to 0.0001% HAIgG

Neutrophils suspended in either 0.0001% (w/v) HAIgG, remained >95% spherical throughout the duration of the experiment.

5. 2. 3. 6. Morphological responses to normal and heated HSA

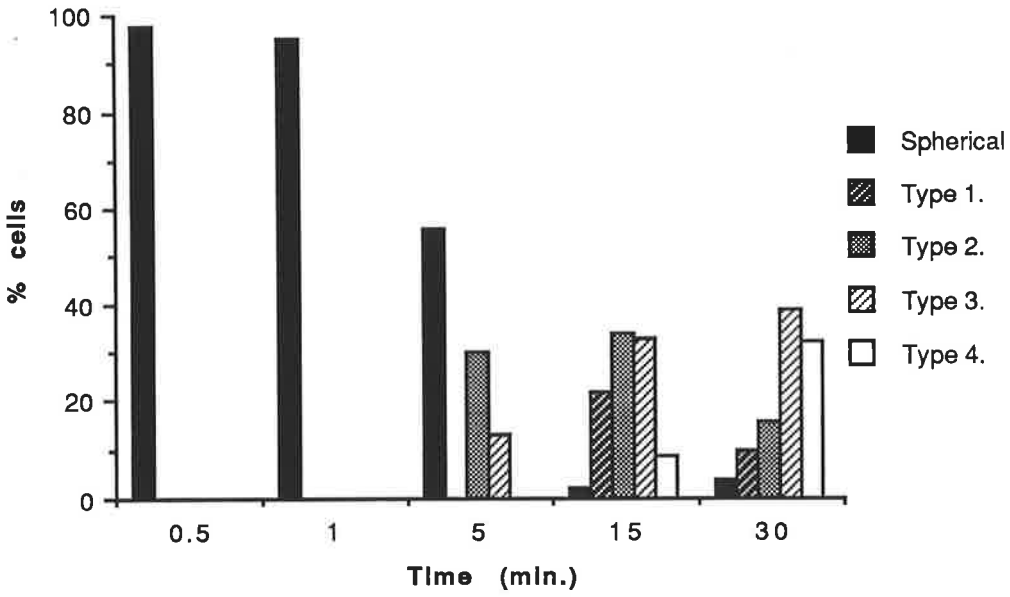
Neutrophils suspended in either 1.0% (w/v) HSA or 1.0% (w/v) heated HSA, remained >95% spherical throughout the duration of the experiment.

5. 2. 4. Investigation of delay period during the morphological response of neutrophils to IgG I: response to different commercial preparation

5. 2. 4. 1. Design of experiment

The purpose of the following experiment was to confirm the existence of a delay period during the morphological response of neutrophils to IgG by using a different commercial preparation of immunoglobulin to that used in the preceding studies. Neutrophils were suspended for 0.5, 1, 5, 15 and 30 minutes at 37 °C in a 0.01% (w/v) solution of IgG which was prepared from a 20% solution obtained from the Commonwealth Serum Laboratories (CSL). The morphological response of neutrophils at each time point was assessed by visual classification of morphological subtypes displayed.

Fig. 5. 4. Time course of the morphological response of neutrophils in suspension to 0.01% IgG ('CSL preparation')



5. 2. 4. 2. Response of neutrophils to CSL preparation of IgG (Fig. 5. 4.)

Neutrophils suspended in 0.01% (w/v) IgG (CSL) did not display a morphological response after 0.5 minutes incubation or at 1 minute, but at 5 minutes only 56% of cells remained spherical and at 15 and 30 minutes <5% of cells remained spherical. Cells displaying extensions of the cytoplasm were not present in sufficient quantity to be classified into types 1, 2, 3, and 4 at 0.5 minutes and 1 minute but were so at later time intervals. The number of type 1 cells produced rose from a minimum of 0% at 5 minutes to a maximum of 22% at 15 minutes then declined to 10% at 30 minutes. The number of type 2 cells produced rose from 30% at 5 minutes to a maximum of 34% at 15 minutes then declined to a minimum of 16% at 30 minutes. The number of type 3 cells produced gradually rose from a minimum of 13% at 5 minutes to a maximum of 39% by 30 minutes. The number of type 4 cells produced gradually rose from a minimum of 0% at 5 minutes to a maximum of 32% by 30 minutes.

5. 2. 5. Investigation of the delay period during the morphological response of neutrophils to IgG. 2: responses to supernatants obtained from IgG treated cells

5. 2. 5. 1. Design of experiments

In the preceding experiments, morphological responses of neutrophils to commercial and heat aggregated preparations of IgG were found to be consistently characterised by little or no change to the morphology of the cells during the first minute followed by a large increase in the proportion of cells displaying pseudopodia at 5 minutes. One explanation for this delay period is that an interaction between neutrophils and IgG during the first 5 minutes may introduce a secondary factor into the suspension medium which then induces the major part of the cells' morphological response. To examine this hypothesis, the morphological responses of neutrophils to

two preparations of IgG were examined; (1) a solution of 0.01% HAIgG pre-incubated for 5 minutes at 37 °C, and (2) the supernatant obtained following incubation of neutrophils (10^6/ml) for 5 minutes at 37 °C in 0.01% HAIgG. Fresh neutrophils (10^6/ml) were then incubated for 0.5, 1, 5, 15 and 30 minutes at 37 °C in each preparation of IgG and their morphology assessed by visual classification of morphological subtypes. A control was performed by suspending neutrophils for 30 minutes in a supernatant obtained from cells suspended for 5 minutes in HBSS-Hepes in the absence of HAIgG. This experiment was perfomed three times using cells from a different donor on each occasion.

5. 2. 5. 2. Response to HAIgG pre-incubated in absence of cells (Fig. 5. 5. a.)

Neutrophils suspended in 0.01% HAIgG remained predominantly spherical at 0.5 minutes (89.3 ± 15.9%) and 1 minute (90.0 ± 13.0%), but only 16.0 ± 8.2%, 1.7 ± 1.2% and 6.0 ± 2.0% of cells remained spherical at 5, 15 and 30 minutes respectively. Morphological subtypes 1, 2, 3 and 4 were not consistently observed in sufficient quantities to be individually quantified at 0.5 minutes and 1 minute, but were so at later time intervals. The number of type 1 cells produced rose from a minimum of 9.3 ± 4.6% at 5 minutes to a maximum of 26.7 ± 13.7% at 15 minutes then declined to 14.0 ± 1.7% at 30 minutes. The number of type 2 cells produced gradually declined from a maximum of 43.0 ± 9.5% at 0.5 minutes to a minimum of 4.3 ± 1.2% by 30 minutes. The number of type 3 cells produced gradually declined from a maximum of 22.7 ± 5.0% at 0.5 minutes to a minimum of 13.7 ± 4.2% by 30 minutes. The number of type 4 cells produced gradually rose from a minimum of 5.3 ± 1.5% at 0.5 minutes to a maximum of 61.7 ± 1.5% at 30 minutes.

Fig. 5. 5. a. Time course of the morphological response of neutrophils in suspension to 0.01% heat aggregated IgG preincubated for 5 minutes in the absence of cells

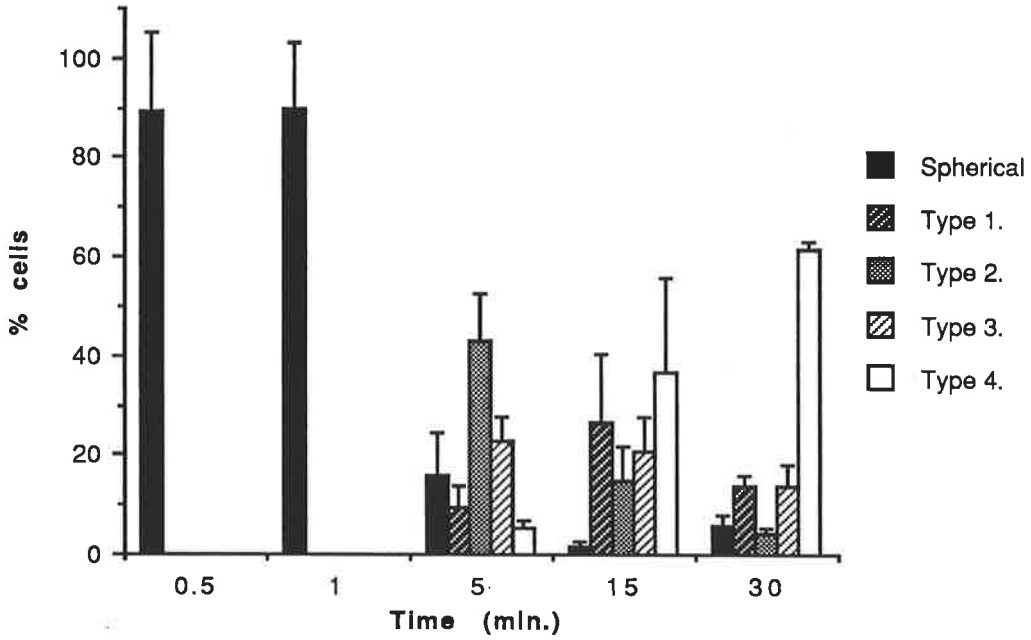
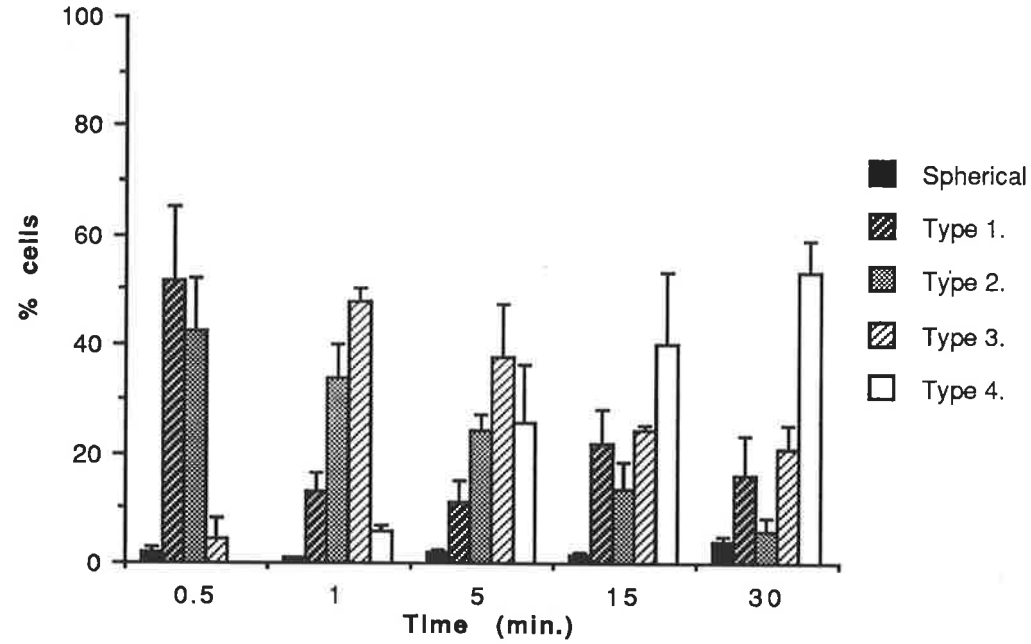


Fig. 5. 5. b. Time course of the morphological response of neutrophils in suspension to the supernatant obtained from cells incubated in 0.01% HAIgG for 5 minutes



5. 2. 5. 3. Response to supernatant from cells treated with HAIgG (Fig. 5. 5. b.)

Neutrophils suspended in the supernatant from cells treated with HAIgG displayed an immediately large morphological response with only 2.0 ± 1.0% of cells retaining a spherical shape after 0.5 minutes and <5% of cells remaining spherical at later time points. The number of type 1 cells produced gradually declined from a maximum of 51.7 ± 13.6% at 0.5 minutes to a minimum of 11.3 ± 3.8% by 5 minutes then rose to 21.7 ± 6.5% at 15 minutes and declined to 16.0 ± 7.0% at 30 minutes. The number of type 2 cells produced gradually declined from a maximum of 42.3 ± 9.9% at 0.5 minutes to a minimum of 6.0 ± 2.0% by 30 minutes. The number of type 3 cells produced rose from a minimum of 4.3 ± 4.0% at 0.5 minutes to a maximum of 47.7 ± 2.5% at 1 minute then gradually declined to 20.7 ± 4.6% by 30 minutes. The number of type 4 cells produced gradually rose from a minimum of 0.0 ± 0.0% at 0.5 minutes to a maximum of 53.3 ± 5.7% by 30 minutes.

5. 2. 5. 4. Response to supernatant from cells suspended in HBSS-Hepes

Neutrophils suspended for 30 minutes in the supernatant from cells incubated in the absence of IgG were 98.3 ± 2.1% spherical.

5. 2. 6. Effect of PIPLC digestion on the morphological response of neutrophils to IgG

5. 2. 6. 1. Design of experiments

The purpose of this experiment was to examine the role of the neutrophil's type III receptor for IgG (FcRIII; CD16) during the cell's morphological response to this immunoglobulin. Since this receptor is attached to the neutrophil's surface via a phosphoinositol linkage, an attempt was made to remove it from the cell's surface

using the enzyme, phosphoinositol specific phospholipase C (PIPLC). The extent of digestion was assessed by measuring the degree of binding of the following monoclonal antibodies to the neutrophil's surface using FACS analysis; (1) VIFcRIII, a mouse IgM class monoclonal antibody to the neutrophil's type III receptor for IgG, (2) VIBE3, a mouse IgM class monoclonal antibody which binds to another phosphoinositol-linked structure on the neutrophil's surface known as CD24 (positive control), and (3) VIM12, a mouse IgG1 class monoclonal antibody which binds to the complement type 3 receptor (CD11b or iC3b receptor), which is not linked to the neutrophil's surface via a phosphoinositol linkage (negative control).

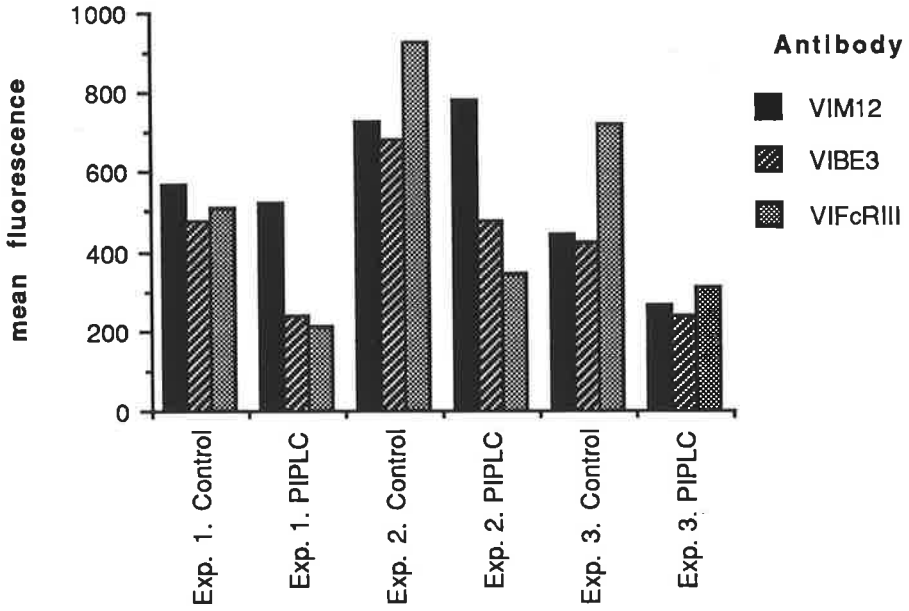
The effect of PIPLC digestion on the morphological response of neutrophils to IgG was assessed by comparing the responses of digested and non-digested cells to suspension in 0.05%, 0.005% and 0.0005% IgG (w/v in HBSS-Hepes) for 30 minutes. All responses were assessed by visual classification of morphological subtypes and statistical differences between responses of PIPLC treated and untreated neutrophils were determined by applying a chi-square goodness-of-fit test to the mean proportions of morphological subtypes obtained from three experiments. Cells from a different donor were used during each experiment.

5. 2. 6. 2. Binding of monoclonal antibodies to neutrophil's digested with PIPLC (Fig. 5. 6. a.)

Neutrophils treated with PIPLC consistently displayed a 60% reduction in their binding to VIFcRIII and a 30% to 50% reduction in their binding to VIBE3. Binding of VIM12 to neutrophils was not markedly altered in the first two experiments, but a 40% reduction in binding was observed in the third experiment.

5. 2. 6. 3. Morphological responses of PIPLC treated and untreated neutrophils to various concentrations IgG in suspension

Fig. 5. 6. a. Effect of PIPLC digestion on the degree of binding of fluorescein-conjugated monoclonal antibodies to the surface of neutrophils. ('Control' represents degree of binding to un-digested cells)



5. 2. 6. 3. 1. Responses to 0.05% IgG (Fig. 5. 6. b. i.)

The proportions of morphological subtypes displayed by untreated neutrophils after suspension 0.05% IgG were: 15.0 ± 10.8% spherical, 13.3 ± 11.2% type 1, 14.3 ± 8.5% type 2, 16.0 ± 6.1% type 3, and 41.0 ± 27.9% type 4.

The proportions of morphological subtypes displayed by PIPLC treated neutrophils after suspension in 0.05% IgG were: 12.7 ± 10.6% spherical, 14.0 ± 7.9% type 1, 13.0 ± 9.5% type 2, 17.3 ± 8.4% type 3, and 43.7 ± 29.3% type 4. These proportions of morphological subtypes were not significantly different to those displayed by untreated neutrophils towards 0.05% IgG (X^2= 0.08).

5. 2. 6. 3. 2. Responses to 0.005% IgG (Fig. 5. 6. b. ii.)

The proportions of morphological subtypes displayed by untreated neutrophils after suspension in 0.005% IgG were: 14.7 ± 12.5% spherical, 10.7 ± 1.5% type 1, 15.7 ± 10.3% type 2, 24.3 ± 15.9% type 3, 34.3 ± 22.2% type 4.

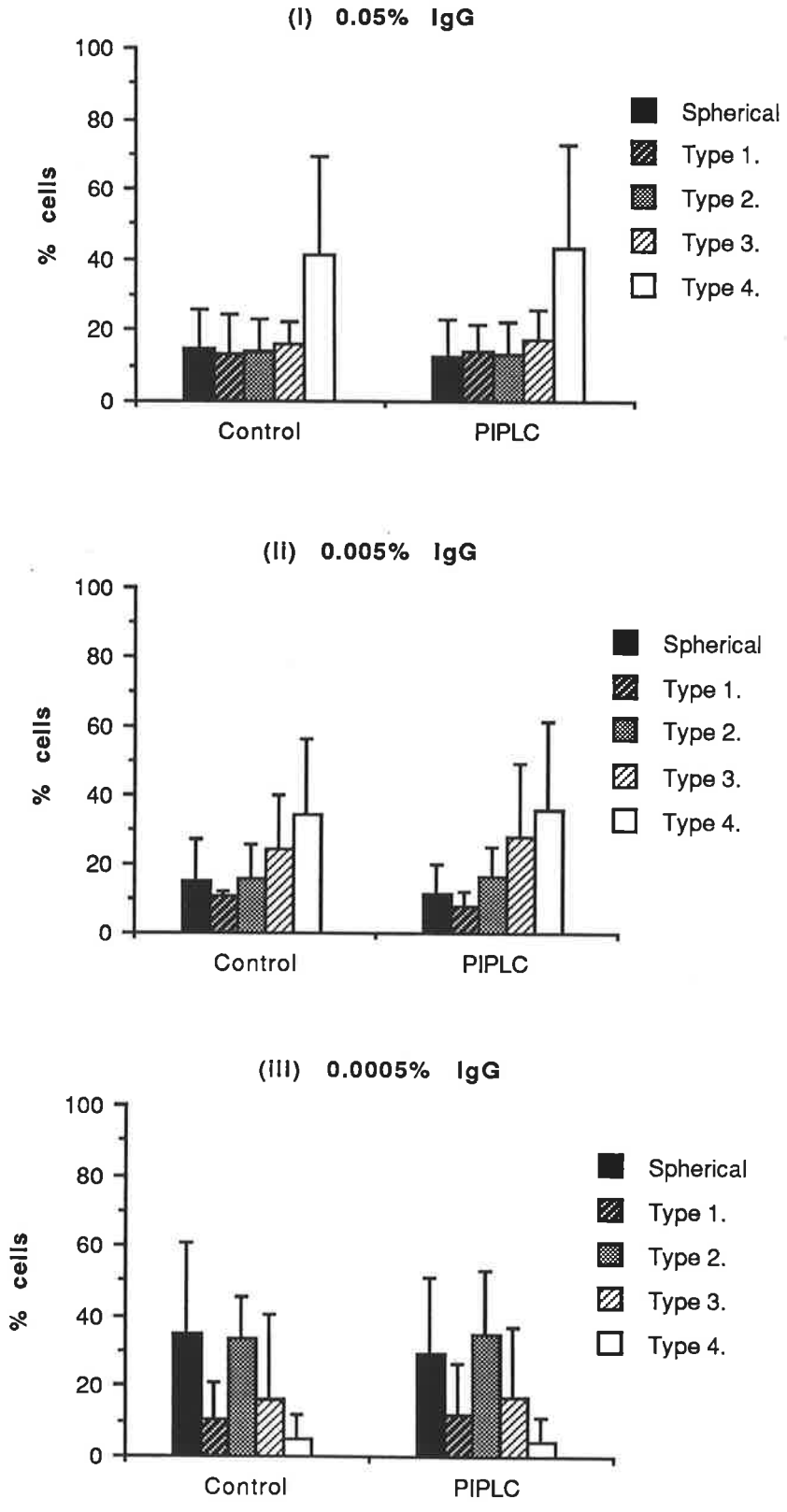
The proportions of morphological subtypes displayed by PIPLC treated neutrophils after suspension in 0.05% IgG were: 11.3 ± 8.5% spherical, 8.0 ± 4.0% type 1, 16.7 ± 8.5% type 2, 28.0 ± 21.2% type 3, and 35.7 ± 25.5% type 4. These proportions of morphological subtypes were not significantly different to those displayed by untreated neutrophils towards 0.005% IgG (X^2= 2.15).

5. 2. 6. 3. 3. Responses to 0.0005% IgG (Fig. 5. 6. b. iii.)

The proportions of morphological subtypes displayed by untreated neutrophils after suspension in 0.0005% IgG were: 35.3 ± 25.8% spherical, 10.3 ± 10.7% type 1, 33.3 ± 12.1% type 2, 16.3 ± 24.0% type 3, and 4.7 ± 7.2% type 4.

The proportions of morphological subtypes displayed by PIPLC treated neutrophils after suspension in 0.05% IgG were: 29.7 ± 21.1% spherical, 12.0 ±

Fig. 5. 6. b. Effect of PIPLC digestion on the morphological responses of neutrophils in suspension to various concentrations of IgG ('Control' values represent responses of undigested cells)



14.9% type 1, 35.0 ± 18.1% type 2, 17.0 ± 20.1% type 3, and 4.3 ± 6.7% type 4. These proportions of morphological subtypes were not significantly different to those displayed by untreated neutrophils towards 0.0005% IgG (X^2= 1.32).

5. 2. 7. Effects of cation chelating agents on the morphological response of neutrophils to IgG

5. 2. 7. 1. Design of experiments

Since morphological responses of neutrophils to stimuli examined in previous chapters appear to be affected by the availability of extracellular divalent cations, the aim of these experiments was to investigate the role of these cations during the morphological response of neutrophils to IgG.

Neutrophils were suspended for 30 minutes at 37 °C in 0.1%, 0.01% and 0.001% HAIgG (w/v in HBSS-Hepes) in the presence and absence of 5 mM EDTA or 5 mM EGTA. This experiment was performed four times using cells from a different donor on each occasion. The mean proportions of morphological subtypes displayed in the presence of chelating agents were compared with those obtained in their absence by using a chi-square goodness-of-fit test.

5. 2. 7. 2. Effects on response to 0.1% HAIgG (Fig. 5. 7. a.)

The proportions of morphological subtypes displayed by neutrophils after suspension in 0.1 % HAIgG, in the absence of chelating agents, were: 3.0 ± 1.2% spherical, 25.5 ± 8.5% type 1, 12.8 ± 4.3% type 2, 12.8 ± 5.9% type 3, and 46.5 ± 14.1% type 4.

The proportions of morphological subtypes displayed by neutrophils after suspension in 0.1% HAIgG, in the presence of 5 mM EDTA, were: 37.5 ± 19.1% spherical, 3.5 ± 1.3% type 1, 20.8 ± 6.8% type 2, 14.0 ± 5.0% type 3, and 24.3 ±

19.3% type 4. These proportions were significantly different to those displayed by cells in response to 0.1% HAIgG in the absence of chelating agents (X^2= 431.44).

The proportions of morphological subtypes displayed by neutrophils after suspension in 0.1% HAIgG, in the presence of 5 mM EGTA, were: 2.8 ± 1.0% spherical, 21.0 ± 11.2% type 1, 16.3 ± 4.3% type 2, 16.3 ± 4.3% type 3, and 41.5 ± 15.7% type 4. These proportions of morphological subtypes were not significantly different to those displayed by cells in response to 0.1% HAIgG in the absence of chelating agents (X^2= 3.26).

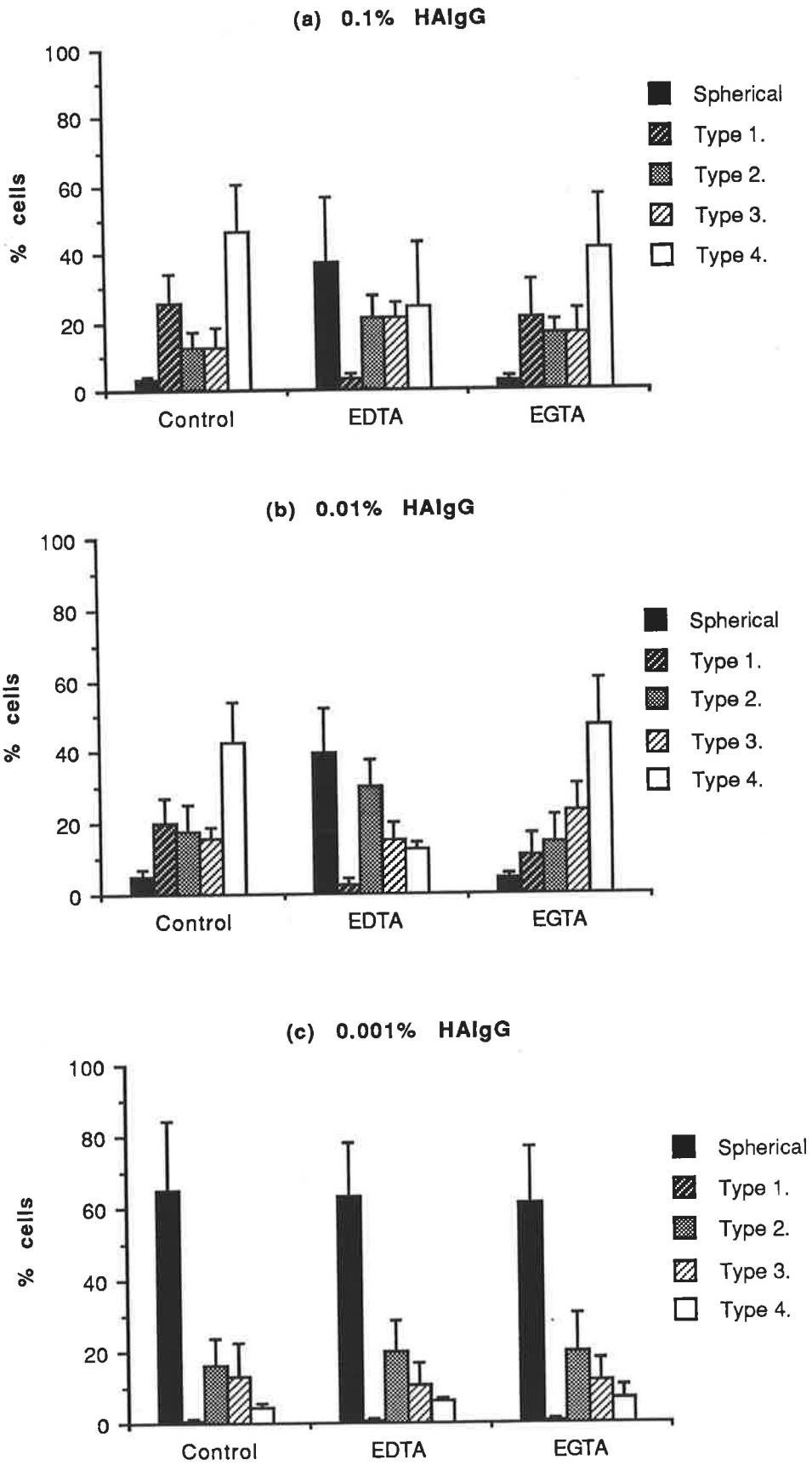
5. 2. 7. 3. Effects on response to 0.01% HAIgG (Fig. 5. 7. b.)

The proportions of morphological subtypes displayed by neutrophils after suspension in 0.01% HAIgG, in absence of chelating agents, were: 5.0 ± 1.8% spherical, 20.3 ± 6.8% type 1, 17.5 ± 7.4% type 2, 15.8 ± 2.9% type 3, and 42.5 ± 11.6% type 4.

The proportions of morphological subtypes displayed by neutrophils after suspension in 0.01% HAIgG, in the presence of 5 mM EDTA, were: 39.8 ± 12.5% spherical, 2.8 ± 1.3% type 1, 30.5 ± 7.2% type 2, 15.0 ± 5.0% type 3, and 12.3 ± 2.4% type 4. These proportions of morphological subtypes were significantly different to those displayed by cells suspended in 0.01% HAIgG in the absence of chelating agents (X^2= 288.46).

The proportions of morphological subtypes displayed by neutrophils after suspension in 0.01% HAIgG, in the presence of 5 mM EGTA, were: 4.5 ± 1.0% spherical, 11.0 ± 5.9% type 1, 14.5 ± 7.5% type 2, 23.3 ± 7.4% type 3, and 47.0 ± 13.2% type 4. These proportions of morphological subtypes were not significantly different to those displayed by cells suspended in 0.01% HAIgG in the absence of chelating agents (X^2= 8.86).

Fig. 5. 7. Effects of cation chelating agents on the morphological responses of neutrophils in suspension to various concentrations of heat aggregated IgG (HAIgG)



5. 2. 7. 4. Effects on response to 0.001% HAIgG (Fig. 5. 7. c.)

The proportions of morphological subtypes displayed by neutrophils after suspension in 0.001% HAIgG, in the absence of chelating agents, were: 65.3 ± 19.0% spherical, 0.7 ± 0.6% type 1, 16.3 ± 7.6% type 2, 13.0 ± 9.2% type 3, and 4.3 ± 1.5% type 4.

The proportions of morphological subtypes displayed by neutrophils after suspension in 0.001% HAIgG, in the presence of 5 mM EDTA, were: 63.3 ± 14.8% spherical, 0.7 ± 0.6% type 1, 20.0 ± 8.5% type 2, 10.7 ± 6.4% type 3, 6.0 ± 1.0% type 4. These proportions of morphological subtypes were not significantly different to those displayed by cells suspended in 0.001% HAIgG in the absence of chelating agents (X^2= 1.98).

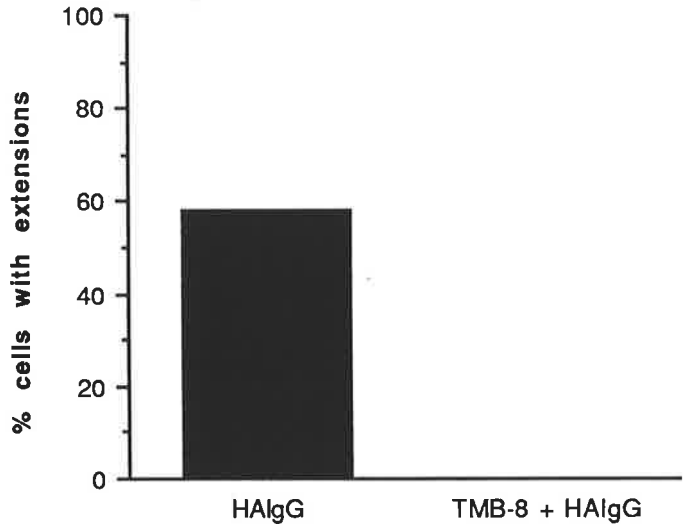
The proportions of morphological subtypes displayed by neutrophils after suspension in 0.001% HAIgG, in the presence of 5 mM EGTA, were: 61.0 ± 15.6% spherical, 0.7 ± 0.6% type 1, 20.3 ± 10.3% type 2, 11.7 ± 6.4% type 3, and 6.7 ± 4.0% type 4. These proportions of morphological subtypes were not significantly different to those displayed by cells suspended in 0.001% HAIgG in the absence of chelating agents (X^2= 2.73).

5. 2. 8. Effect of TMB-8 on the morphological response of neutrophils to IgG

5. 2. 8. 1. Design of experiments

The role of intracellular calcium was assessed by examining the effect of pre-incubation for 10 minutes at 37 °C in HBSS-Hepes containing 5 x 10-4 M TMB-8, on the subsequent morphological response of neutrophils to 0.01 % HAIgG for 30 minutes at 37 °C.

Fig. 5. 8. Effect of TMB-8 on the morphological response of neutrophils to 0.01% HAIgG



5. 2. 8. 2. Effect of TMB-8

Neutrophils incubated for 10 minutes at 37 °C in HBSS-Hepes developed extensions of their cytoplasm following addition of 0.01% HAIgG with 58% of cells displaying extensions after 30 minutes. In contrast, neutrophils incubated for 10 minutes at 37 °C in HBSS-Hepes containing 5 x 10-4 M TMB-8 remained >95% spherical following the addition of 0.01% HAIgG.

5. 2. 9. Effect of IgG on the F-actin distribution of neutrophils in suspension

5. 2. 9. 1. Design of experiments

Neutrophils were suspended in 0.01% IgG/4% human serum albumin for 1, 15 and 30 minutes before being fixed with 8% formalin and stained with the fluorescent F-actin probe, rhodamine phalloidin. The distribution of staining for F-actin within the cytoplasm of cells with each morphological subtype was then examined by fluorescence microscopy.

5. 2. 9. 2. F-actin distribution of cells

Cells treated with IgG consistently displayed intense staining for F-actin within cytoplasmic extensions in a similar manner to cells treated with FMLP (see chapter 3., Fig. 3. 5.).

5. 3. Summary

Commercial preparations of purified IgG (containing polymeric IgG) induced neutrophils to develop cytoplasmic extensions at concentrations of 0.005% and 0.0005% (w/v), but not at 0.00005% (w/v). These extensions enabled classification of neutrophils into types 1, 2 and 3 morphological subtypes, but type 4 cells were rarely observed. However, no change in the morphology of cells was observed until after 5 minutes incubation. When the content of polymeric IgG in these preparations was reduced by ultra-centrifugation, responses were either further delayed or abolished entirely. A similar delay period was observed when a different commercial preparation of IgG was used.

Removal of approximately 60% of the neutrophil's type III receptors for IgG by PIPLC digestion did not affect the response of these cells to commercial IgG. In addition, Fc and Fab fragments of IgG prepared by papain digestion produced little or no change in neutrophil shape.

Heat aggregated preparations of IgG (HAIgG) induced neutrophils to develop cytoplasmic extensions at 0.1%, 0.01% and 0.001% (w/v) but not at 0.0001% (w/v). These extensions enabled classification of cells into types 1, 2, 3 and 4 morphological subtypes, with the number of type 4 cells increasing in a concentration dependent manner. The order in which these morphological subtypes appeared during responses to IgG was slightly different to those observed in previous chapters as the proportion of type 1 cells often peaked after the peak proportion of type 2 cells was reached. Furthermore, no change in the morphology of the cells was generally observed until after 5 minutes incubation. In contrast, supernatants from cells suspended in 0.01% HAIgG for 5 minutes induced an immediate (within 30 seconds) response in fresh cells.

EDTA (5 mM) markedly reduced the responses of neutrophils to 0.1% and 0.01% HAIgG, but did not affect the response of neutrophils to 0.001% HAIgG.

However, responses to all concentrations of HAIgG tested were unaltered in the presence of EGTA (5 mM).

Pre-treatment of neutrophils with TMB-8 abolished the response of these cells to HAIgG (0.01%).

Actin microfilaments (F-actin) accumulated within the extensions of cells polarised in response to 0.01% IgG.

CHAPTER 6.

THE MORPHOLOGICAL RESPONSES OF HUMAN NEUTROPHILS IN SUSPENSION TO C5a AND INTERLEUKIN-8

6. 1. Introduction

This chapter examines the morphological responses of human neutrophils in suspension to the endogenous chemotactic peptides, C5a and interleukin-8 (IL-8). Responses of neutrophils to each peptide are characterised by the proportions of morphological subtypes induced with time by various peptide concentrations. Furthermore, the possible roles of extracellular and intracellular divalent cations during these responses are examined. In addition, the effects of each peptide on the F-actin distribution of neutrophils is assessed.

6. 2. Results

6. 2. 1. Time courses of the morphological responses of neutrophils in suspension to various concentrations of C5a

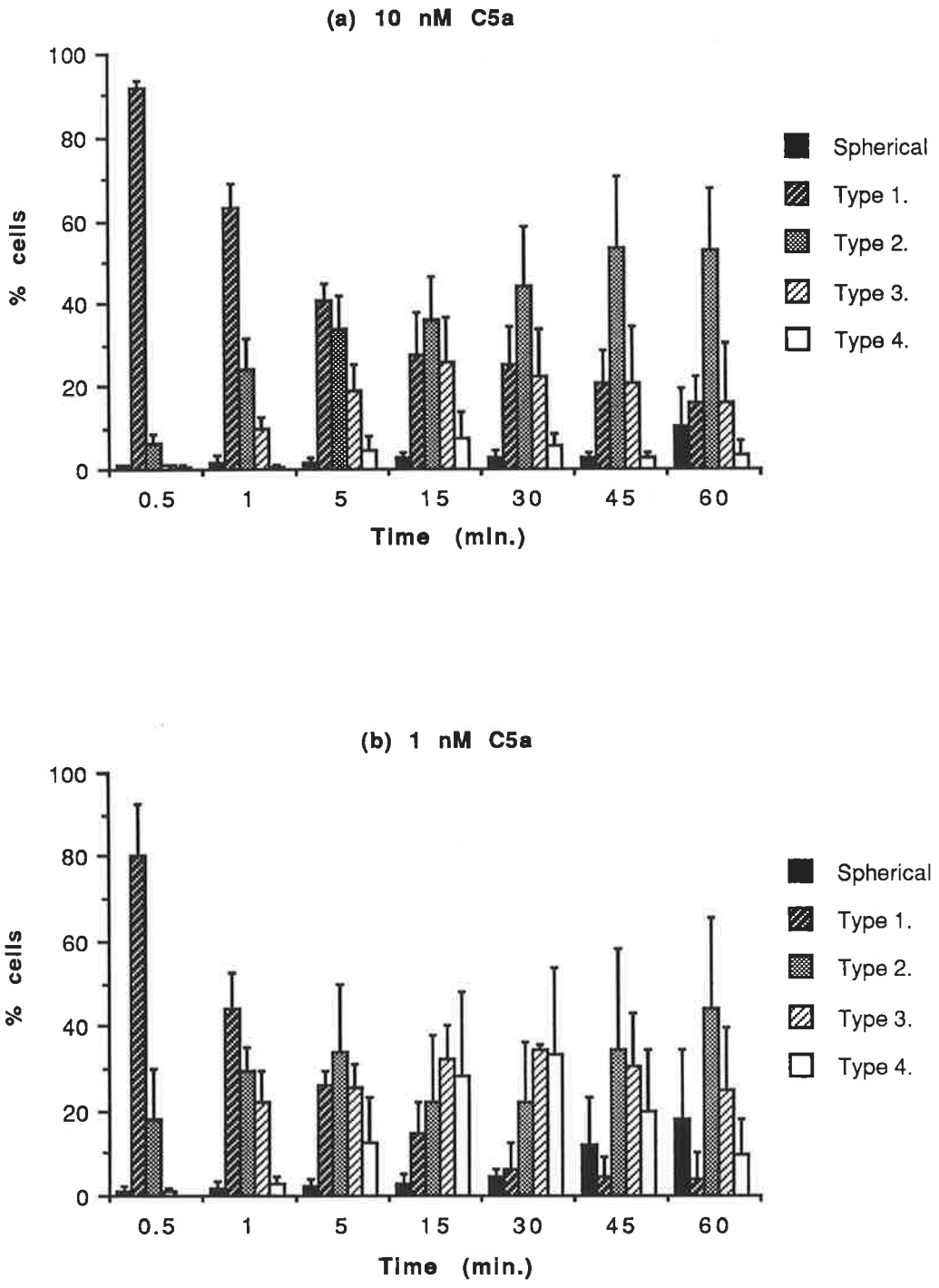
6. 2. 1. 1. Design of experiments

These experiments were designed to characterise the changes in morphology displayed by neutrophils with time following addition of different concentrations of C5a. Neutrophils were suspended in 10, 1, 0.1 and 0.01 nM C5a (in HBSS-Hepes at 37 °C) for 0.5, 1, 5, 15, 30, 45 and 60 minutes. At the end of each incubation, cells were fixed with glutaraldehyde and their morphology assessed by visual classification of morphological subtypes. Results are expressed as the mean ± standard deviation for the proportions of morphological subtypes obtained from three experiments. Cells from a different donor were used during each experiment.

6. 2. 1. 2. Response to 10 nM C5a (Fig. 6. 1. a.)

Neutrophils suspended in 10 nM C5a displayed an immediate morphological response with only 1.0 ± 0.0% of cells remaining spherical at 0.5 minutes after commencing incubation. The number of spherical cells remained < 5% for 45 minutes then rose to 10.3 ± 9.2% at 60 minutes. The number of type 1 cells produced gradually declined from a maximum of 91.7 ± 2.1% at 0.5 minutes to a minimum of 16.3 ± 6.0% by 60 minutes. The number of type 2 cells produced gradually rose from a minimum of 6.3 ± 2.1% at 0.5 minutes to a maximum of 53.3 ± 17.6% by 45 minutes then declined to 52.7 ± 15.4% at 60 minutes. The number of type 3 cells produced gradually rose from a minimum of 1.0 ± 0.0% at 0.5 minutes to a maximum of 26.0 ± 10.8% by 15 minutes then gradually declined to 16.3 ± 14.0% by 60

Fig. 6. 1. Time courses of the morphological responses of neutrophils in suspension to various concentrations of C5a



minutes. The number of type 4 cells produced gradually rose from a minimum of 0.3 ± 0.6% at 0.5 minutes to a maximum of 7.7 ± 6.0% then gradually declined to < 5% by 45 minutes onwards.

6. 2. 1. 3. Response to 1 nM C5a (Fig. 6. 1. b.)

Neutrophils suspended in 1 nM C5a displayed an immediate morphological response with only 1.0 ± 1.0% of cells remaining spherical at 0.5 minutes after commencing incubation. The number of spherical cells remained < 5% for 30 minutes then gradually increased to a maximum of 18.0 ± 16.5% by 60 minutes. The number of type 1 cells produced gradually declined from a maximum of 80.0 ± 12.5% at 0.5 minutes to a minimum of 4.0 ± 6.1% by 60 minutes. The number of type 2 cells produced gradually rose from a minimum of 18.0 ± 12.1% at 0.5 minutes to 33.7 ± 16.0% by 5 minutes then declined to 22.0 ± 15. 6% at 15 minutes before gradually rising to a maximum of 44.3 ± 21.4% by 60 minutes. The number of type 3 cells produced gradually rose from a minimum of 1.3 ± 0.6% at 0.5 minutes to a maximum of 34.3 ± 1.5% by 30 minutes then gradually declined to 24.7 ± 14.8% by 60 minutes. The number of type 4 cells produced gradually rose from a minimum of 0.0 ± 0.0% at 0.5 minutes to a maximum of 33.3 ± 20.4% by 30 minutes then gradually declined to 9.7 ± 8.1% by 60 minutes.

6. 2. 1. 4. Response to 0.1 nM C5a

Neutrophils suspended in 0.1 nM C5a displayed an immediate morphological response in one experiment, but this observation could not be repeated. During the experiment in which a response was observed, the number of spherical cells was reduced to 5% at 0.5 minutes but then gradually rose to >95% by 30 minutes onwards. Cell types 1, 2, 3, and 4 were only observed in sufficient quantities to be individually quantified during the first 15 minutes. The number of type 1 cells

produced gradually declined from a maximum of 39% at 0.5 minutes to 0% by 15 minutes. The number of type 2 cells produced remained at approximately 50% for the first 5 minutes then declined to 7% at 15 minutes. The number of type 3 cells produced rose from 1% at 0.5 minutes to 32% at 1 minute then gradually declined to 1% by 15 minutes. The number of type 4 cells produced remained ≤5% during the entire experiment. During the two experiments in which responses were not observed the number of spherical cells remained ≥95% throughout the incubation.

6. 2. 1. 5. Response to 0.01 nM C5a

Neutrophils suspended in 0.01 nM C5a displayed a delayed morphological response in one experiment, an immediate but low response in a second experiment and no response in a third experiment. In the experiment during which a delayed response was observed the number of spherical cells remained >90% for the first 5 minutes then declined to 8% at 15 minutes before gradually rising to 69% by 60 minutes. Cells types 1, 2, 3, and 4 were only present in sufficient numbers to be individually quantified from 1 minute onwards. The number of type 1 cells produced declined from 7% at 1 minute to 5% at 5 minutes then rose to 11% at 15 minutes before declining to < 5% at 30 minutes onwards. The number of type 2 cells produced gradually rose from a minimum of 0% at 1 minute to a maximum of 60% by 15 minutes then gradually declined to 23% by 45 minutes onwards. The number of type 3 cells produced gradually rose from a minimum of 0% at 1 minute to a maximum of 25% by 30 minutes then gradually declined to 6% by 60 minutes. The number of type 4 cells produced remained ≤1% except for at 30 minutes when 6% of cells were of this subtype. In the experiment during which a low response was observed the number of spherical cells remained at approximately 90% for the first 15 minutes then declined to approximately 80% from 30 minutes onwards. In the experiment during which no response was observed the number of spherical cells remained ≥98% for the entire experiment.

6. 2. 2. Effects of cation chelating agents on the morphological response of neutrophils to C5a

6. 2. 2. 1. Design of experiments

The aim of these experiments was to examine the possible role of extracellular Ca^{2+} and Mg^{2+} ions during the morphological response of neutrophils in suspension to C5a. Morphological responses to each chemotactic factor were studied by suspending neutrophils for 30 minutes at 37 °C in HBSS-Hepes containing either 10 nM or 1nM C5a. The role of extracellular Ca^{2+} and Mg^{2+} ions during each response was examined by including either 5 mM EDTA or 5 mM EGTA in the suspension medium. Controls were perfomed by suspending cells in the absence of cation chelating agents. At the end of each incubation, cells were fixed with glutaraldehyde and their morphology assessed by visual classification. Responses in the presence of cation chelating agents were compared with control responses by using a Chi-square goodness-of-fit test on the mean proportions of morphological subtypes calculated from three experiments (significantly different when $X^2 \geq 9.488$). Cells from a different donor were used during each experiment.

6. 2. 2. 2. Effects on response to 10 nM C5a (Fig. 6. 2. a.)

The proportions of morphological subtypes displayed by neutrophils after suspension in 10 nM C5a, in the absence of chelating agents, were: 4.0 ± 0.0% spherical, 19.0 ± 11.3% type 1, 63.5 ± 7.8% type 2, 11.0 ± 2.8% type 3, and 2.5 ± 0.7% type 4 cells.

The proportions of morphological subtypes displayed by neutrophils after suspension in 10 nM C5a, in the presence of 5 mM EDTA, were: 1.5 ± 0.7% spherical, 10.0 ± 2.8% type 1, 37.0 ± 11.3% type 2, 37.5 ± 2.1% type 3, 14.8 ±

9.2% type 4. These proportions of morphological subtypes were significantly different to those displayed by cells suspended in 10 nM C5a in the absence of chelating agents (X^2= 141.24).

The proportions of morphological subtypes displayed by neutrophils after suspension in 10 nM M C5a, in the presence of 5 mM EGTA, were: 4.0 ± 1.4% spherical, 5.5 ± 3.5% type 1, 48.5 ± 7.8% type 2, 33.0 ± 0.0% type 3, and 9.5 ± 4.9% type 4. These proportions of morphological subtypes were significantly different to those displayed by cells suspended in 10 nM C5a in the absence of chelating agents (X^2= 76.73).

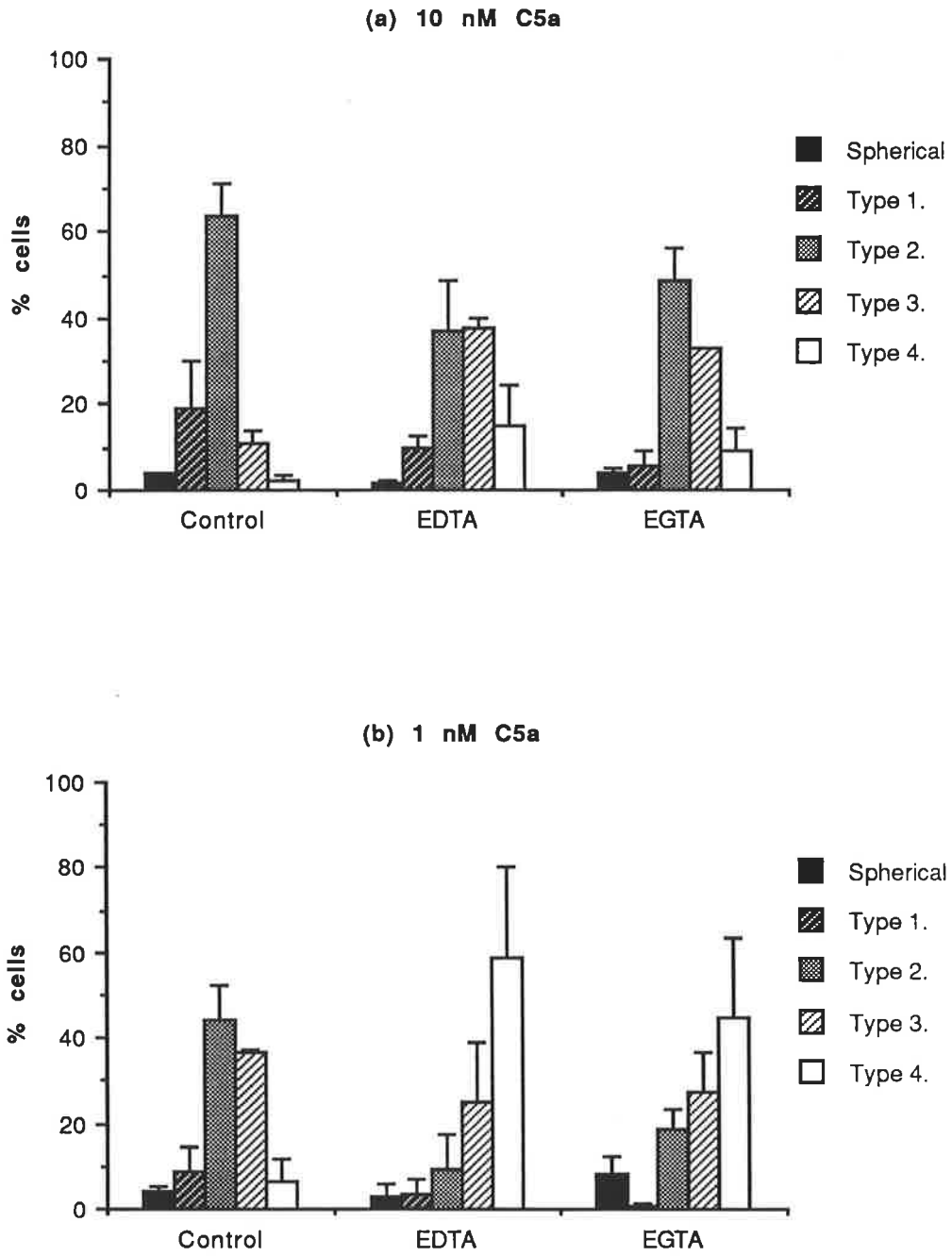
6. 2. 2. 3. Effects on response to 1 nM C5a (Fig. 6. 2. b.)

The proportions of morphological subtypes displayed by neutrophils after suspension in 1 nM C5a, in the absence of chelating agents, were: 4.0 ± 1.4% spherical, 9.0 ± 5.7% type 1, 44.0 ± 8.5% type 2, 36.5 ± 0.7% type 3, 6.5 ± 4.9% type 4.

The proportions of morphological subtypes displayed by neutrophils after suspension in 1 nM C5a, in the presence of 5 mM EDTA, were: 3.0 ± 2.8% spherical, 3.5 ± 3.5% type 1, 9.5 ± 7.8% type 2, 25.0 ± 14.1% type 3, and 59.0 ± 21.2 type 4. These proportions of morphological subtypes were significantly different to those displayed by cells suspended in 1 nM C5a in the absence of chelating agents (X^2= 458.30).

The proportions of morphological subtypes displayed by neutrophils after suspension in 1 nM M C5a, in the presence of 5 mM EGTA, were: 8.0 ± 4.2% spherical, 0.5 ± 0.7% type 1, 18.5 ± 4.9% type 2, 27.5 ± 9.2% type 3, and 45.0 ± 18.4% type 4. These proportions of morphological subtypes were significantly different to those displayed by cells suspended in 1 nM C5a in the absence of chelating agents (X^2= 257.07).

Fig. 6. 2. Effects of cation chelating agents on the morphological responses of neutrophils to various concentrations of C5a



6. 2. 3. Effect of TMB-8 on the morphological response of neutrophils to C5a

6. 2. 3. 1. Design of experiments

The purpose of these experiments was to examine the possible role of the release of Ca^{2+} ions from intracellular stores during the morphological response of neutrophils in suspension to C5a. The morphological responses to C5a was studied by suspending neutrophils for 30 minutes at 37 °C in HBSS-Hepes containing 1 nM C5a. The role of intracellular calcium release was examined by pretreating neutrophils for 10 minutes at 37 °C with 5×10^{-4} M TMB-8 before addition of chemotactic factors. Controls were perfomed by pre-incubating cells in the absence of TMB-8. At the end of each incubation, cells were fixed with glutaraldehyde and their morphology assessed by visual classification of morphological subtypes. This experiment was perfomed once.

6. 2. 3. 2. Effect of TMB-8 on response to C5a (Fig. 6. 3.)

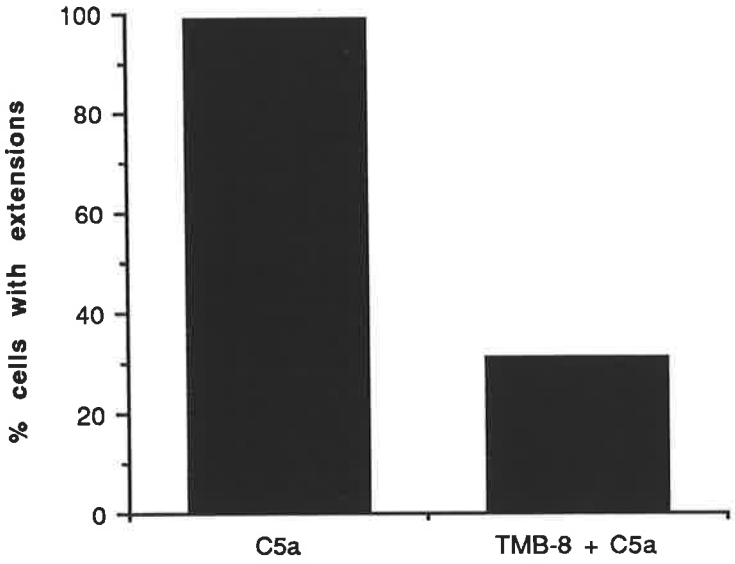
While 97% of neutrophils pre-incubated in HBSS-Hepes displayed extensions of the cytoplasm following addition of C5a, only 30% of neutrophils pre-incubated with TMB-8 displayed pseudopodia following subsequent incubation with C5a.

6. 2. 4. Effect of C5a on the F-actin distribution of neutrophils in suspension (Fig. 6. 4.)

6. 2. 4. 1. Design of experiments

Neutrophils were suspended in 1 nM/4% human serum albumin at 37 °C for 1, 15 and 30 minutes before being fixed with 8% formalin and stained with the

Fig. 6. 3. Effect of TMB-8 on the morphological response of neutrophils to 1 nM C5a



fluorescent F-actin probe, rhodamine phalloidin. The distribution of staining for F-actin within the cytoplasm of cells with each morphological subtype was then examined by fluorescence microscopy.

6. 2. 4. 2. F-actin distribution of cells

Cells treated with C5a consistently displayed intense staining for F-actin within cytoplasmic extensions in a similar manner to cells treated with FMLP (see chapter 3., Fig. 3. 5.).

6. 2. 5. Time courses of the morphological responses of neutrophils in suspension to various concentrations of IL-8

6. 2. 5. 1. Design of experiments

These experiments were designed to characterise the changes in morphology displayed by neutrophils with time following addition of different concentrations of IL-8. Neutrophils were suspended in 125, 12.5, 1.25 and 0.125 nM IL-8 (in HBSS-Hepes at 37 °C) for 0.5, 1, 5, 15, 30, 45 and 60 minutes. At the end of each incubation, cells were fixed with glutaraldehyde and their morphology assessed by visual classification of morphological subtypes. Results are expressed as the mean ± standard deviation for the proportions of morphological subtypes obtained from three experiments. Cells from a different donor were used during each experiment.

6. 2. 5. 2. Response to 125 nM IL-8 (Fig. 6. 5. a.)

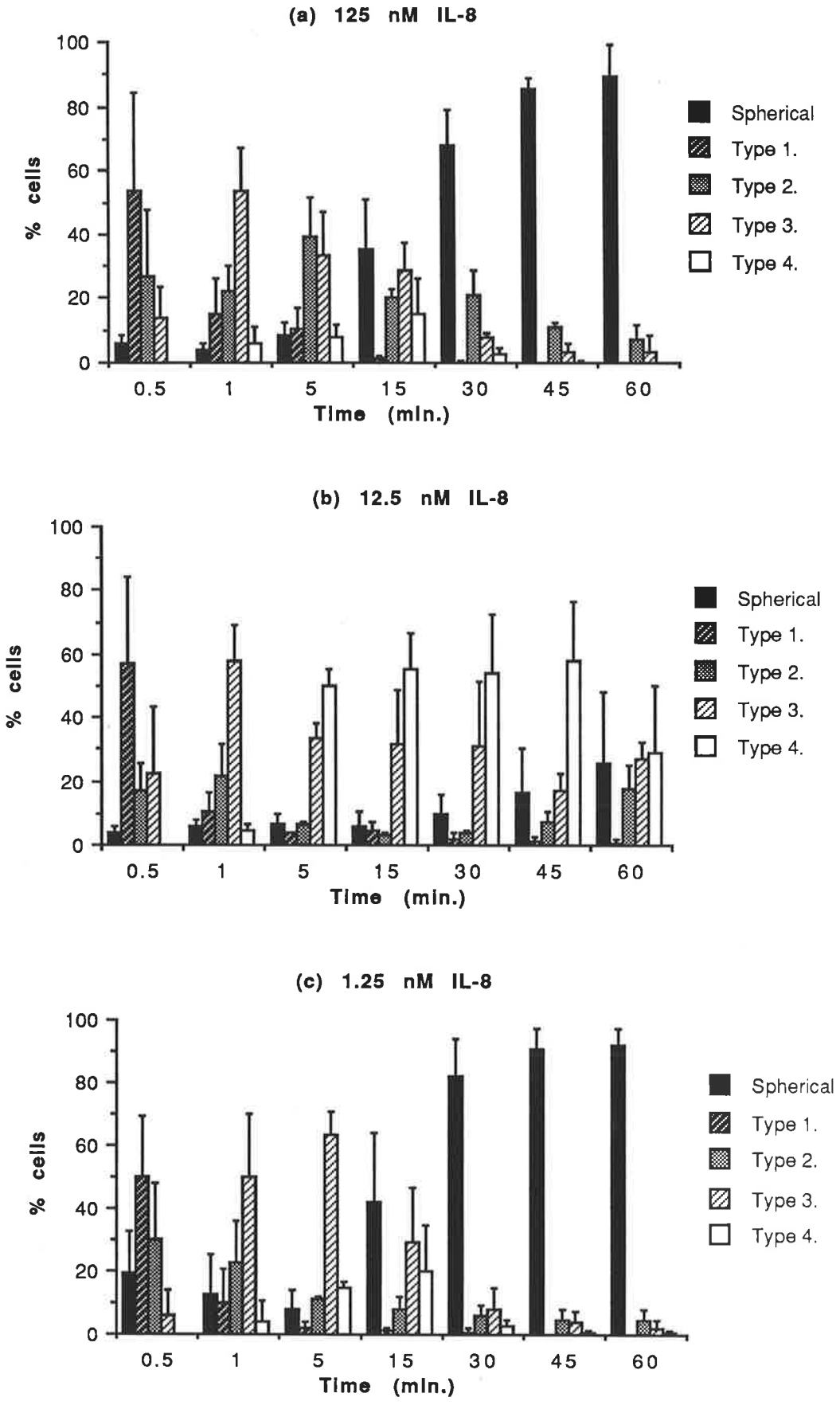
Neutrophils suspended in 125 nM IL-8 displayed an immediate morphological response with only 5.7 ± 2.5% of cells remaining spherical at 0.5 minutes after commencing incubation. The number of spherical cells further declined to a minimum

value of 3.7 ± 2.1% at 1 minute then gradually rose to a maximum of 89.3 ± 10.0% by 60 minutes. The number of type 1 cells produced gradually declined from a maximum of 53.7 ± 30.4% at 0.5 minutes to <5% by 15 minutes onwards. The number of type 2 cells produced declined from 27.0 ± 21.0% at 0.5 minutes to 22.3 ± 7.6% at 1 minute ,then rose to a maximum of 39.3 ± 12.3% at 5 minutes and gradually declined to a minimum of 7.0 ± 4.6% by 60 minutes. The number of type 3 cells produced rose from 14.0 ± 9.5% at 0.5 minutes to a maximum of 53.7 ± 13.3% at 1 minute then gradually declined to < 5% by 30 minutes onwards. The number of type 4 cells produced gradually rose from 0.0 ± 0.0% at 0.5 minutes to a maximum of 15.3 ± 10.7% at 15 minutes then declined to <5% at 30 minutes.

6. 2. 5. 3. Response to 12.5 nM IL-8 (Fig. 6. 5. b.)

Neutrophils suspended in 12.5 nM IL-8 displayed an immediate morphological response with only 4.0 ± 2.0% of cells remaining spherical at 0.5 minutes after commencing incubation. The number of spherical cells remained close to 5% until 15 minutes then gradually rose to a maximum of 25.7 ± 22.5% by 60 minutes. The number of type 1 cells produced gradually declined from a maximum of 57.3 ± 27.0% at 0.5 minutes to <5% by 5 minutes onwards. The number of type 2 cells produced rose from 17.0 ± 8.7% at 0.5 minutes to a maximum of 21.7 ± 10.1% at 1 minute, then gradually declined to <5% by 15 minutes and rose to 18.0 ± 7.0% by 60 minutes. The number of type 3 cells produced rose from 22.3 ± 20.8% at 0.5 minutes to a maximum of 57.7 ± 11.7% at 1 minute, then gradually declined to a minimum of 17.3 ± 5.1% by 45 minutes and rose to 26.7 ± 5.5% at 60 minutes. The number of type 4 cells produced gradually rose from a minimum of 0.0 ± 0.0% at 0.5 minutes to 55.0 ± 11.3% by 15 minutes, then declined to 54.0 ± 18.4% at 30 minutes, rose to a maximum of 58.0 ± 18.3% at 45 minutes and declined to 28.7 ± 21.1% at 60 minutes.

Fig. 6. 4. Time courses of the morphological responses of neutrophils in suspension to various concentrations of IL-8



6. 2. 5. 4. Response to 1.25 nM IL-8 (Fig. 6. 5. c.)

Neutrophils suspended in 1.25 nM IL-8 displayed an immediate morphological response with only 19.3 ± 13.1% of cells remaining spherical at 0.5 minutes after commencing incubation. The number of spherical cells gradually declined further to a minimum of 8.3 ± 5.5% by 5 minutes then gradually rose to a maximum of 92.3 ± 5.1% by 60 minutes. The number of type 1 cells produced gradually declined from a maximum of 50.3 ± 18.9% at 0.5 minutes to <5% by 5 minutes onwards. The number of type 2 cells produced gradually declined from a maximum of 30.3 ± 17.7% at 0.5 minutes to <5% by 45 minutes onwards. The number of type 3 cells produced gradually rose from 6.0 ± 7.9% at 0.5 minutes to a maximum of 63.3 ± 7.5% by 5 minutes then gradually declined to <5% by 45 minutes onwards. The number of type 4 cells produced gradually increased from 0.0 ± 0.0% at 0.5 minutes to a maximum of 19.7 ± 15.1% by 15 minutes then declined to <5% for the duration of the experiment.

6. 2. 5. 5. Response to 0.125 nM IL-8

Neutrophils suspended in 0.125 nM IL-8 did not display any noticeable morphological response since the percentage of spherical cells remained greater than 95% throughout the hour incubation.

6. 2. 6. Effects of cation chelating agents on the morphological response of neutrophils to IL-8

6. 2. 6. 1. Design of experiments

The aim of these experiments was to examine the possible role of extracellular Ca^{2+} and Mg^{2+} ions during the morphological response of neutrophils in suspension to

IL-8. The morphological response to IL-8 were studied by suspending neutrophils for 30 minutes at 37 °C in HBSS-Hepes containing various concentrations of IL-8 (125, 12.5 and 1.25 nM). The role of extracellular Ca^{2+} and Mg^{2+} ions during each response was examined by including either 5 mM EDTA or 5 mM EGTA in the suspension medium. Controls were perfomed by suspending cells in the absence of cation chelating agents. At the end of each incubation, cells were fixed with glutaraldehyde and their morphology assessed by visual classification. Responses in the presence of cation chelating agents were compared with control responses by using a Chi-square goodness-of-fit test on the mean proportions of morphological subtypes calculated from three experiments (significantly different when $X^2 \geq 9.488$). Cells from a different donor were used during each experiment.

6. 2. 6. 2. Effects on response 125 nM IL-8 (Fig. 6. 6. a.)

The proportions of morphological subtypes displayed by neutrophils after suspension in 125 nM IL-8, in the absence of chelating agents, were: 51.0 ± 17.5% spherical, 2.0 ± 1.0% type 1, 31.7 ± 11.4% type 2, 7.7 ± 2.9% type 3, and 10.0 ± 5.3% type 4.

The proportions of morphological subtypes displayed by neutrophils after suspension in 125 nM IL-8, in the presence of 5 mM EDTA, were: 40.0 ± 18.7% spherical, 2.3 ± 1.5% type 1, 41.3 ± 11.9% type 2, 10.0 ± 4.6% type 3, and 6.0 ± 2.6% type 4. These proportions of morphological subtypes were not significantly different to those displayed by cells suspended in 125 nM in the absence of chelating agents (X^2= 7.62).

The proportions of morphological subtypes displayed by neutrophils after suspension in 125 nM IL-8, in the presence of 5 mM EGTA, were: 48.7 ± 11.5% spherical, 1.3 ± 0.6% type 1, 35.7 ± 10.3% type 2, 8.0 ± 4.6% type 3, and 6.3 ± 3.5% type 4 cells. These proportions of morphological subtypes were not

significantly different to those displayed by cells suspended in 125 nM in the absence of chelating agents (X^2= 2.23).

6. 2. 6. 3. Effects on response to 12.5 nM IL-8 (Fig. 6. 6. b.)

The proportions of morphological subtypes displayed by neutrophils after suspension in 12.5 nM IL-8, in the absence of chelating agents, were: 8.7 ± 5.0% spherical, 4.3 ± 1.5% type 1, 14.0 ± 6.2% type 2, 37.0 ± 4.4% type 3, and 36.7 ± 5.5% type 4.

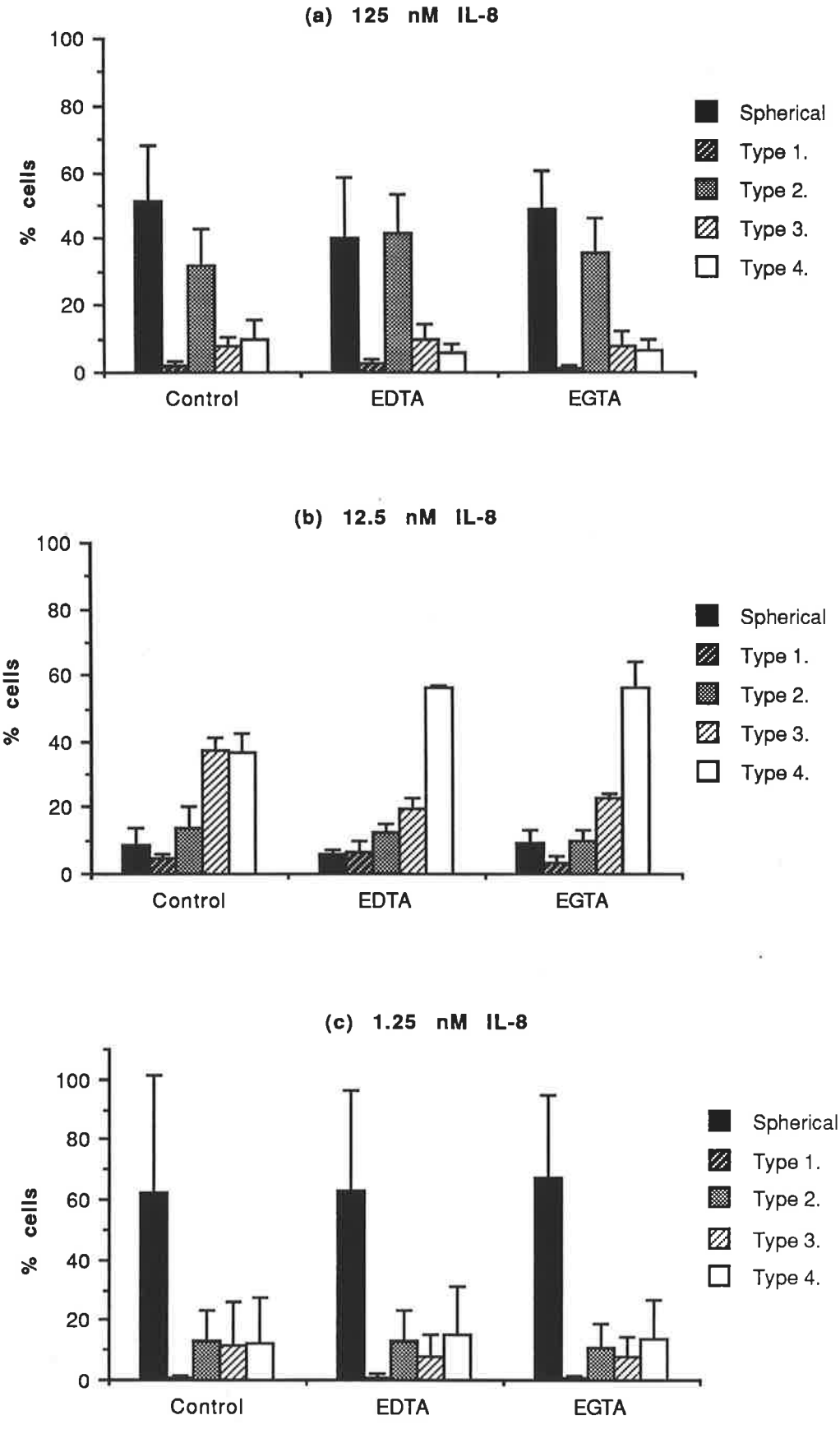
The proportions of morphological subtypes displayed by neutrophils after suspension in 12.5 nM IL-8, in the presence of 5 mM EDTA, were: 5.7 ± 1.2% spherical, 6.3 ± 3.5% type 1, 12.7 ± 2.1% type 2, 19.7 ± 3.1% type 3, 56.3 ± 0.6% type 4. These proportions of morphological subtypes were significantly different to those displayed by cells suspended in 12.5 nM in the absence of chelating agents (X^2= 20.61).

The proportions of morphological subtypes displayed by neutrophils after suspension in 12.5 nM IL-8, in the presence of 5 mM EGTA, were: 9.0 ± 4.0% spherical, 3.0 ± 2.0% type 1, 9.7 ± 3.1% type 2, 22.7 ± 1.5% type 3, and 56.0 ± 8.2% type 4. These proportions of morphological subtypes were significantly different to those displayed by cells suspended in 12.5 nM in the absence of chelating agents (X^2= 17.40).

6. 2. 6. 4. Effects on response to 1.25 nM IL-8 (Fig. 6. 6. c.)

The proportions of morphological subtypes displayed by neutrophils after suspension in 1.25 nM IL-8, in the absence of chelating agents, were: 62.3 ± 38.9% spherical, 0.7 ± 0.6% type 1, 13.0 ± 10.5% type 2, 11.7 ± 14.2% type 3, 12.3 ± 15.3% type 4.

Fig. 6. 5. Effects of cation chelating agents on the morphological responses of neutrophils to various concentrations of IL-8



The proportions of morphological subtypes displayed by neutrophils after suspension in 1.25 nM IL-8, in the presence of 5 mM EDTA, were: 62.7 ± 33.3% spherical, 1.0 ± 1.0% type 1, 13.0 ± 10.0% type 2, 8.0 ± 7.2% type 3, and 15.0 ± 15.9% type 4. These proportions of morphological subtypes were not significantly different to those displayed by cells suspended in 1.25 nM in the absence of chelating agents (X^2= 1.89).

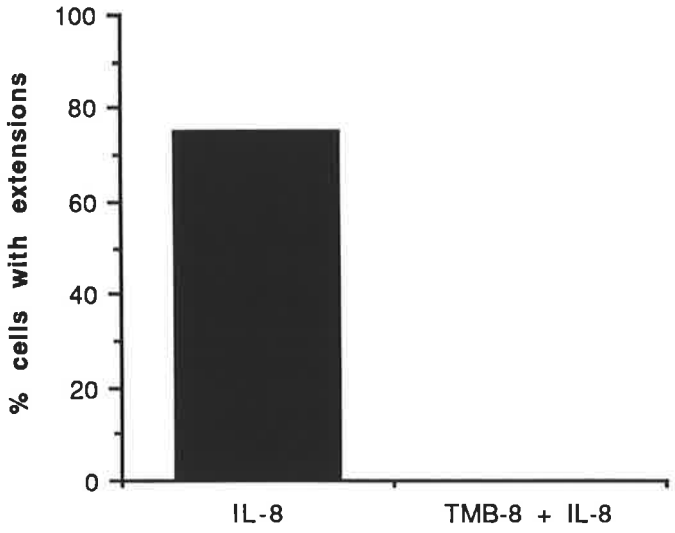
The proportions of morphological subtypes displayed by neutrophils after suspension in 1.25 nM IL-8, in the presence of 5 mM EGTA, were: 67.0 ± 27.6% spherical, 0.7 ± 0.6% type 1, 10.7 ± 8.1% type 2, 8.0 ± 6.6% type 3, and 13.7 ± 12.8% type 4. These proportions of morphological subtypes were not significantly different to those displayed by cells suspended in 1.25 nM in the absence of chelating agents (X^2= 2.09).

6. 2. 7. Effect of TMB-8 on the morphological response of neutrophils to IL-8

6. 2. 7. 1. Design of experiments

The purpose of these experiments was to examine the possible role of the release of Ca^{2+} ions from intracellular stores during the morphological response of neutrophils in suspension IL-8. The morphological response to IL-8 was studied by suspending neutrophils for 30 minutes at 37 °C in HBSS-Hepes containing 12.5 nM IL-8. The role of intracellular calcium release was examined by pretreating neutrophils for 10 minutes at 37 °C with 5 x 10^{-4} M TMB-8 before addition of chemotactic factors. Controls were perfomed by pre-incubating cells in the absence of TMB-8. At the end of each incubation, cells were fixed with glutaraldehyde and their morphology assessed by visual classification of morphological subtypes. This experiment was perfomed once.

Fig. 6. 6. Effect of TMB-8 on the morphological response of neutrophils to 12.5 nM IL-8



6. 2. 7. 3. Effect of TMB-8 on response to IL-8 (Fig. 6. 7.)

While 75% of neutrophils pre-incubated in HBSS-Hepes displayed extensions of the cytoplasm following addition of IL-8, 0% of neutrophils pre-incubated with TMB-8 displayed pseudopodia following subsequent incubation with IL-8.

6. 2. 8. Effect of IL-8 on the F-actin distribution of neutrophils in suspension (Fig. 6. 8.)

6. 2. 8. 1. Design of experiments

Neutrophils were suspended in 12.5 nM IL-8/4% human serum albumin at 37 °C for 1, 15 and 30 minutes before being fixed with 8% formalin and stained with the fluorescent F-actin probe, rhodamine phalloidin. The distribution of staining for F-actin within the cytoplasm of cells with each morphological subtype was then examined by fluorescence microscopy.

6. 2. 8. 2. F-actin distribution of cells

Cells treated with IL-8 generally displayed intense staining for F-actin within cytoplasmic extensions in a similar manner to cells treated with FMLP (see chapter 3., Fig. 3. 5.). However, polarised cells displaying moderate to poor staining throughout their cytoplasm, were often observed at later time intervals. These poorly staining cells were similar in appearance to those poorly staining polarised cells noted after 15 minutes in plasma (see chapter 4., Fig. 4. 15.).

6. 3. Summary

The fragment of the fifth component of complement, C5a consistently polarised neutrophils at 10 nM and 1 nM, but not at lower concentrations. Cytoplasmic extensions were apparent within 30 seconds and persisted throughout the 60 minutes incubation. The greatest proportion of type 4 cells (fully polarised with uropod) were observed when cells were incubated in 1 nM C5a. Supra-optimal concentrations of C5a (10 nM) maintained higher numbers of cells with non-polarised extensions (type 1 cells) and induced formation of low numbers of type 4 cells.

Interleukin-8 polarised neutrophils at 125 nM, 12.5 nM and 1.25 nM, but not at 0.125 nM. Cytoplasmic extensions were apparent within 30 seconds and persisted throughout the 60 minutes incubations, but disappeared from the majority of cells by 30 minutes in 125 nM and 1.25 nM IL-8. The greatest proportion of type 4 cells were observed when cells were incubated in 12.5 nM IL-8. Supra-optimal concentrations of IL-8 (125 nM) induced formation of very low numbers of type 4 cells.

The orders in which each morphological subtype reached its highest proportion during responses to C5a and IL-8 were generally similar to that displayed during responses to FMLP with type 1 cells appearing early and type 4 cells appearing last.

Responses of neutrophils to optimal and supra-optimal concentrations of C5a were enhanced in the presence of either EDTA or EGTA with EDTA having a slightly greater effect. In contrast, while responses of neutrophils to optimal concentrations of IL-8 were slightly enhanced in the presence of chelating agents, responses to supra-optimal and sub-optimal concentrations were unaffected.

TMB-8 greatly reduced the morphological response of neutrophils to C5a and abolished the response of neutrophils to IL-8.

F-actin generally accumulated within the pseudopodia of cells treated with either C5a or IL-8 in a similar manner to those treated with FMLP. However, cells treated with IL-8 occasionally displayed pseudopodia with weak staining for F-actin in a similar manner to cells treated with plasma.

CHAPTER 7.

THE MORPHOLOGICAL RESPONSES OF HUMAN NEUTROPHILS IN SUSPENSION TO LEUKOTRIENE-B_4 AND PLATELET ACTIVATING FACTOR

7. 1. Introduction

This chapter examines the morphological responses of human neutrophils in suspension to the endogenous chemotactic lipids leukotriene B_4 (LTB_4) and platelet activating factor (PAF). Responses of neutrophils to each lipid are characterised by the proportions of morphological subtypes induced with time by various concentrations of each lipid. Furthermore, the possible roles of extracellular and intracellular divalent cations during these responses are examined. In addition, the effects of each peptide on the F-actin distribution of neutrophils is assessed.

7. 2. Results

7. 2. 1. Time courses of the morphological responses of neutrophils in suspension to various concentrations of LTB_4

7. 2. 1. 1. Design of experiments

These experiments were designed to characterise the changes in morphology displayed by neutrophils with time following addition of different concentrations of LTB_4. Neutrophils were suspended in 100, 10, 1 and 0.1 nM LTB_4 (in HBSS-Hepes at 37 °C) for 0.5, 1, 5, 15, 30, 45 and 60 minutes. At the end of each incubation, cells were fixed with glutaraldehyde and their morphology assessed by visual classification of morphological subtypes. Results are expressed as the mean ± standard deviation for the proportions of morphological subtypes obtained from three experiments. Cells from a different donor were used during each experiment.

7. 2. 1. 2. Response to 100 nM LTB_4 (Fig. 7. 1. a.)

Neutrophils suspended in 100 nM LTB_4 displayed an immediate morphological response with only 3.3 ± 2.3% of cells retaining a spherical shape at 0.5 minutes after commencing incubation. The number of spherical cells remained approximately ≤ 5% for 30 minutes then gradually rose to a maximum of 8.7 ± 9.1% by 60 minutes. The number of type 1 cells produced declined from a maximum of 34.0 ± 9.5% at 0.5 minutes to 10.7 ± 5.5% at 1 minute then rose to 13.7 ± 5.5% at 5 minutes before gradually declining to ≤ 6% by 45 minutes onwards. The number of type 2 cells produced rose from 48.0 ± 7.0% at 0.5 minutes to a maximum of 50.3 ± 4.5% at 1 minute then gradually declined to a minimum of 12.7 ± 4.2% by 60 minutes. The number of type 3 cells produced rose from a minimum of 14.0 ± 4.4%

at 0.5 minutes to 24.7 ± 3.8% at 1 minute, declined to 22.0 ± 1.0% at 5 minutes, then rose to 27.7 ± 3.2% at 15 minutes and gradually declined to 21.7 ± 15.0% by 60 minutes. The number of type 4 cells produced gradually rose from a minimum of 0.7 ± 0.6% at 0.5 minutes to a maximum of 51.0 ± 11.3% by 60 minutes.

7. 2. 1. 3. Response to 10 nM LTB_4 (Fig. 7. 1. b.)

Neutrophils suspended in 10 nM LTB_4 displayed an immediate morphological response with only 2.0 ± 1.0% of cells retaining a spherical shape at 0.5 minutes after commencing incubation. The number of spherical cells remained < 5% for the remainder of the experiment. The number of type 1 cells produced declined from a maximum of 34.3 ± 11.0% at 0.5 minutes to 6.3 ± 3.2% at 1 minutes before rising to 13.7 ± 3.2% at 5 minutes and gradually declined to a minimum of 4.3 ± 3.1% by 60 minutes. The number of type 2 cells produced declined from a maximum of 56.3 ± 7.6% at 0.5 minutes to 27.3 ± 5.9% at 1 minute before rising to 32.3 ± 10.7% at 5 minutes and gradually declining to a minimum of 4.0 ± 1.0% by 60 minutes. The number of type 3 cells produced rose from a minimum of 7.0 ± 2.0% at 0.5 minutes to a maximum of 39.0 ± 1.0% at 1 minute then gradually declined to 16.0 ± 4.4% by 60 minutes. The number of type 4 cells produced rose from a minimum of 0.3 ± 0.6% at 0.5 minutes to 27.3 ± 8.3% at 1 minute then declined to 20.3 ± 14.9% at 5 minutes before gradually rising to a maximum of 72.3 ± 2.9% by 60 minutes.

7. 2. 1. 4. Response to 1 nM LTB_4 (Fig. 7. 1. c.)

Neutrophils suspended in 1 nM LTB_4 displayed an immediate morphological response with only 3.3 ± 2.3% of cells retaining a spherical shape at 0.5 minutes after commencing incubation. The number of spherical cells remained < 5% for 30 minutes then gradually rose to a maximum of 12.7 ± 8.6% by 60 minutes. The number of type 1 cells produced declined from a maximum of 43.3 ± 10.0% at 0.5

minutes to 6.7 ± 3.2% at 1 minute then rose to 11.3 ± 3.1% at 5 minutes before gradually declining to a minimum of 2.7 ± 1.5% by 60 minutes. The number of type 2 cells produced gradually declined from a maximum of 44.3 ± 6.7% at 0.5 minutes to a minimum of 11.7 ± 7.3% by 30 minutes then gradually rose to 22.3 ± 20.3% by 60 minutes. The number of type 3 cells produced rose from a minimum of 10.7 ± 3.1% at 0.5 minutes to a maximum of 41.3 ± 3.2% at 1 minute before gradually declining to 17.7 ± 15.0% by 15 minutes, gradually rising to 27.3 ± 14.0% by 45 minutes and declining to 20.3 ± 7.5% at 60 minutes. The number of type 4 cells produced gradually rose from a minimum of 0.3 ± 0.6% at 0.5 minutes to a maximum of 55.3 ± 18.2% by 30 minutes then gradually declined to 42.0 ± 30.5% by 60 minutes.

7. 2. 1. 5. Response to 0.1 nM LTB_4 (Fig. 7. 1. d.)

Neutrophils suspended in 0.1 nM LTB_4 displayed an immediate morphological response with only 9.0 ± 2.6% of cells retaining a spherical shape at 0.5 minutes after commencing incubation. The number of spherical cells further declined to a minimum of 4.3 ± 1.2% at 1 minute then gradually rose to >95% by 45 minutes onwards. The number of type 1 cells produced gradually declined from a maximum of 27.7 ± 4.5% at 0.5 minutes to <5% by 5 minutes onwards. The number of type 2 cells produced declined from a maximum of 58.7 ± 0.6% at 0.5 minutes to 41.7 ± 9.0% at 1 minute then rose to 44.7 ± 10.7% at 5 minutes before gradually declining to <5% by 30 minutes onwards. The number of type 3 cells produced rose from 3.7 ± 2.1% at 0.5 minutes to a maximum of 38.7 ± 11.2% at 1 minute then gradually declined to ≤5% by 15 minutes onwards. The number of type 4 cells produced gradually rose from 0.7 ± 0.6% at 0.5 minutes to a maximum of 8.7 ± 8.0% by 5 minutes and then declined to ≤5% by 15 minutes onwards.

Fig. 7. 1. Time courses of the morphological responses of neutrophils in suspension to various concentrations of LTB4

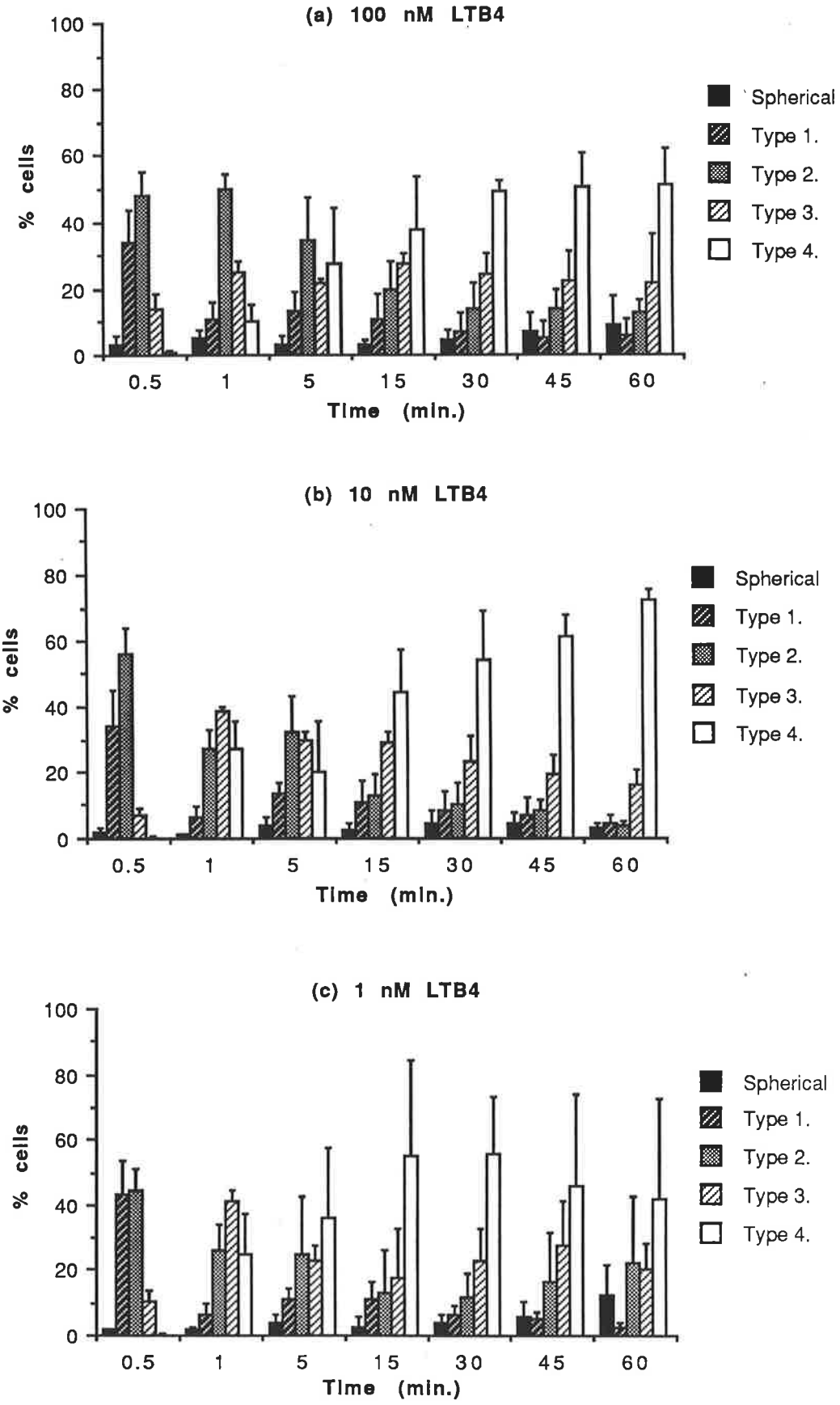
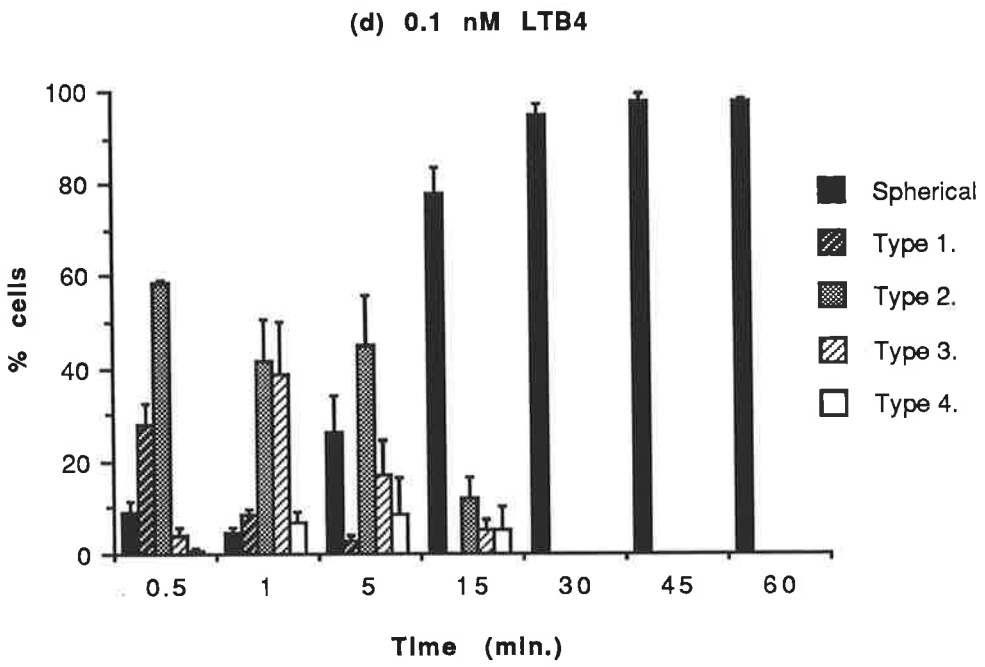


Fig. 7. 1. (continued)



7. 2. 2. Effects of cation chelating agents on the morphological response of neutrophils to LTB_4

7. 2. 2. 1. Design of experiments

The aim of these experiments was to examine the possible role of extracellular Ca^{2+} and Mg^{2+} ions during the morphological response of neutrophils in suspension to LTB_4. The morphological responses to LTB_4 were studied by suspending neutrophils for 30 minutes at 37 °C in HBSS-Hepes containing 10 or 1 nM LTB_4. The role of extracellular Ca^{2+} and Mg^{2+} ions during these responses was examined by including either 5 mM EDTA or 5 mM EGTA in the suspension medium. Controls were perfomed by suspending cells in the absence of cation chelating agents. At the end of each incubation, cells were fixed with glutaraldehyde and their morphology assessed by visual classification of morphological subtypes. Responses in the presence of cation chelating agents were compared with control responses by using a Chi-square goodness-of-fit test on the mean proportions of morphological subtypes calculated from three experiments (significantly different when $X^2 \geq 9.488$). Cells from a different donor were used during each experiment.

7. 2. 2. 2. Effects on response to 10 nM LTB_4 (Fig. 7. 2. a.)

The proportions of morphological subtypes displayed by neutrophils after suspension in 10 nM LTB_4, in the absence of chelating agents, were: 2.7 ± 0.6% spherical, 4.7 ± 2.1% type 1, 6.0 ± 2.6% type 2, 24.3 ± 13.7% type 3, and 63.3 ± 9.0% type 4.

The proportions of morphological subtypes displayed by neutrophils after suspension in 10 nM LTB_4 in the presence of 5 mM EDTA were: 1.3 ± 0.6% spherical, 6.0 ± 2.0% type 1, 5.7 ± 0.6% type 2, 16.7 ± 5.1% type 3, and 71.0 ± 4.0% type 4. These proportions of morphological subtypes were not significantly

different to those those displayed by cells suspended in 10 nM LTB_4 in the absence of chelating agents (X^2= 4.43).

The proportions of morphological subtypes displayed by neutrophils after suspension in 10 nM LTB_4 in the presence of 5 mM EGTA were: 2.0 ± 1.0% spherical, 4.0 ± 2.6% type 1, 6.0 ± 2.6% type 2, 15.7 ± 6.4% type 3, and 72.3 ± 8.1% type 4. These proportions of morphological subtypes were not significantly different to those those displayed by cells suspended in 10 nM LTB_4 in the absence of chelating agents (X^2= 4.60).

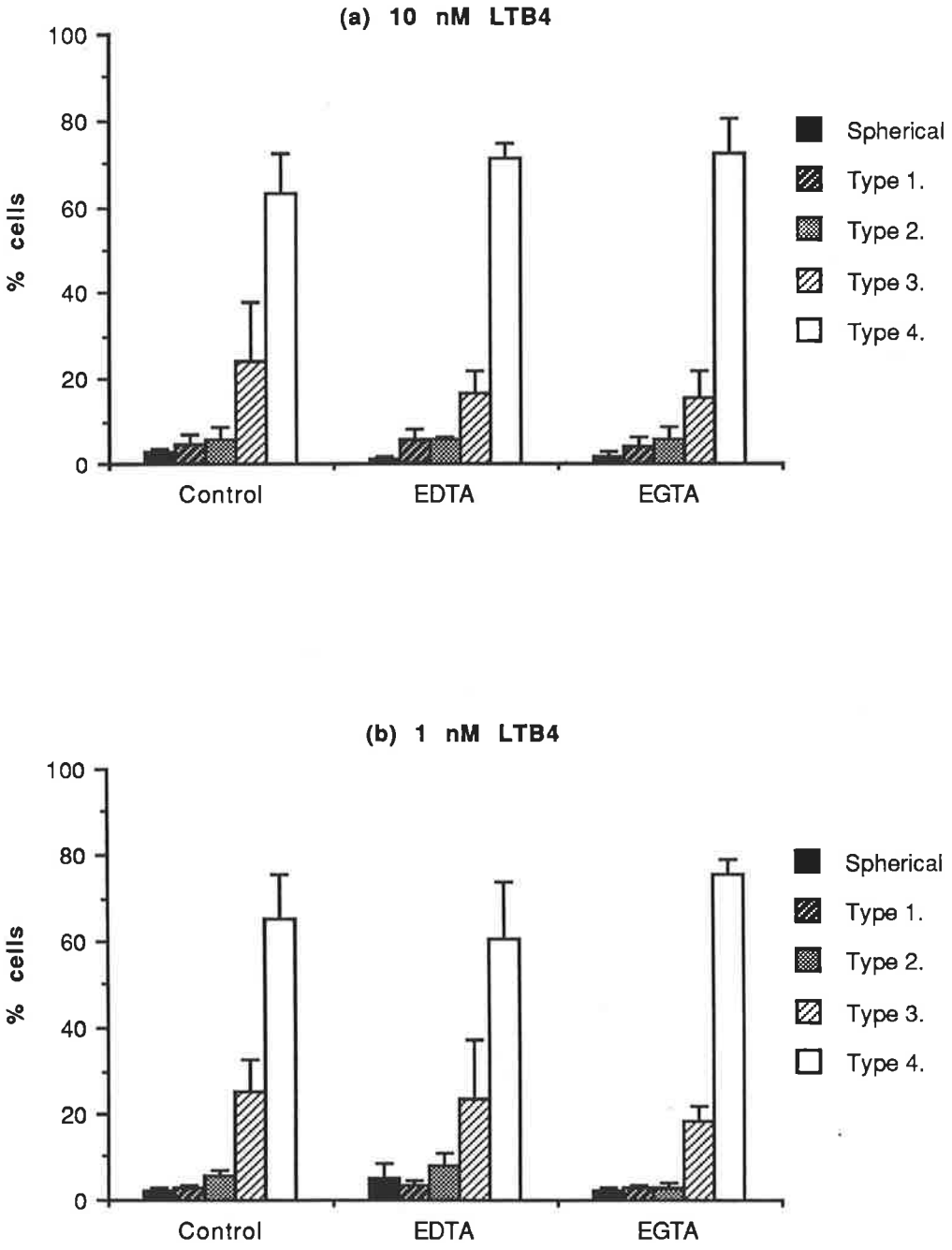
7. 2. 2. 3. Effects on response to 1 nM LTB_4 (Fig. 7. 2. b.)

The proportions of morphological subtypes displayed by neutrophils after suspension in 1 nM LTB_4, in the absence of chelating agents, were: 2.3 ± 0.6% spherical, 2.7 ± 0.6% type 1, 6.0 ± 1.0% type 2, 25.0 ± 7.8% type 3, and 65.0 ± 10.4% type 4.

The proportions of morphological subtypes displayed by neutrophils after suspension in 1 nM LTB_4 in the presence of 5 mM EDTA were: 5.3 ± 3.2% spherical, 3.3 ± 1.2% type 1, 8.0 ± 2.6% type 2, 23.7 ± 13.3% type 3, and 60.3 ± 13.6% type 4. These proportions of morphological subtypes were not significantly different to those displayed by cells suspended in 1 nM LTB_4 in the absence of chelating agents (X^2= 5.12).

The proportions of morphological subtypes displayed by neutrophils after suspension in 1 nM LTB_4 in the presence of 5 mM EGTA were: 2.0 ± 1.0% spherical, 2.7 ± 0.6% type 1, 3.0 ± 1.0% type 2, 18.0 ± 3.6% type 3, 75.7 ± 2.9% type 4. These proportions of morphological subtypes were not significantly different to those displayed by cells suspended in 1 nM LTB_4 in the absence of chelating agents (X^2= 5.26).

Fig. 7. 2. Effects of cation chelating agents on the morphological responses of neutrophils to various concentrations of LTB4



7. 2. 3. Effect of TMB-8 on the morphological response of neutrophils to LTB_4

7. 2. 3. 1. Design of experiments

The purpose of these experiments was to examine the possible role of the release Ca^{2+} ions from intracellular stores during the morphological response of neutrophils in suspension to LTB_4. The morphological response to LTB4 was studied by suspending neutrophils for 30 minutes at 37 °C in HBSS-Hepes containing 10 nM LTB_4. The role of intracellular calcium release was examined by pretreating neutrophils for 10 minutes at 37 °C with 5 x 10^{-4} M TMB-8 before addition of chemotactic factors. Controls were perfomed by pre-incubating cells in the absence of TMB-8. At the end of each incubation, cells were fixed with glutaraldehyde and their morphology assessed by visual classification of morphological subtypes. This experiment was perfomed once.

7. 2. 3. 2. Effect of TMB-8 on response LTB_4 (Fig. 7. 3.)

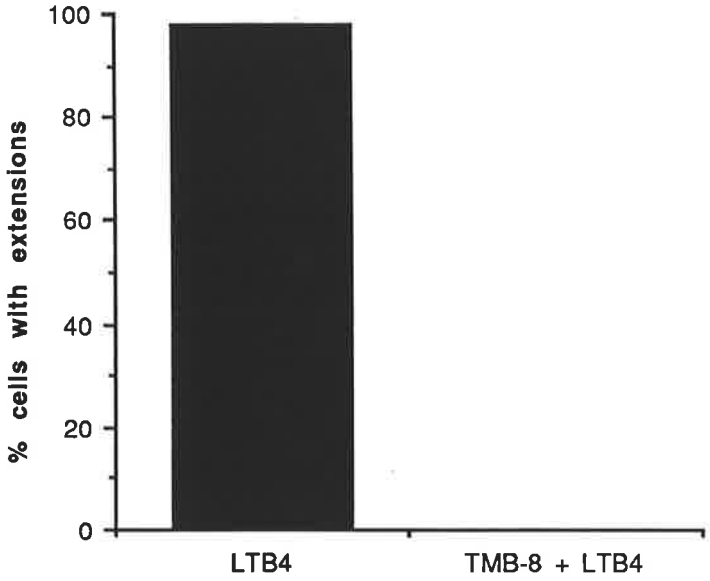
While 98% of neutrophils pre-incubated in HBSS-Hepes displayed extensions of the cytoplasm following addition of LTB_4, 0% of neutrophils pre-incubated with TMB-8 displayed pseudopodia following subsequent incubation with LTB_4.

7. 2. 4. Effect of LTB_4 on the F-actin distribution of neutrophils in suspension (Fig. 7. 4.)

7. 2. 4. 1. Design of experiments

Neutrophils were suspended in 10 nM LTB_4/4% human serum albumin for 1, 15 and 30 minutes before being fixed with 8% formalin and stained with the fluorescent F-actin probe, rhodamine phalloidin. The distribution of staining for F-

Fig. 7. 3. Effect of TMB-8 on the morphological response of neutrophils to 10 nM LTB4



actin within the cytoplasm of cells with each morphological subtype was then examined by fluorescence microscopy.

7. 2. 4. 2. F-actin distribution of cells

Cells treated with LTB_4 consistently displayed intense staining for F-actin within cytoplasmic extensions in a similar manner to cells treated with FMLP (see chapter 3., Fig. 3. 5.).

7. 2. 5. Time courses of the morphological responses of neutrophils in suspension to various concentrations of PAF

7. 2. 5. 1. Design of experiments

These experiments were designed to characterise the changes in morphology displayed by neutrophils with time following addition of different concentrations of PAF. Neutrophils were suspended in 4000, 400, 40 and 4 nM PAF (in HBSS-Hepes at 37 °C) for 0.5, 1, 5, 15, 30, 45 and 60 minutes. At the end of each incubation, cells were fixed with glutaraldehyde and their morphology assessed by visual classification of morphological subtypes. Results are expressed as the mean ± standard deviation for the proportions of morphological subtypes obtained from three experiments. Cells from a different donor were used during each experiment.

7. 2. 5. 2. Response to 4000 nM PAF (Fig. 7. 5. a.)

Neutrophils suspended in 4000 nM PAF displayed an immediate morphological response with only 2.3 ± 0.6% of cells retaining a spherical shape at 0.5 minutes after commencing incubation. The number of spherical cells further declined to a minimum of 0.3 ± 0.6% at 1 minute then gradually rose to a maximum of 57.7 ±

41.9% by 60 minutes. The number of type 1 cells produced declined from a maximum of 60.7 ± 20.3% at 0.5 minutes to 29.3 ± 9.3% at 1 minute, then rose to 42.3 ± 6.5% at 5 minutes before gradually declining to a minimum of 5.3 ± 2.3% by 60 minutes. The number of type 2 cells produced rose from 33.0 ± 15.7% at 0.5 minutes to a maximum of 33.3 ± 4.2% at 1 minute, then gradually declined to 18.3 ± 3.5% by 15 minutes before rising to 28.7 ± 12.7% at 30 minutes and gradually declining to 18.3 ± 18.0% by 60 minutes. The number of type 3 cells produced rose from a minimum of 4.0 ± 5.2% at 0.5 minutes to 36.0 ± 12.5% at 1 minute, then declined to 26.0 ± 8.9% at 5 minutes before rising to 50.0 ± 12.8% at 15 minutes and gradually declining to 17.3 ± 25.0% by 60 minutes. The number of type 4 cells produced gradually rose from a minimum of 0.0 ± 0.0% at 0.5 minutes to a maximum of 8.7 ± 9.1% by 5 minutes then remained <5% for the remainder of the experiment.

7. 2. 5. 3. Response to 400 nM PAF (Fig. 7. 5. b.)

Neutrophils suspended in 400 nM PAF displayed an immediate morphological response with only 1.7 ± 0.6% of cells retaining a spherical shape at 0.5 minutes after commencing incubation. The number of spherical cells further declined to a minimum of 1.0 ± 0.0% at 1 minute and then gradually rose to a maximum of 27.0 ± 41.6% by 60 minutes. The number of type 1 cells produced gradually declined from a maximum of 67.7 ± 31.1% at 0.5 minutes to close to 5% by 5 minutes onwards. The number of type 2 cells produced gradually declined from a maximum of 27.7 ± 26.0% at 0.5 minutes to a minimum of 12.7 ± 9.6% by 15 minutes before rising to 20.7 ± 21.5% at 30 minutes and gradually declining to 13.0 ± 8.5% by 60 minutes. The number of type 3 cells produced gradually rose from a minimum of 3.0 ± 5.2% at 0.5 minutes to a maximum of 39.7 ± 22.8% by 15 minutes then gradually declined to 20.3 ± 22.2% by 60 minutes. The number of type 4 cells produced gradually rose from a minimum of 0.0 ± 0.0% at 0.5 minutes to a maximum of 47.0

± 28.2% by 5 minutes before gradually declining to 35.3 ± 40.2% by 45 minutes and rising to 36.0 ± 37.0% at 60 minutes.

7. 2. 5. 4. Response to 40 nM PAF (Fig. 7. 5. c.)

Neutrophils suspended in 40 nM PAF displayed an immediate morphological response with only 2.3 ± 1.5% of cells retaining a spherical shape at 0.5 minutes after commencing incubation. The number of spherical cells further declined to a minimum of 1.0 ± 0.0% at 1 minute and then gradually rose to a maximum of 16.0 ± 17.3% by 60 minutes. The number of type 1 cells produced declined from a maximum of 41.3 ± 9.3% at 0.5 minutes to 14.0 ± 6.1% at 1 minute then rose to 20.0 ± 10.4% at 5 minutes before declining to 11.3 ± 3.5% at 15 minutes, rising to 11.7 ± 1.5% at 30 minutes and gradually declining to a minimum of 7.0 ± 3.0% by 60 minutes. The number of type 2 cells produced declined from a maximum of 49.3 ± 8.1% at 0.5 minutes to 26.0 ± 3.6% at 1 minute and then rose to 34.3 ± 11.1% at 5 minutes before gradually declining to a minimum of 16.3 ± 13.6% by 60 minutes. The number of type 3 cells produced rose from a minimum of 8.0 ± 5.0% at 0.5 minutes to a maximum of 51.3 ± 7.2% at 1 minute then gradually declined to 31.1 ± 4.5% by 30 minutes before rising to 37.7 ± 9.6% at 45 minutes and declining to 34.0 ± 13.2% at 60 minutes. The number of type 4 cells produced rose from a minimum of 0.0 ± 0.0% at 0.5 minutes to 8.0 ± 3.6% at 1 minute then declined to 3.7 ± 2.1% at 5 minutes before gradually rising to a maximum of 35.3 ± 26.3% by 30 minutes and declining to 27.3 ± 21.4% by 60 minutes.

7. 2. 5. 5. Response to 4 nM PAF (Fig. 7. 5. d.)

Neutrophils suspended in 4 nM PAF displayed an immediate morphological response with only 17.0 ± 19.2% of cells retaining a spherical shape at 0.5 minutes after commencing incubation. The number of spherical cells then gradually rose to

Fig. 7. 4. Time courses of the morphological responses of neutrophils in suspension to various concentrations of PAF

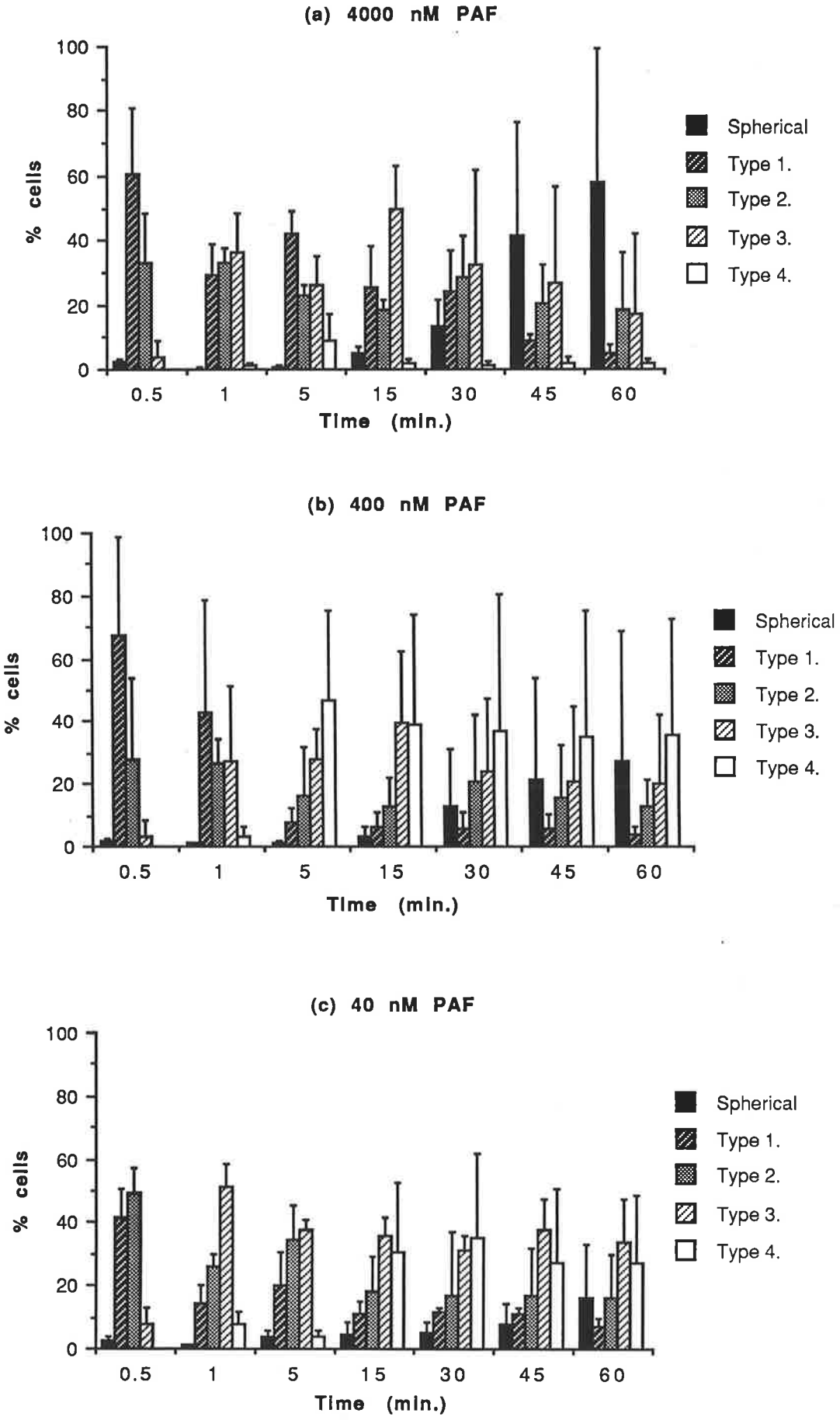
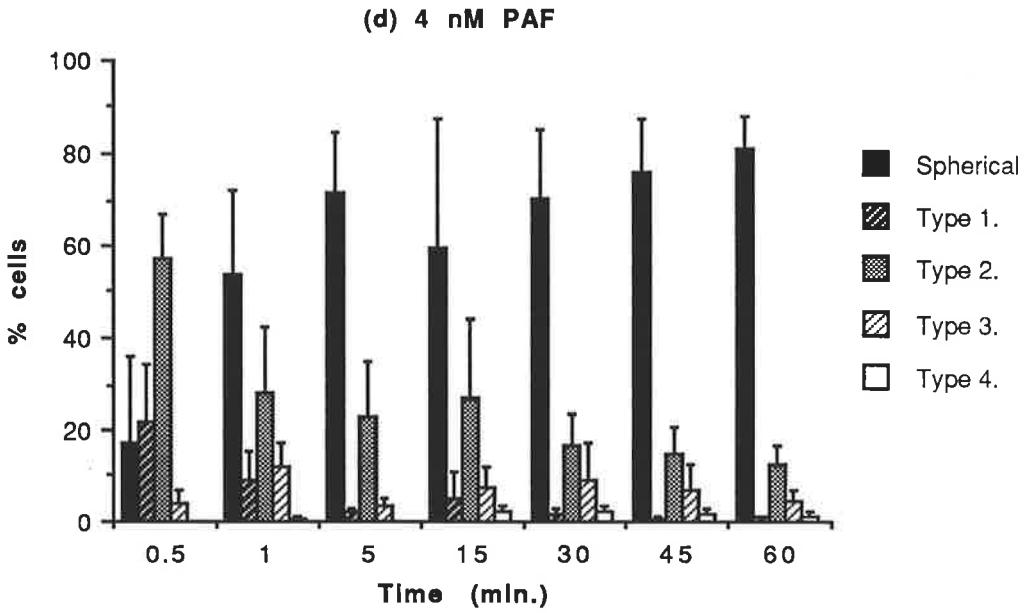


Fig. 7. 4. (continued)



71.7 ± 13.0% by 5 minutes, declined to 59.3 ± 28.0% at 15 minutes and then gradually rose to a maximum of 81.0 ± 7.2% by 60 minutes. The number of type 1 cells produced gradually declined from a maximum of 22.0 ± 12.1% at 0.5 minutes to ≤5% by 5 minutes onwards. The number of type 2 cells produced gradually declined from a maximum of 57.0 ± 9.8% at 0.5 minutes to 22.7 ± 12.1% by 5 minutes then rose to 27.0 ± 17.1% at 15 minutes before gradually declining to a minimum of 12.7 ± 4.0% by 60 minutes. The number of type 3 cells produced rose from 4.0 ± 2.6% at 0.5 minutes to a maximum of 12.0 ± 5.3% at 1 minute then declined to a minimum of 3.7 ± 1.5% at 5 minutes before gradually rising to 9.3 ± 7.6% by 30 minutes and gradually declining to 4.3 ± 2.5% by 60 minutes. The number of type 4 cells produced was always <5% and ranged from a minimum of 0.0 ± 0.0% at 0.5 minutes and 5 minutes to a maximum of 2.0 ± 1.7% at 15 and 30 minutes.

7. 2. 6. Effects of cation chelating agents on the morphological response of neutrophils to PAF

7. 2. 6. 1. Design of experiments

The aim of these experiments was to examine the possible role of extracellular Ca^{2+} and Mg^{2+} ions during the morphological response of neutrophils in suspension to PAF. The morphological responses to PAF were studied by suspending neutrophils for 30 minutes at 37 °C in HBSS-Hepes containing 4000, 400, 40 and 4 nM PAF. The role of extracellular Ca^{2+} and Mg^{2+} ions during these responses was examined by including either 5 mM EDTA or 5 mM EGTA in the suspension medium. Controls were perfomed by suspending cells in the absence of cation chelating agents. At the end of each incubation, cells were fixed with glutaraldehyde and their morphology assessed by visual classification of morphological subtypes. Responses in the presence of cation chelating agents were compared with control responses by

using a Chi-square goodness-of-fit test on the mean proportions of morphological subtypes calculated from three experiments (significantly different when $X^2 \geq 9.488$). Cells from a different donor were used during each experiment.

7. 2. 6. 2. Effects on response to 4000 nM PAF (Fig. 7. 6. a.)

The proportions of morphological subtypes displayed by neutrophils after suspension in 4000 nM PAF, in the absence of chelating agents were: 24.0 ± 10.1% spherical, 38.7 ± 9.6% type 1, 32.0 ± 15.9% type 2, 4.7 ± 3.8% type 3, and 0.0 ± 0.0% type 4.

The proportions of morphological subtypes displayed by neutrophils after suspension in 4000 nM PAF, in the presence of 5 mM EDTA were: 18.7 ± 5.7% spherical, 41.7 ± 10.7% type 1, 25.7 ± 3.1% type 2, 14.0 ± 3.6% type 3, and 1.0 ± 0.0% type 4. These proportions of morphological subtypes were significantly different to those displayed by cells suspended in 4000 nM PAF in the absence of chelating agents ($X^2 = 27.49$).

The proportions of morphological subtypes displayed by neutrophils after suspension in 4000 nM PAF, in the presence of 5 mM EGTA were: 24.3 ± 12.9% spherical, 38.3 ± 10.8% type 1, 27.7 ± 4.0% type 1, 9.7 ± 3.1% type 2, 0.3 ± 0.6% type 4. These proportions of morphological subtypes were not significantly different to those displayed by cells suspended in 4000 nM PAF in the absence of chelating agents ($X^2 = 5.90$).

7. 2. 6. 3. Effects on response to 400 nM PAF (Fig. 7. 6. b.)

The proportions of morphological subtypes displayed by neutrophils after suspension in 400 nM PAF, in the absence of chelating agents were: 7.0 ± 3.5% spherical, 13.3 ± 4.2% type 1, 29.7 ± 6.4% type 2, 41.3 ± 9.1% type 3, and 9.3 ± 2.5% type 4.

The proportions of morphological subtypes displayed by neutrophils after suspension in 400 nM PAF, in the presence of 5 mM EDTA were: 25.3 ± 3.5% spherical, 4.0 ± 1.0% type 1, 38.0 ± 6.1% type 2, 21.7 ± 4.0% type 3, and 10.7 ± 1.5% type 4. These proportions of morphological subtypes were significantly different to those displayed by cells suspended in 400 nM PAF in the absence of chelating agents (X^2= 66.17).

The proportions of morphological subtypes displayed by neutrophils after suspension in 400 nM PAF, in the presence of 5 mM EGTA were: 38.7 ± 1.5% spherical, 2.0 ± 1.0% type 1, 36.0 ± 2.0% type 2, 16.0 ± 2.6% type 3, and 7.0 ± 1.0% type 4. These proportions of morphological subtypes were significantly different to those displayed by cells suspended in 400 nM PAF in the absence of chelating agents (X^2= 170.56).

7. 2. 6. 4. Effects on response to 40 nM PAF (Fig. 7. 6. c.)

The proportions of morphological subtypes displayed by neutrophils after suspension in 40 nM PAF, in the absence of chelating agents were: 20.0 ± 14.3% spherical, 6.0 ± 2.0% type 1, 38.7 ± 6.7% type 2, 26.0 ± 6.2% type 3, and 10.0 ± 2.6% type 4.

The proportions of morphological subtypes displayed by neutrophils after suspension in 40 nM PAF, in the presence of 5 mM EDTA were: 27.0 ± 5.3% spherical, 3.0 ± 1.0% type 1, 23.0 ± 13.0% type 2, 22.0 ± 2.6% type 3, and 25.0 ± 9.6% type 4. These proportions of morphological subtypes were significantly different to those displayed by cells suspended in 40 nM PAF in the absence of chelating agents (X^2= 33.40).

The proportions of morphological subtypes displayed by neutrophils after suspension in 40 nM PAF, in the presence of 5 mM EGTA were: 33.0 ± 7.9% spherical, 2.7 ± 2.1% type 1, 26.0 ± 14.0% type 2, 18.7 ± 5.1% type 3, and 20.0 ± 0.0% type 4. These proportions of morphological subtypes were significantly

Fig. 7. 5. Effects of cation chelating agents on the morphological responses of neutrophils to various concentrations of PAF

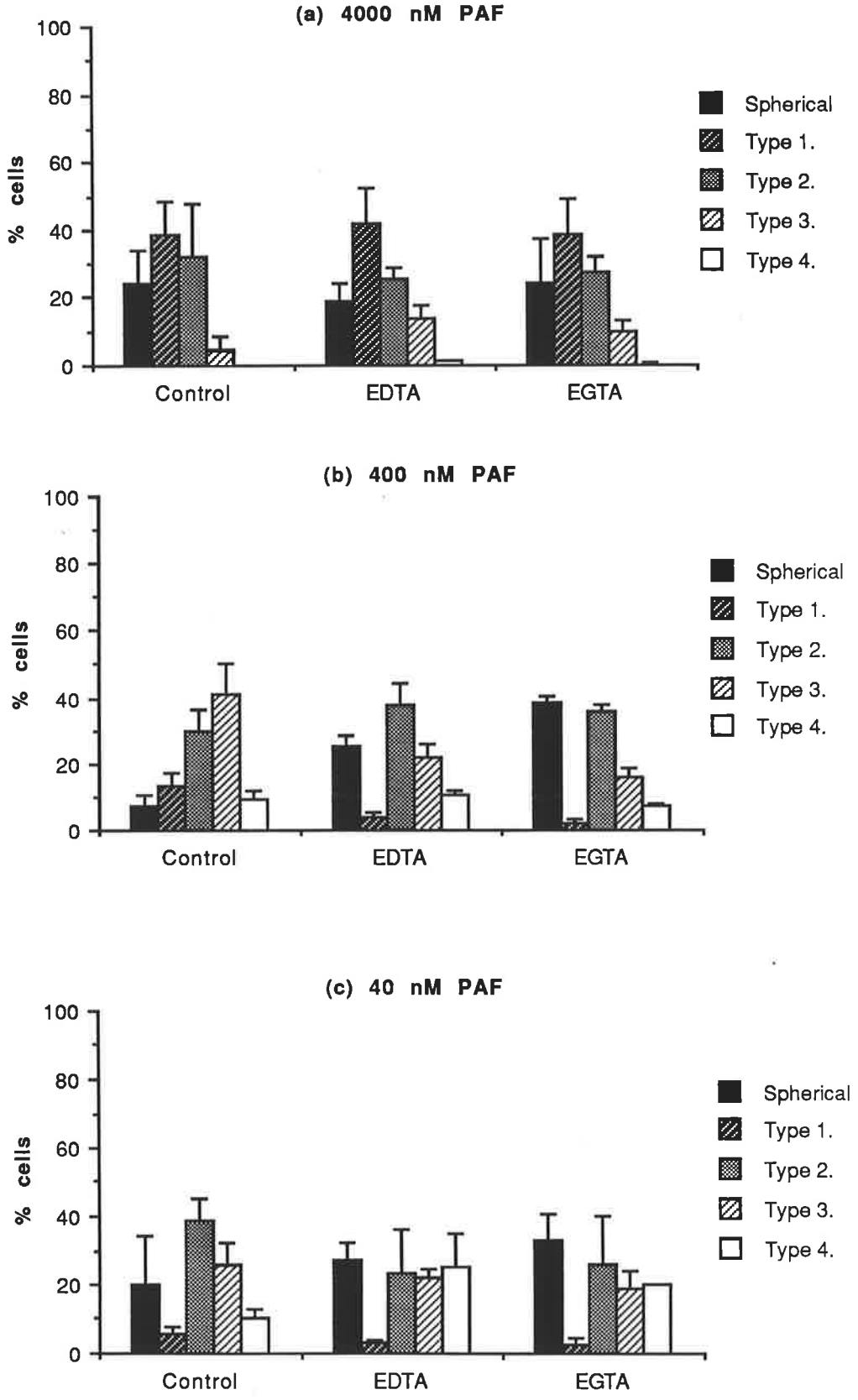
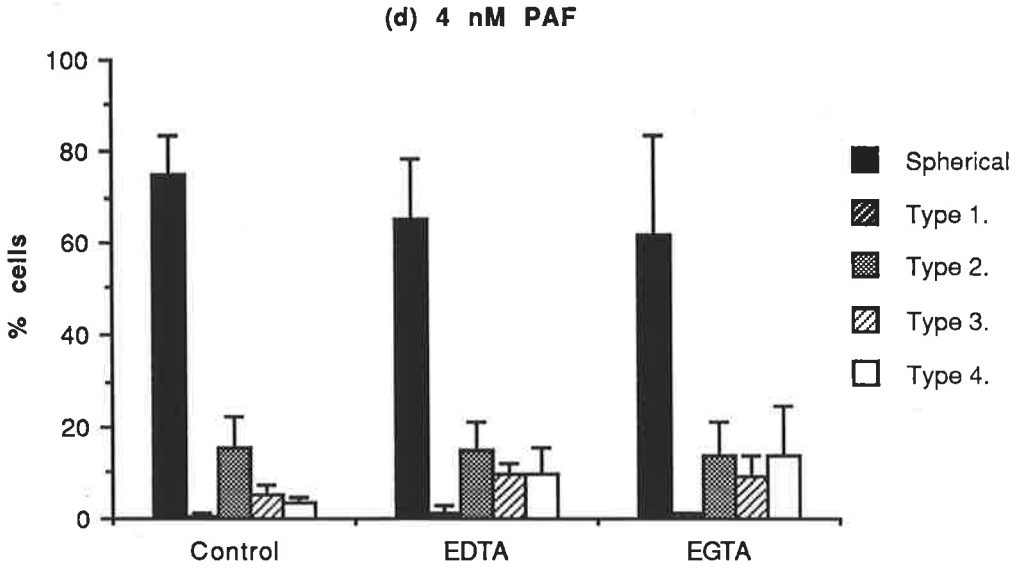


Fig. 7. 5. (continued)



different to those displayed by cells suspended in 40 nM PAF in the absence of chelating agents (X^2= 26.49).

7. 2. 6. 5. Effects on response to 4 nM PAF (Fig. 7. 6. d.)

The proportions of morphological subtypes displayed by neutrophils after suspension in 4 nM PAF, in the absence of chelating agents were: 75.0 ± 8.2% spherical, 0.7 ± 0.6% type 1, 15.7 ± 6.5% type 2, 5.3 ± 2.3% type 3, and 3.3 ± 1.5% type 4.

The proportions of morphological subtypes displayed by neutrophils after suspension in 4 nM PAF, in the presence of 5 mM EDTA were: 65.0 ± 13.2% spherical, 1.3 ± 1.5% type 1, 14.7 ± 6.5% type 2, 9.7 ± 2.1% type 3, and 9.7 ± 5.5% type 4. These proportions of morphological subtypes were significantly different to those displayed by cells suspended in 4 nM PAF in the absence of chelating agents (X^2= 17.96).

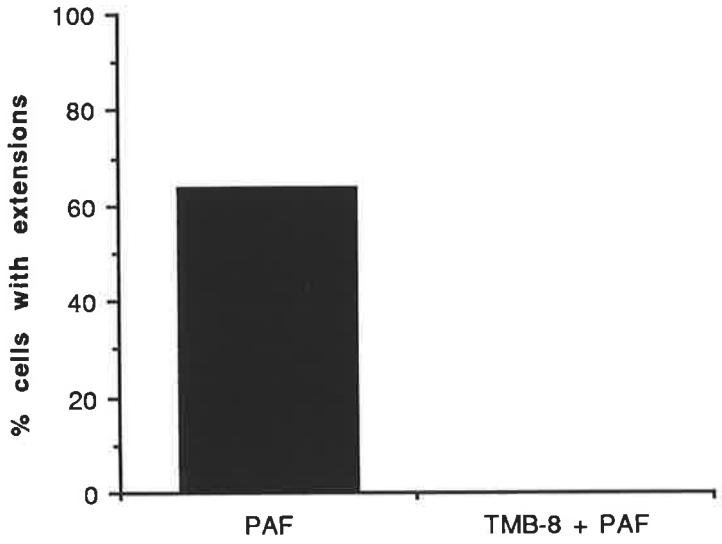
The proportions of morphological subtypes displayed by neutrophils after suspension in 4 nM PAF, in the presence of 5 mM EGTA were: 62.0 ± 21.7% spherical, 1.0 ± 0.0% type 1, 14.0 ± 7.2% type 2, 9.3 ± 4.2% type 3, and 13.7 ± 11.0% type 4. These proportions of morphological subtypes were significantly different to those displayed by cells suspended in 4 nM PAF in the absence of chelating agents (X^2= 38.38).

7. 2. 7. Effect of TMB-8 on the morphological response of neutrophils to PAF

7. 2. 7. 1. Design of experiments

The purpose of these experiments was to examine the possible role of the release Ca^{2+} ions from intracellular stores during the morphological response of neutrophils in suspension to PAF. The morphological response to PAF was studied by

Fig. 7. 6. Effect of TMB-8 on the morphological response of neutrophils to 40 nM PAF



suspending neutrophils for 30 minutes at 37 °C in HBSS-Hepes containing 40 nM M PAF. The role of intracellular calcium release was examined by pretreating neutrophils for 10 minutes at 37 °C with 5 x 10-4 M TMB-8 before addition of chemotactic factors. Controls were perfomed by pre-incubating cells in the absence of TMB-8. At the end of each incubation, cells were fixed with glutaraldehyde and their morphology assessed by visual classification of morphological subtypes. This experiment was perfomed once.

7. 2. 7. 2. Effect of TMB-8 on response to PAF (Fig. 7. 7.)

While 64% of neutrophils pre-incubated in HBSS-Hepes displayed extensions of the cytoplasm following addition of PAF, 0% of neutrophils pre-incubated with TMB-8 displayed pseudopodia following subsequent incubation with PAF.

7. 2. 8. Effect of PAF on the F-actin distribution of neutrophils (Fig. 7. 8.)

7. 2. 8. 1. Design of experiments

Neutrophils were suspended in 40 nM PAF/4% human serum albumin for 1, 15 and 30 minutes before being fixed with 8% formalin and stained with the fluorescent F-actin probe, rhodamine phalloidin. The distribution of staining for F-actin within the cytoplasm of cells with each morphological subtype was then examined by fluorescence microscopy.

7. 2. 8. 2. F-actin distribution of cells

Cells treated with PAF consistently displayed intense staining for F-actin within cytoplasmic extensions in a similar manner to cells treated with FMLP (see chapter 3., Fig. 3. 5.).

7. 3. Summary

Leukotriene-B_4 induced polarisation of neutrophils in suspension at concentrations of 100, 10, 1 and 0.1 nM. During responses to LTB_4 cells generally displayed cytoplasmic extensions within 30 seconds which remained for the duration of the 60 minutes incubation. However, extensions disappeared from the surface of cells suspended in 0.1 nM LTB_4 after 30 minutes. The greatest proportion of fully polarised cells (type 4 cells) were observed when cells were incubated in 10 nM LTB_4. Supra-optimal concentrations of LTB_4 induced formation of low numbers of type 4 cells, but did not maintain higher numbers of non-polarised cells (type 1) as noted during responses to supra-optimal concentrations of FMLP (Chapter 3) and C5a (Chapter 6).

Platelet activating factor induced polarisation of neutrophils in suspension at concentrations of 4000, 400, 40 and 4 nM. During responses to PAF cytoplasmic extensions were apparent within 30 seconds and remained for the 60 minutes incubation. However, the number of cells displaying extensions declined during the incubation with highest numbers remaining in 40 nM PAF. The greatest proportion of type 4 cells were induced by PAF at concentrations of 40 nM to 400 nM. Supra-optimal concentrations of PAF (4000 nM) maintained higher numbers of non-polarised cells and induced formation of few type 4 cells.

The orders in which each morphological subtype reached its highest proportion during responses to LTB_4 and PAF were generally similar to that observed in previous chapters during responses to other chemotactic factors, with type 1 cells appearing early and type 4 cells appearing last.

While responses of neutrophils to optimal and supra-optimal concentrations of LTB_4 were unaffected by chelators of extracellular Ca^{2+} and Mg^{2+} ions, responses to various concentrations of PAF were significantly affected by these agents. Responses to supra-optimal concentrations (4000 nM) were slightly enhanced

(more elongated cells, i.e. type 3 cells) in the presence of EDTA, but were unaffected by EGTA. Fewer cells displayed extensions of the cytoplasm in response to optimal concentrations of PAF (40 nM and 400 nM) in the presence of EDTA or EGTA with EGTA having the greater inhibitory effect. However, the proportions of type 4 cells induced by optimal concentrations were either slightly increased (40 nM) or unaffected (400 nM). Finally, responses of cells to sub-optimal concentrations of PAF (4 nM) were slightly enhanced (more cells with extensions and more type 4 cells) in the presence of chelating agents, but these effects appeared small.

TMB-8 abolished the morphological responses of neutrophils to both LTB_4 and PAF.

F-actin accumulated within the pseudopodia of neutrophils treated with either LTB_4 or PAF in a similar manner to those treated with FMLP.

CHAPTER 8.

GENERAL DISCUSSION

8. 1. Introduction

Throughout all studies of the present work, neutrophils remained spherical when suspended in Hanks' balanced salt solution (HBSS) alone, but developed polarised extensions in the presence of a variety of factors including the synthetic bacterial peptide FMLP, plasma, immunoglobulin type G and inflammatory mediators. Abnormal morphological changes, such as zeiosis (Bessis, 1976) were rarely observed and all cell preparations remained viable for the periods of the experiments. The consistency with which neutrophils remained spherical in HBSS is similar to observations by Shields and Haston (1985), Haston and Shields (1986) and Bignold and Ferrante (1988). Early studies by Lichtman et al. (1976) had indicated that neutrophils developed cytoplasmic extensions when suspended in HBSS alone. However, although this difference has not been discussed at length in the literature, it seems likely that the observations of Lichtman et al. (1976) may have been influenced by technical difficulties such as those encountered in the methods chapter of this thesis (see section, 2. 1. 3., HBSS-Hepes). The findings of the present work therefore demonstrate neutrophil polarisation in suspension to be a morphological response to external stimuli rather than an intrinsic activity of the cells.

The major issues raised by the present work are: how neutrophil polarisation should be measured, what is the nature of the factors which stimulate this response in plasma, and how this response compares to that induced by FMLP and chemotactic inflammatory mediators. In addition, data has been accumulated concerning the cell biological basis of polarisation of neutrophils in suspension.

8. 2. How should neutrophil polarisation be measured?

Three methods were examined in this thesis as techniques for the analysis of neutrophil polarisation: visual classification, morphometry and fluorescence activated cell sorter (FACS) analysis. These techniques were chosen on the basis of the following observations. Visual classification had previously been used by authors to establish that variations occur in the morphological response of neutrophils to FMLP according to time and FMLP concentration (Keller, 1983; Keller et al., 1983; Shields and Haston, 1985; Bignold and Ferrante, 1988). Morphometry had been used to obtain a more reliable index of these variations in cell shape because of the less subjective nature of this technique compared with visual analysis (Shields and Haston, 1985). Finally, FACS analysis had recently provided authors with a simple and rapid method for analysing large numbers of cells (Meshulam et al., 1986; Donabedian et al., 1987). This last technique seemed particularly attractive because of the numerous samples required when studying the effects of time and stimulus concentration on morphological responses of neutrophils (Roos et al., 1987).

When visual classification, morphometry and FACS analysis were compared as techniques for analysis of neutrophil polarisation induced by FMLP, all three techniques were found to be sensitive to variations in the morphology of cells with time, but each technique provided different indications of the nature of this shape change. Visual classification allowed the formation, distribution and type of cytoplasmic extensions on neutrophils to be detected. Morphometry allowed an increase in cell size related to area and perimeter to be detected and also demonstrated an increase in the ellipticity of the cells, but details on cytoplasmic extensions were not provided from this data. Finally FACS analysis allowed an increase in cell size related to mean forwards light scatter (FSC) to be detected, but this parameter provided no indication of changes in cell shape. Furthermore, the lack of a visual component during FACS analysis meant that contaminating particulate material such as red blood cells,

aggregates of cells or damaged cells were difficult to totally exclude from analysis even after application of a "granulocyte gate" (see section, 2. 2. 9.). On the basis of these observations visual classification provided a more detailed and more reliable analysis of neutrophil polarisation than the other techniques tested.

The use of visual classification in preference to the other techniques described in this thesis is also supported on the basis of cell biological aspects of neutrophil motility. Firstly, although various chemicals including PMA and chemotactic factors (Roos et al., 1987) induce neutrophils to develop cytoplasmic extensions, motility of these cells is only observed when these extensions are polarised to one side of the cell. Secondly, Keller (1983) and Keller et al. (1983) have reported the appearance of a uropod or rear extension on neutrophils to correlate with optimal motility of these cells. Since, neutrophil polarisation assays are intended to provide an index of neutrophil motility, consideration of the above indicates that these assays should be sensitive to the formation, distribution and type of cytoplasmic extensions displayed by neutrophils.

Visual classification therefore appeared to be the most appropriate method for assessing morphological responses in neutrophils, but there is some uncertainty in the literature concerning which categories of morphological subtype should be used. From the above considerations of motility it is clear that categories of non-polarised, polarised and polarised with uropod (type 4 cells) should be included. In addition, non-polarised cells should be separated into categories of spherical and "with extensions" (type 1 cells) to differentiate between responding and non-responding cells. However, a reason for further categorising polarised cells according to whether their body is round (type 2 cells) or elongated (type 3 cells) has yet to be established on the basis of motility studies. Nevertheless, Haston and Shields (1986) have demonstrated elongation of cells during time course studies of polarisation of individual cells. While continuous analysis of individual cells was not practicable in the present work (see section 1. 5. 6. 1.), the sequence in which each morphological subtype predominated (subtype 2 followed by 3) is consistent with observations made

by Haston and Shields (1986) of individual cells. Distinction between morphological subtypes 2 and 3 therefore contributes to providing a measurement of the degree of polarity displayed by neutrophils.

In the current studies, visual classification of cells into the morphological subtypes spherical, type 1, type 2, type 3 and type 4 is supported on the basis that no additional subtypes were required during assessment of responses to all stimuli examined. Furthermore, the distinction between these categories of morphological subtype allowed differences between various responses to be detected. In particular the absence of type 4 cells in plasma illustrated a difference between this response and that induced by chemotactic factors. Simpler visual classification methods which only differentiate between polarised and non-polarised cells (Lichtman et al., 1976; Keller and Cottier, 1981; Haston and Shields, 1985; 1986; Lopez et al., 1986; Bignold, 1987a; Haston and Wilkinson, 1988; Lord and Roath, 1990) would not have detected this difference.

A possible disadvantage of visual classification is that this method is more subjective than morphometric and FACS analysis techniques. However, the criteria used to differentiate between morphological subtypes in this thesis were objectively validated by morphometric analysis.

Nevertheless, visual classification of cells into morphological subtypes is a time consuming process and at times can become tedious for the observer. An answer to this problem might be to use a computer software application which can be programmed to automatically recognise and calculate proportions of different morphological subtypes displayed by cells. Image processing systems for studying motility and chemotaxis of various cell types have been described by Donovan et al (1987), Pedersen et al (1988) and Fisher et al (1989).

8. 3. Are products of the plasma activation systems the cause of neutrophil polarisation in plasma?

The coagulation, fibrinolytic, kinin and complement systems of plasma can be activated by contact with a variety of synthetic materials in vitro and peptides produced by these systems have been reported to be chemotactic for neutrophils (Stecher et al., 1971; Skogen et al.,1988; Kaplan et al., 1972; Hugli and Morgan, 1984). Since the plasma used in this thesis was unavoidably exposed to synthetic materials such as plastics and siliconised glass during it's preparation, it was considered that the neutrophil-polarising activity of this plasma may have been induced by chemotactic peptides derived from either one of the activation systems of plasma. This explanation is supported by the discovery of polarisation activity in the lowest molecular weight (≤10 kDa) fraction of plasma proteins separated by chromatography on Sephacryl 300, which was similar to that of whole plasma with respect to its sensitivity to extracellular cations. Furthermore, the morphological subtypes displayed by neutrophils were similar to those produced by low concentrations of chemotactic factors (i.e. low numbers of type 4 cells). The low number of type 4 cells induced by plasma and low concentrations of chemotactic factors in HBSS may explain the lack of chemotactic activity in these media.

The notion that coagulation products might be responsible for polarising neutrophils suspended in heparinised plasma is supported by the finding that heparin reduced the polarisation of neutrophils induced by plasma and did not affect polarisation induced by FMLP. However, if coagulation products were responsible for neutrophil polarisation in plasma during these experiments they must have been formed at an early stage during the coagulation pathway because fibrin clots were never observed even when the lowest concentration of heparin was used to anticoagulate whole blood (5 I.U/ml). Nevertheless, a small quantity of intermediary coagulation cascade products may have been formed between drawing of blood and

addition of heparin. The greater shape change displayed by neutrophils suspended in serum compared with those suspended in plasma, also suggests that products of coagulation might be responsible for polarising neutrophils in plasma. However, the markedly different responses of neutrophils to plasma anticoagulated with different Ca^{2+} chelating agents suggest that the activity of these plasmas was dependent upon the ability of these agents to remove Mg^{2+} ions rather than inhibit coagulation. In addition, serum fractionated on S300 failed to display the low molecular weight fraction observed in plasma, which displayed similar activity to whole plasma.

Fibrinolytic products seem not to be relevant to the polarisation of neutrophils by plasma because the short time interval between adding anticoagulant to the fresh blood, confirmed by the absence of fibrin clots throughout, indicates that products of fibrinolysis were unlikely to have been formed.

With regard to the kinin system, kallikrein is unlikely to be directly responsible for polarising neutrophils suspended in plasma since soybean trypsin inhibitor (STI), which has been demonstrated to block the chemotactic activity of this enzyme for neutrophils, failed to reduce the polarisation of neutrophils in plasma. However, since STI dramatically enhanced the activity of plasma, it is possible that kallikrein or some other plasma protease inhibited by STI such as plasmin, may regulate neutrophil polarisation in plasma by degrading substrates which are required for the formation and/or activity of the stimulatory factor(s) responsible for this response.

With regard to the complement system, the low responses of neutrophils towards plasma anticoagulated with either EDTA or citrate suggest that activation of this system may be involved. In addition, the response of neutrophils to plasma anticoagulated with EGTA is still consistent with activation of the complement system since this chelating agent inhibits the classical but not the alternative pathway of complement activation (see section,1. 3. 6. 1.). Furthermore, the larger response of neutrophils towards plasma preincubated with Mg^{2+} ions before addition of EDTA is consistent with the possibility that a Mg^{2+} dependent enzyme such as the C3 convertase

of the complement system may mediate formation of active factors in plasma. However, since the effects of chelating agents on the morphological response of neutrophils to purified C5a were opposite to those observed during neutrophil polarisation in heparinised plasma, the factor responsible for polarising neutrophils in plasma is unlikely to be derived from complement activation. Nevertheless, studies are required to examine the responses of neutrophils to other derivatives of the complement system such as C3a and C3bi.

Taken together, the above observations are consistent with the notion that the factors responsible for polarising neutrophils in plasma are formed in a protease/Mg^{2+} dependent manner following the removal of blood from the body, but since the interactions between the activation systems of plasma involving proteases and cations are complex the identity of the specific factor(s) responsible for stimulating this response remain unresolved. In addition this issue is complicated by the fact that inhibitors such as chelating agents which are added to plasma may affect either the formation or activity of factors responsible for polarising neutrophils in plasma.

Further analysis of neutrophil polarisation in plasma might therefore involve separating processes required for the formation of stimulatory factors from those required during the response of neutrophils to these factors. In addition, future studies could include examine the morphological responses of neutrophils to combinations of plasma derived factors such as C5a, IgG (see below) and albumin. This might cast light on why there was a greater response in 50% plasma compared to 90%, since an inhibitor could conceivably be acting in the higher concentration of plasma but be less effective in lower concentrations.

8. 4. Does IgG polarise neutrophils in plasma and what is the mechanism of IgG-induced neutrophil polarisation?

Considering that commercial preparations of IgG are potent stimuli of neutrophil polarisation (active at 0.0005%), that high concentrations of IgG are present in plasma (up to 1.6% w/v) and that IgG is a known stimulus of neutrophils, it appears likely that this immunoglobulin may be responsible for polarising neutrophils suspended in plasma. However, three observations prevent such a direct conclusion being made. Firstly, whereas neutrophils developed cytoplasmic extensions within seconds of their suspension in plasma, neutrophils suspended in solutions of IgG displayed little or no change in shape until at least 5 minutes. Secondly, the reduced responsiveness of neutrophils towards ultra-centrifuged preparations of IgG indicates that aggregated rather than native monomeric IgG is the stimulus of neutrophil polarisation. Thirdly, while EDTA reduced responses of neutrophils to plasma and IgG, EGTA only reduced the response to plasma.

Since IgG has not been previously reported to stimulate neutrophil polarisation, the opportunity was taken to examine the mechanism of this morphological response further. The ability of supernatants from IgG-treated neutrophils to stimulate an immediate morphological response in untreated neutrophils suggests that a stimulus of neutrophil polarisation is either released or produced during the interaction between neutrophils and polymeric IgG. This additional factor may be the chemotactic substance described by Hayashi et al. (1974; "leucoegressin") formed during the limited proteolysis of IgG by proteases released during neutrophil degranulation. This issue might be further investigated by examining whether the preparations of IgG used in this thesis stimulate neutrophil degranulation within a time period which is appropriate to the appearance of the cell's morphological response. In addition, the role of degranulation alone could be assessed by studying the effect of other stimuli of this activity such as IgA on neutrophil shape.

Alternatively, because the preparations used contained a proportion of aggregated IgG as well as monomeric IgG, this aggregated component could have been responsible for the neutrophil polarisation indirectly by stimulating LTB_4 and PAF synthesis by neutrophils. The possible role of LTB_4 and other products of arachidonate

metabolism during IgG-induced polarisation could be further examined by pretreating cells with inhibitors of the arachidonic acid's metabolic pathway such as corticosteroids. The possible role of PAF could be examined by pretreating cells with PAF antagonists such as WEB 2086 (Kroegel et al., 1989). Irrespective of what factor is responsible for IgG-induced neutrophil polarisation, the supernatants from these studies could be tested for chemotactic activity.

Given the substantial literature regarding the relative roles of the neutrophils type II and type III receptor for IgG during responses to this immunoglobulin, the role of the type III receptor during polarisation was assessed by removing it from the neutrophil's surface by digestion with the enzyme PIPLC. Although the removal of 60% of the neutrophil's type III receptors had no effect on the responses to even the lowest concentrations of IgG tested, the remaining 40% may have been sufficient to mediate normal responses owing to the fact that the number of type III receptors on neutrophils is ten times greater than that for type II receptors (Tosi and Berger, 1988; Huizinga et al., 1989). Future studies of the relative roles of these receptors during IgG-induced neutrophil polarisation could involve digesting the neutrophil's type III IgG receptor with other enzymes such as elastase (Tosi and Berger, 1988) or by examining the morphological responses of neutrophils from patients with paroxymal nocturnal hemoglobinuria, an inherited condition in which phosphoinositol linked structures are absent from the neutrophil's surface (Fries and Frank, 1988).

The ability of polymeric IgG to stimulate neutrophil polarisation in vitro suggests that phagocytosis of IgG opsonised material during an inflammatory response may induce the release or formation of factors which stimulate the migration of additional neutrophils to the site of inflammation.

8. 5. What factors may contribute to the different morphological responses of neutrophils to supra-optimal concentrations of inflammatory mediators and FMLP?

In the present studies, different morphological responses were observed towards supra-optimal concentrations of inflammatory mediators and FMLP. While lower numbers of type 4 cells was a common characteristic of responses to each stimulus, higher numbers of type 1 cells were only observed during responses to FMLP, C5a and PAF. With IL-8, supra-optimal concentrations caused more spherical cells and less type 4 cells, while LTB_4 decreased type 4 cells, without specifically raising any one of the other subtypes.

The significance of these observations is that it is evidence of different shape changes in response to particular stimuli, rather than the similarity of response which was suggested in the time courses of responses to optimal concentrations. One possible explanation for the different responses of neutrophils according to stimulus concentration, may involve high and low affinity receptors as have been reported for FMLP, LTB_4 and PAF (Snyderman and Uhing, 1988; Goldman and Goetzl, 1984; Besemer et al., 1989). During responses to low and medium concentrations of these chemotactic factors, the high affinity receptors are more likely to be occupied than the low affinity receptors and may therefore be primarily responsible for the morphological responses observed. Nevertheless, high concentrations of chemotactic factor would enable greater numbers of low affinity receptors to be occupied. The subsequent signals transduced via these low affinity receptors may then contribute to the morphological response of the cell. For example, an inhibitory signal transduced via these receptors may reduce the ability of each cell to produce pseudopodia. This explanation may therefore be sufficient to explain the decrease in number of cells displaying extensions in high concentrations of IL-8. Similarly, signals transduced via low affinity receptors for FMLP and PAF may reduce the ability of neutrophils to polarise their extensions, hence the higher numbers of cells with non-polarised extensions.

Another explanation involving membrane receptors is that high concentrations of chemotactic factors may have saturated receptors present at all points on the neutrophil's surface thus some how interfere with the mechanisms which are

normally responsible for polarising pseudopodia to one side of the cell. This explanation may be particularly appropriate for the response to C5a since studies have suggested the existence of only one population of receptors for this peptide on neutrophils (Huey and Hugli, 1985).

A further explanation for the morphological responses observed to high concentrations of chemotactic factors is the ability of these concentrations to induce other activities of the cell such as degranulation and superoxide production. Degranulation, in particular may alter the polarised morphology of neutrophils as cytoplasmic granules are released from the surface of the cell. Nevertheless, chemotactic factors usually require cytochalasin B to induce degranulation of neutrophils in cell suspensions. An alternative activity, which may have contributed to the morphological responses of neutrophils towards chemotactic factors is the ability of these stimuli to induce neutrophils to synthesize and, or release additional stimuli of these cells. For example, the morphological responses of neutrophils towards FMLP and PAF, may have been affected by the ability of these stimuli to activate synthesis and release of LTB_4 by neutrophils. Since LTB_4 production by neutrophils stimulated with high concentrations of FMLP is observed within a few minutes (McColl, 1987) morphological responses to both stimuli, if present, would have been too close together in their development to observe a biphasic change in the morphology of the cells.

8. 6. What could be the role of extracellular divalent cations during neutrophil polarisation?

Previous studies of the roles of extracellular Ca^{2+} and Mg^{2+} ions during neutrophil polarisation have generally indicated that this shape change is independent of the availability of these cations (Smith et al. ,1979; Marasco et al., 1980; Haston and Shields, 1986). This is in direct contrast to the findings of this study where responses to plasma, FMLP, IgG, C5a, IL-8 and PAF were all found to be affected by the

removal of extracellular Ca^{2+} and Mg^{2+} ions by cation chelating agents. This different observation may be explained by the fact that previous studies have largely been limited to polarisation induced by either FMLP or C5a. Furthermore, the techniques used to measure neutrophil polarisation in these previous studies (Smith et al.,1979; Marasco et al., 1980; Haston and Shields, 1986) did not distinguish between different morphological subtypes of polarised cells as was done in the present study.

The morphological responses of neutrophils to FMLP, C5a, IL-8 and PAF were generally enhanced in the presence of chelating agents, but variable results were often obtained according to different concentrations of stimulus used. In contrast, the responses of neutrophils to plasma and IgG were reduced by chelating agents. However, the requirement for extracellular Ca^{2+} and Mg^{2+} ions during responses to plasma and IgG are complicated by the fact that these cations may have been required during the formation of existing or additional factors responsible for the polarisation of neutrophils (see sections 8. 3. and 8. 4.). The morphological response of neutrophils to LTB_4 was unique in that it was the only response unaffected by chelating agents.

With regard to responses to FMLP, C5a, IL-8 and PAF, experiments involving inclusion of additional cations (similar to those performed during studies with chelating agents in plasma; see section 4. 2. 6.) are required to confirm a role for extracellular divalent cations during these responses. However, since EDTA (which binds to Ca^{2+} and Mg^{2+} ions with similar affinity) generally enhanced responses to a more significant degree than EGTA (which binds primarily to Ca^{2+} ions) it appears that both Ca^{2+} and Mg^{2+} ions may have contributed to the responses observed.

In considering how extracellular divalent cations may have reduced the polarisation of neutrophils in suspension the following explanations are possible. Since extracellular divalent cations are considered to support adhesion of neutrophils to each other and other cells (Jones, 1970), contact between cells during this process may have affected the morphology of the cells. However, clumps of cells were rarely observed in the fixed preparations of neutrophils regardless of whether or not chelating agents were used.

The binding of agonists to their specific receptors may also have been influenced by extracellular cations. Differences between the binding characteristics of the neutrophil's receptors for LTB_4 compared with receptors for other stimuli, may explain why chelating agents did not specifically affect the polarisation induced by LTB_4. Future studies may therefore investigate the role of extracellular cations in binding of agonists to cell receptors by the use of cation chelating agents in the manner used in the present work.

Alternatively, the effects of extracellular Ca^{2+} and Mg^{2+} ions may have been mediated intracellularly following fluxes of these cations into the cell (see below).

8. 7. What could be the role of intracellular divalent cations during neutrophil polarisation?

In view of the multiple roles which intracellular calcium is reported to play within the cytoplasm (translocation of protein kinase C to the inner aspect of the plasma membrane, actin polymerisation, opening of membrane ion channels) the role of these cations during polarisation is a potentially complex issue. Nevertheless, TMB-8, an inhibitor of the release of Ca^{2+} ions from intracellular stores, was found throughout these studies to abolish morphological responses of neutrophils to all agonists examined. This result suggests that the release of Ca^{2+} ions from intracellular stores may be a ubiquitous aspect of neutrophil polarisation. Nevertheless, further studies are required to confirm, firstly, that the agonists used in this thesis induce the release of calcium from intracellular stores under the conditions studied, and secondly, that TMB-8 was successful in preventing this release. These studies of intracellular calcium could involve use of fluorescent intracellular Ca^{2+} chelators such as quin-2 (Tsien et al., 1982).

Whereas intracellular Ca^{2+} ions have been extensively studied in neutrophils, the role of intracellular Mg^{2+} ions is less clear due to the lack of techniques for specificly studying this cation. However, fluorescent probes have recently become

available for measuring intracellular Mg^{2+} ion concentrations in cells (Murphy et al., 1989). Nevertheless, recent studies of intracellular cations in neutrophils have continued to focus primarily on Ca^{2+} ions (Marks and Maxfield, 1990a; 1990b). The novel effects of extracellular Mg^{2+} ions and their chelating agents observed in this thesis indicate that more attention should be paid to the possible intracellular roles of these cations during neutrophil function particularly during polarisation.

8. 8. What could be role of the cytoskeleton, especially microfilaments during neutrophil polarisation?

In the present studies, F-actin was consistently noted within the pseudopodia of polarised neutrophils (types 2, 3 and 4). These observations are consistent with findings of previous studies (Haston, 1987; Roos et al., 1987) and support the opinion that pseudopod production by neutrophils is related to polymerisation and redistribution of actin microfilaments within these cells. Nevertheless, the inability to demonstrate accumulation of F-actin within the markedly extended bodies of types 3 and 4 cells indicates that other cytoskeletal elements such as microtubules may contribute to this aspect of the cells morphological response. Future studies could therefore examine the distribution of microtubules within the elongated bodies of neutrophils by using fluorescent probes as previously described (Malawista and Bensch, 1967; Anderson, 1982).

With regard to the microfilaments within pseudopodia, the present microscopical techniques did not allow a detailed interpretation of the arrangement of these structures, including in relation to the plasma membrane, due to an inadequate depth of field at high magnifications. However, recently developed confocal microscopical techniques which construct detailed images of cells from different planes within a specimen may provide a clearer interpretation of these arrangements (Shotton, 1989). Future studies could also examine changes in the quantity of F-actin present in neutrophils during their exposure to plasma and inflammatory mediators

by FACS analysis of cells stained with fluorescent-phalloidin probes (Howard and Oresajo, 1985).

8. 9. How might polarisation of neutrophils be studied in relation to other activities of these cells in vitro and in vivo?

Polarised morphology occurs in vivo in a complex micro-environment of blood components, vascular endothelia, connective tissues, inflammatory mediators and invading bacteria. In addition, polarisation of neutrophils both in vivo and and in vitro occurs in conjunction with a variety of related activities of the cell including adhesion to substrata, locomotion, chemotaxis and phagocytosis.

In the present studies polarisation responses of neutrophils to FMLP, plasma, IgG and inflammatory mediators were studied within the simplified enviroment of cell suspensions. However, further studies are required to examine the relationships between these responses and the other activities displayed by the cell, particularly those perfomed within the micro-enviroment of inflamed tissues in vivo.

With regard to neutrophil adhesion, future studies may include analysis of the morphological responses of neutrophils to inflammatory mediators while in contact with monolayers of cultured endothelial cells or within three dimensional arrangements of connective tissue components such as collagen, and proteoglycans such as those secreted by neutrophils (Bartold et al., 1989).

With regard to neutrophil polarisation in suspension, future studies may investigate the possible effects of other endogenous and bacterial factors either alone or in combination with inflammatory mediators tested in this thesis. Examples of other factors to be tested may include, prostaglandins and endotoxins as well as products secreted by endothelial cells, fibroblasts and blood cells. Furthermore, neutrophil polarisation assays may provide an important clinical tool for assessing the behaviour of neutrophils during illness, particularly those conditions for which defective motility of neutrophils has been reported such as leukemia, rheumatoid arthritis and

periodontal disease (Pinkerton et al., 1978; Klebanoff and Clark, 1978; Harris, 1988).

With regard to neutrophil motility and chemotaxis, future studies could examine whether or not the concentrations of inflammatory mediators required for optimal production of type 4 cells in the present study, correlate with those which produce optimal motile and chemotactic responses.

Finally, with regard to the neutrophil's other responses such as degranulation and respiratory burst, future studies may document the morphological subtypes displayed by neutrophils during these activities.

8. 10. Conclusions

The major conclusions of this study are summarised as follows:

Visual classification of neutrophils into morphological subtypes provides a sensitive, reliable and appropriate technique for measuring polarisation of these cells in cell suspensions.

C5a, IgG and products of the coagulation system of plasma are potent stimuli of neutrophil polarisation. However, it is possible that the response of the cells to plasma is not due to only one of these factors since none of them precisely reproduced the response to whole fresh plasma in vitro. In particular, the response to IgG could be an indirect effect of a prior interaction between polymeric IgG and neutrophils.

The polarisation responses of neutrophils to optimal concentrations of FMLP, C5a, IL-8, LTB_4 and PAF are similar, but the responses of neutrophils to supra-optimal concentrations of each chemotactic factor vary. This raises the possibility that polarisation responses in inflammatory lesions could vary according to the mediation and nature of the injury.

The factors responsible for polarisation in plasma appear to be dependent upon the availability of extracellular divalent cations especially Mg^{2+} and may be influenced by plasma proteases. The enhancing effects of cation chelating agents on

polarisation of neutrophils in cell suspensions in response to chemotactic factors suggest that extracellular Ca^{2+} and Mg^{2+} ions may influence aspects of neutrophil motility other than adhesion to substrata.

Release of intracellular Ca^{2+} may be an essential primary step in polarisation of neutrophils, since the response to each stimulus was markedly reduced or abolished by TMB-8.

Finally, while the redistribution of F-actin to within pseudopodia appears to be a consistent and fundamental aspect of neutrophil polarisation in cell suspensions, this event is not associated with the elongation of the cell's body which occurs during this morphological response.

APPENDIX

SIGMA CHEMICAL COMPANY

THE WORLD'S FOREMOST MANUFACTURER OF RESEARCH BIOCHEMICALS AND DIAGNOSTIC REAGENTS

POST OFFICE BOX 14508
SAINT LOUIS, MISSOURI 63178, USA

FAX: USA/CANADA 1-800-325-5052 OUTSIDE USA/CANADA 314-771-5757
TELEX: 910-761-0593 or 434475 ANSWERBACK "SIG CK COLLECT"

TELEPHONE: USA/CANADA 1-800-325-3010
OUTSIDE USA/CANADA call COLLECT 314-771-5750

April 2, 1992

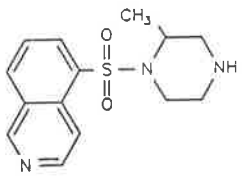
UNIVERSITY OF ADELAIDE
ATTN MRS J HANCOCK
PATHOLOGY DEPARTMENT
G P O BOX 498
ADELAIDE
5001 SOUTH AUSTRALIA

Dear Customer:

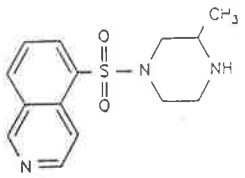
Important information regarding I 0260, 1-(5-Isoquinolinylsulfonyl)-2-methylpiperazine Free Base

See page 2 for your Order Summary.

Recent reevaluation of this material in our laboratories has shown that the methyl group is located at the 3 position of the piperazine ring, not the 2 position as indicated by the name in our catalog and on the label. The structures for the 2-methyl and the 3-methyl products are as follows:



1-(5-Isoquinolinylsulfonyl)-2-methylpiperazine Free Base



1-(5-Isoquinolinylsulfonyl)-3-methylpiperazine Free Base

Since we cannot be certain of your application for this product nor the effect that the position of the methyl group may have, we suggest that you review any research that you may have done with I 0260.

We are unable to supply 1-(5-Isoquinolinylsulfonyl)-2-methylpiperazine Free Base at this time, but we expect to have it available in the near future. Please complete the information on the following page and return it in the enclosed envelope so that we may take the appropriate action.

We will continue to offer 1-(5-Isoquinolinylsulfonyl)-3-methylpiperazine Free Base which is Product Number I 6266 at the following package sizes and prices:

5 mg	$ 24.75	25 mg	$ 96.00	100 mg	$ 310.50

If you have any questions concerning this matter, please contact our Technical Service Department at 314-771-5765.

We apologize for any inconvenience this may have caused.

Sincerely,

SIGMA CHEMICAL COMPANY
Customer Service Department

BIBLIOGRAPHY

Ackerley W. L., Guyre P. M. and Davis B. H. (1991) Neutrophil activation through high-affinity Fc-gamma receptor using a monomeric antibody with unique properties. Blood, 77, 607-615.

Albrechtsen M., Yeaman G. R. and Kerr M. A. (1988) Characterisation of the IgA receptor from human polymorphonuclear leukocytes. Immunology, 64, 201-205.

Alexander E. L., Titus J. A. and Segal D. M. (1979) Human leukocyte Fc (IgG) receptors: quantitation and affinity with radiolabelled affinity cross-linked rabbit IgG. The Journal of Immunology, 123, 295-302.

Allan R. B. and Wilkinson P. C. (1978) A visual analysis of chemotactic and chemokinetic locomotion of human neutrophil leukocytes. Experimental Cell Research, 111, 191-203.

Anderson C. L. (1989) Human IgG Fc receptors. Clinical Immunology and Immunopathology, 53, S63-S71.

Anderson C. L., Shen L., Eicher D. M., Wewers M. D. and Gill J. K. (1990) Phagocytosis mediated by three distinct Fc gamma receptor classes on human leukocytes. Journal of Experimental Medicine, 171, 1333-1345.

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.

Anderson R., Glover A. and Rabson A. R. (1977) The in vitro effects of histamine and metiamide on neutrophil motility and their relationship to intracellular cyclic nucleotide levels. The Journal of Immunology, 118, 1690-1696.

Andersson T., Dahlgren C., Pozzan T., Stendahl O. and Lew P. D. (1986) Characterisation of fMet-Leu-Phe receptor-mediated Ca^{2+} influx across the

plasma membrane of human neutrophils. Molecular Pharmacology, 30, 437-443.

Baggiolini M., Walz A. and Kunkel S. L. (1989) Neutrophil-activating peptide-1/Interleukin 8, a novel cytokine that activates neutrophils. Journal of Clinical Investigation, 84, 1045-1049.

Bainton D. F. (1988) Phagocytic cells: developmental biology of neutrophils and eosinophils. In, Inflammation: Basic Principles and Clinical Correlates, pp 265-280, editors, Gallin J. I., Goldstein I. M. and Snyderman R., Raven Press Ltd., New York.

Bartold P. M., Harkin D. G. and Bignold L. P. (1989) Proteoglycans synthesized by human polymorphonuclear leukocytes in vitro. Immunology and Cell Biology, 67, 9-17.

Bassoe C-F. (1989) Neutrophils studied by flow cytometry, in Flow Cytometry: Advanced Research and Clinical Applications, Volume 2, Yen A., Ed., CRC Press, Florida.

Becker E. L. and Showell H. J. (1972) The effect of Ca^{2+} and Mg^{2+} on the chemotactic responsiveness and spontaneous motility of rabbit polymorponuclear leukocytes. Zeitschrift fur Immunitaetsforschung Experimentelle und Klinische Immunologie, 143, 466-476.

Becker E. L. (1990) The short and happy life of neutrophil activation. Journal of Leukocyte Biology. 47, 378-389.

Belloc F., Vincendeau P., Freyburger G., Dumain P. and Boisseau M. R. (1990) Flow cytometric study of the activation of polymorphonuclear cells. Journal of Leukocyte Biology, 48, 353-358.

Berridge M. J. (1986) Cell signalling through phospholipid metabolism. Journal of Cell Science, Supplement, 4, 137-153.

Besemer J., Hujber A. and Kuhn B. (1989) Specific binding, internalization, and degradation of human neutrophil activating factor by human polymorphonuclear leukocytes. The Journal of Biological Chemistry, 264, 17409-17415.

Bessis M. and de Boisfleury A. (1976) A catalogue of white blood cell movements (normal and pathologic). Blood Cells, 2, 365-410.

Bignold L. P. (1986) Crawling-like movements of polymorphonuclear leukocytes in plasma are not a good index of their motility in microporous cellulose acetate membrane. Cell Biology International Reports, 10, 535-543.

Bignold L. P. (1987a) Effects of preparative technique on the rate of adoption of crawling-like movements by polymorphonuclear leukocytes. Cell Biology International Reports, 11, 19-25.

Bignold L. P. (1987b) Amoeboid movement: a review and proposal of a 'membrane ratchet' model. Experientia, 43, 860-868.

Bignold L. P. (1988) Measurement of chemotaxis of polymorponuclear leukocytes in vitro. Journal of Immunological Methods, 108, 1-18.

Bignold L. P. (1989) Cytokines and inflammation: modified "Miles-Wilhelm" criteria for assessing the likely roles of these substances in vivo. Pathology, 21, 200-206.

Bignold L. P. and Ferrante A. (1987) Mechanism of separation of polymorphonuclear leukocytes from whole blood by the one-step Hypaque-Ficoll method. Journal of Immunological Methods, 96, 29-33.

Bignold L. P. and Ferrante A. (1988) Effects of cytochalasin B, N-formyl peptide and plasma on polarisation, zeiosis (blebbing) and degranulation of polymorphonuclear leukocytes in suspension. Cell Biology International Reports, 12, 195-203.

Bignold L. P., Ferrante A. and Haynes D. R. (1990a) Studies of chemotactic, chemotactic movement-inhibiting and random movement-inhibiting effects of interleukin-1 alpha and beta, tumour necrosis factors alpha and beta and interferon gamma on human neutrophils in assays using 'sparse-pore' polycarbonate (Nuclepore) membranes in the Boyden chamber. International Archives of Allergy and Applied Immunology, 91, 1-7.

Bignold L. P., Rogers S. D. and Harkin D. G. (1990b) Effects of plasma proteins on the adhesion, spreading, polarization in suspension, random motility and chemotaxis of neutrophil leukocytes on polycarbonate (Nuclepore) filtration membrane. European Journal of Cell Biology, 53, 27-34.

Bignold L. P., Rogers S. D., Siaw T. M. and Bahnisch J. (1991) Inhibition of chemotaxis of neutrophil leukocytes to interleukin-8 by endotoxins of various bacteria. Infection and Immunity, 59, 4255-4258.

Bignold L. P., Harkin D. G. and Rogers S. D. (1992) Interleukin-8 and neutrophil leukocytes: adhesion, spreading, polarisation, random motility, chemotaxis and deactivation in assays using 'sparse-pore' polycarbonate (Nuclepore) membranes in the Boyden chamber. International Archives of Allergy and Applied Immunology, (in press).

Blackburn W. D. and Heck L. W. (1988) Neutrophil activation by surface bound IgG: pertussis toxin insensitive activation. Biochemical and Biophysical Research Communications, 152, 136-142.

Boggs J. M., Koo C. H. and Goetzl E. J. (1991) Down-regulation of receptor antigen in leukocyte B_4-induced chemotactic deactivation of human polymorphonuclear leukocytes. Immunology, 73, 212-216.

Bokoch G. M. (1990) Signal transduction by GTP binding proteins during leukocyte activation: phagocytic cells. Current Topics in Membranes and Transport, 35, 65-101.

Borish L. A., Rosenbaum R. and Rocklin R. (1987) Role of calcium in the activation of neutrophils by leukocyte inhibitory factor (LIF). Lymphokine Research, 6, 341-349.

Borish L., Rosenbaum R., Albury L. and Clark S. (1989) Activation of neutrophils by recombinant interleukin 6. Cellular Immunology, 121, 280-289.

Boucek M. M. and Snyderman R. (1976) Calcium influx requirement for human neutrophil chemotaxis: inhibition by lanthanum chloride. Science, 193, 905-907

Boyd W., and Sheldon H. (1980) Introduction to the study of disease., eighth edition, Lea and Febiger, Philadelphia.

Boyden S. (1962) The chemotactic effect of mixtures of antibody and and antigen on polymorphonuclear leukocytes. Journal of Experimental Medicine, 115, 453-466.

Boyles J. and Bainton D. F. (1979) Changing patterns of plasma membrane -associated filaments during the initial phases of polymorphonuclear leukocyte adherence. Journal of Cell Biology, 82, 347-368.

Braquet P., Hosford D., Braquet M., Bourgain R. and Bussolino F. (1989) Role of cytokines and and platelet-activating factor in microvascular immune injury. International Archives of Allergy and Applied Immunology, 88, 88-100.

Bray D., Heath J. and Moss D. (1986) The membrane-associated 'cortex' of animal cells: it's structure and mechanical properties. Journal of Cell Science, Supplement, 4, 71-88.

Bryant R. E., Des Prez R. M., Van Way M. H. and Rogers D. E. (1966) Studies on human leukocyte motility, I: effects of alterations in pH, electrolyte concentration, and phagocytosis on leukocyte migration, adhesiveness and aggregation. Journal of Experimental Medicine, 124, 483-499.

Buckley I. K. (1963) Microscopic morphology of injured living tissue. International Review of Experimental Pathology, 2, 241-310.

Butcher E. C. (1990) Cellular and molecular mechanisms that direct leukocyte traffic. American Journal of Pathology, 136, 3-11.

Campbell A. K. (1986) What does calcium do in cells?. Cell Calcium, 7, 285-286.

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

Charo I. F., Yuen C. Perez H. H. and Goldstein I. M. (1986) Chemotactic peptides modulate adherence of human polymorphonuclear leukocytes to monolayers of cultured endothelial cells. Journal of Immunology, 136, 3412-3419.

Chenoweth D. E., Cooper S. W., Hugli T. E., Stewart R. W., Blackstone E. H. and Kirklin J. W. (1981) Complement activation during cardiopulmonary bypass: evidence for generation of C3a and C5a anaphylatoxins. The New England Journal of Medicine, 304, 497-503.

Cheung W. Y. (1981) Calmodulin and the adenylate cyclase phospho-diesterase system. Cell Calcium, 2, 263-280.

Chilton F. H. and Lichtenstein L. M. (1990) Lipid mediators of the allergic reaction. Chemical Immunology, 49, 173-205.

Christiansen N. O. (1990) Pertussis toxin inhibits the FMLP-induced membrane association of protein kinase C in human neutrophils. Journal of Leukocyte Biology, 47, 60-63.

Clark E. R. and Clark E. L. (1935) Observations on changes in blood vascular endothelium in living animals. American Journal of Anatomy, 57, 385-438.

Colditz I. G. (1987) Early accumulation of neutrophils in acute inflammatory lesions. In, Leukocyte Emigration and It's Sequelae, pp 14-23, Ed. Movat H. Z., Karger, Basel.

Colditz I., Zwahlen R., Dewald B. and Baggiolini M. (1989) In vivo inflammatory activity of neutrophil-activating factor, a novel chemotactic peptide derived from human monocytes. American Journal of Pathology, 134, 755-760.

Colman R. W. (1986) Regulation of the plasma-kallikrein-kininogen system. Advances in Experimental Medicine and Biology, 198B, 1-9.

Cooke E., Al-Mohanna F. A. and Hallett M. B. (1989) Calcium-dependent and independent mechanisms of cellular control within neutrophils: The roles of kinase C, diacylglycerol, and unidentified intracellular messengers, in The Neutrophil: Cellular Biochemistry and Physiology, pp 219-241, Ed. Hallett M. B., CRC Press Inc., Florida.

Craddock P. R., Fehr J., Dalmasso A. P., Brigham K. L. and Jacob H. S. (1977) Hemodialysis leukopenia: pulmonary vascular leukostasis resulting from

complement activation of dialyser cellophane membranes. The Journal of Clinical Investigation, 59, 879-888.

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

Curnette J. T. (1988a) Phagocytic defects I. abnormalities outside of the respiratory burst. Hematology/Oncology Clinics of North America, Vol. 2., No. 1., W. B. Saunders Co., Philadelphia.

Curnette J. T. (1988b) Phagocytic defects II. abnormalities of the respiratory burst. Hematology/Oncology Clinics of North America, Vol. 2., No. 2., W. B. Saunders Co., Philadelphia.

Cybulsky M. I., Chan M. K. W. and Movat H. Z. (1988) Acute inflammation and microthrombosis induced by endotoxin, interleukin-1 and tumour necrosis factor and their implications in Gram negative infection. Laboratory Investigation, 58, 365-378.

Davis J. M. and Gallin J. I. (1981) The Neutrophil. In, Cellular Functions in Immunity and Inflammation, pp 77-102, editors, Oppenheim J. J., Rosenstreich D. L. and Potter M., Elsevier, New York.

Dawson R. M. C., Elliott D. C., Elliott W. H. and Jones K. M. (1986) Stability constants of metal complexes. In, Data for Biochemical Research, Third Edition, pp 399-415, Clarendon Press, Oxford.

De Bruyn P. P. H. (1944) Locomotion of blood cells in tissue cultures. The Anatomical Record, 89, 43-63.

De Bruyn P. P. H. (1946) The amoeboid movement of the mammalian leukocyte in tissue culture. The Anatomical Record, 95, 177-192.

Dedman J. R. (1986) Mediation of intracellular calcium: variances on a common theme. Cell Calcium, 7, 297-307.

Detmers P. A., Lo S. K., Olsen-Egbert E., Walz A., Baggiolini M. and Cohn Z. A. (1990) Neutrophil-activating protein 1/ Interleukin 8 stimulates the binding activity of

the leukocyte adhesion receptor CD11b/CD18 on human neutrophils. Journal of Experimental Medicine, 171, 1155-1162.

Devreotes P. N. and Zigmond S. H. (1988) Chemotaxis in eukaryotic cells: a focus on leukocytes and dictyostelium. Annual Review of Cell Biology, 4, 649-686.

Dewald B., Bretz U., Baggiolini M. (1982) Release of gelatinase from a novel secretory compartment of human neutrophils. Journal of Clinical Investigation, 70, 518-525.

Dewald B., Thelen M., Wymann M. P. and Baggiolini M. (1989) Staurosporine inhibits the respiratory burst and induces exocytosis in human neutrophils. Biochemical Journal, 264, 879-884.

Dinarello C. A. (1988) Cytokines: interleukin-1 and tumor necrosis factor (Cachectin). In, Inflammation: Basic Principles and Clinical Correlates, pp 195-208, editors, Gallin J. I., Goldstein I. M. and Snyderman R., Raven Press Ltd., New York.

Di Virgilio F., Treves S., Lew D. and Pozzan T. (1989) Role of calcium in neutrophil activation, in, The Neutrophil: Cellular Biochemistry and Physiology, pp 199-218, Ed. Hallett M. B., CRC Press Inc., Florida.

Di Virgilio F., Stendahl O., Pittet D., Lew P. D. and Pozzan T. (1990) Cytoplasmic calcium in phagocyte activation. Current topics in membranes and transport, 35, 179-205.

Djeu J. Y., Matsushima K., Oppenheim J. J., Shiotsuki K. and Blanchard D. K. (1990) Functional activation of human neutrophils by recombinant monocyte-derived neutrophil chemotactic factor/IL-8. The Journal of Immunology, 144, 2205-2210.

Donabedian H., Sawyer T. and Senitzer D. (1987) Inhibition of neutrophil shape change by an inhibitor of chemotaxis. Journal of Leukocyte Biology, 42, 510-518.

Donovan R. M., Goldstein E, Kim Y., Lippert W., Kailath E., Aoki T. T., Cheung A. T., Miller M. E. and Chang D. P. (1987) A computer-assisted image analysis system

for analysing polymorphonuclear leukocyte chemotaxis in patients with diabetes mellitus. Journal of Infectious Disease, 155, 737-741.

Edelman G. M. and Crossin K. L. (1991) Cell adhesion molecules: implications for a molecular histology. Annual Review of Biochemistry, 60, 155-190.

Eisele S., Lackie J. M., Riedwyl H., Zimmermann A. and Keller H. U. (1991) Analysis of lymphocyte shape by visual classification, calculated measures of shape or light scattering. Journal of Immunological Methods, 138, 103-109.

Elferink J. G. R. and Deierkauf M. (1985) The effect of quin2 on chemotaxis of polymorphonuclear leukocytes. Biochimica Biophysica Acta, 846, 364-369.

Elsbach P. and Weiss J. (1988) Phagocytic cells: Oxygen-independent antimicrobial systems. In, Inflammation: Basic Principles and Clinical Correlates, pp 445-470, editors, Gallin J. I., Goldstein I. M. and Snyderman R., Raven Press Ltd., New York.

Erbe D. V., Collins J. E., Shen L., Graziano R. F. and Fanger M. W. (1990) The effect of cytokines on the expression and function of Fc receptors for IgG on human myeloid cells. Molecular Immunology, 27, 57-67.

Estensen R. D., Reusch M. E., Epstein M. L. and Hill H. R. (1976) Role of Ca^{2+} and Mg^{2+} in some human neutrophil functions as indicated by ionophore A23187. Infection and Immunity, 13, 146-151.

Faucher N. and Naccache P. H. (1987) Relationship between pH, sodium, and shape changes in chemotactic-factor-stimulated human neutrophils. Journal of Cellular Physiology, 132, 483-491.

Figari I. S., Mori N. A. and Palladino M. A. Jr. (1987) Regulation of neutrophil migration and superoxide production by recombinant tumour necrosis factors alpha and beta: comparison to recombinant interferon gamma and interleukin-1 alpha. Blood, 70, 979-984.

Fine D. P., Marney S. R., Colley D. G., Sergent J. S. and Prez R. M. D. (1972) C3 shunt activation in human serum chelated with EGTA. The Journal of Immunology, 807-809.

Fisher PR, Merke R, Gerish G (1989) Quantitative analysis of cell motility and chemotaxis in Dictyostelium discoideum by using an image processing system and a novel chemotaxis chamber providing stationary chemical gradients. Journal of Cell Biology, 108, 973-984.

Fletcher M. P. and Seligmann B. E. (1985) Monitoring human neutrophil granule secretion by flow cytometry: secretion and membrane potential changes assessed by light scatter and a fluorescent probe of membrane potential. Journal of Leukocyte Biology, 37, 431-447.

Florey H. W. (1962) Inflammation: microscopical observations. In, General Pathology (third edition), Ed., H. W. Florey, pp 40-97. Lloyd-Luke, London.

Ford-Hutchinson A. W., Bray M. A., Doig M. V., Shipley M. E. and Smith M. J. H. (1980) Leukotriene B, a potent chemotactic and aggregating substance released from polymorphonuclear leukocytes. Nature, 286, 264-265.

Ford-Hutchinson A. W. (1990) Leukotriene B_4 in inflammation. Critical Reviews in Immunology, 10, 1-12.

Foster S. J., Aked D. M., Schroder J. -M. and Christophers E. (1989) Acute inflammatory effects of a monocyte-derived neutrophil-activating peptide in rabbit skin. Immunology, 67, 181-183.

Fries L. F. and Frank M. M. (1988) Complement and related proteins: inherited deficiencies. In, Inflammation: Basic Principles and Clinical Correlates, pp 89-100, editors, Gallin J. I., Goldstein I. M. and Snyderman R., Raven Press Ltd., New York.

Frosch P. J. and Czarnetski B. M. (1987) Surfactants cause in vitro chemotaxis and chemokinesis of human neutrophils. Journal of Investigative Dermatology, 88, 52s-55s.

Fukui Y., De Lozanne A. and Spudich J. A. (1990) Structure and function of the cytoskeleton of a Dictyostelium myosin-defective mutant. The Journal of Cell Biology, 110, 367-378.

Gadd S. J. and Ashman L. K. (1983) Binding of mouse monoclonal antibodies to human leukaemic cells via the Fc receptor: a possible source of "false positive" reactions in specificity screening. Clinical and Experimental Immunology, 54, 811-818.

Gallin J. I. (1988) Phagocytic cells: disorders of function. In, Inflammation: Basic Principles and Clinical Correlates, pp 493-511, editors, Gallin J. I., Goldstein I. M. and Snyderman R., Raven Press Ltd., New York.

Gallin E. K. and McKinney L. C. (1989) Ion transport in phagocytes, in The Neutrophil: Cellular Biochemistry and Physiology, pp 243-259, editors, Hallett M. B., CRC Press Inc., Florida.

Gallin E. K. and McKinney L. C. (1990) Monovalent ion transport and membrane potential changes during activation in phagocytic leukocytes. Current Topics in Membranes and Transport, 35, 127-152.

Gallin E. K. and Sheehy P. A. (1988) Leukocyte ion channels and their functional implications. In, Inflammation: Basic Principles and Clinical Correlates, pp 651-664, editors, Gallin J. I., Goldstein I. M. and Snyderman R., Raven Press Ltd., New York.

Gallin J. I. and Rosenthal A. S. (1974) The regulatory role of divalent cations in human granulocyte chemotaxis, The Journal of Cell Biology, 62, 594-609.

Gallin J. I., Goldstein I. M. and Snyderman R. (1988) Inflammation: Basic Principles and Clinical Correlates, Raven Press, New York.

Gaudry M., Perianin A., Marquetty C. and Hakim J. (1988) Negative effect of protein kinase C inhibitor (H-7) on human polymorphonuclear neutrophil locomotion. Immunology, 63, 715-719.

Godman G. C. and Miranda A. F. (1978) Cellular contractility and the visable effects of cytochalasin. In, Cytochalasins, Biochemical and Cell Biological Aspects, p 277, Ed., Tanenbaum S. W., North-Holland, Amsterdam.

Goetzl E. J. and Austen K. F. (1974) Stimulation of human neutrophil leukocyte aerobic glucose metabolism by purified chemotactic factors. The Journal of Clinical Investigation, 53, 591-599.

Goetzl E. J. and Pickett W. C. (1980) The human PMN leukocyte chemotactic activity of complex hydroxy-eicosatetraenoic acids (HETEs). The Journal of Immunology, 125, 1789-1791.

Golde D. W. and Gasson J. C. (1988) Cytokines: myeloid growth factors. In, Inflammation: Basic Principles and Clinical Correlates, pp 253-261, editors, Gallin J. I., Goldstein I. M. and Snyderman R., Raven Press Ltd., New York.

Goldman D. W. and Goetzl E. J. (1982) Specific binding of leukotriene B_4 to receptors on polymorphonuclear leukocytes. Journal of Immunology, 129, 1600-1604.

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. Journal of Experimental Medicine, 159, 1027-1041.

Goldstein I. M. (1988) Complement: biologically active products. In, Inflammation: Basic Principles and Clinical Correlates, pp 55-74, editors, Gallin J. I., Goldstein I. M. and Snyderman R., Raven Press Ltd., New York.

Gorevic P. D., Prelli F. C. and Frangione B. (1985) Immunoglobulin G., in, Methods in Enzymology, Volume 116, Immunochemical Techniques, editors Di Sabato G., Langone J. J. and Van Vunakis H., Academic Press, Inc., Orlando.

Gorter A., Hiemstra P. S., Leijh P. C. J., Van Es L. A. and Daha M. R. (1987) Stimulation of human polymorphonuclear leukocytes by serum IgA or secretory IgA. Advances in Experimental Biology and Medicine, 216(B), 1325-1331.

Greene W. C. (1988) Cytokines: interleukin-2 and it's receptor. In, Inflammation: Basic Principles and Clinical Correlates, pp 209-228, editors, Gallin J. I., Goldstein I. M. and Snyderman R., Raven Press Ltd., New York.

Gustafson E. J. and Colman R. W. (1987) Interaction of polymorphonuclear cells with contact activation factors. Seminars in Thrombosis and Hemostasis, 13, 95-105.

Gustafson E. J., Schmaler A. H., Wachtfogel Y. T., Kaufman N., Kucich U. and Colman R. W. (1989) Human neutrophils contain and bind high molecular weight kininogen. Journal of Clinical Investigation, 84, 28-35.

Hallett M. B. and Campbell A. K. (1983) Measurement of intracellular calcium ions and oxygen radicals in polymorphonuclear leukocyte-erythrocyte 'ghost' hybrids, Journal of Physiology, 338, 537-550.

Hallett M. B. and Campbell A. K. (1984) Is intracellular Ca^{2+} the trigger for oxygen radical production by polymorphonuclear leukocytes?, Cell Calcium, 5, 1-19.

Hanahan D. J. (1986) Platelet activating factor: a biologically active phosphoglyceride. Annual Review of Biochemistry, 55, 483-509.

Harlan J., De Chatelet L. R., Iverson D. B. and McCall C. E. (1977) Magnesium-dependent adenosine triphosphatase as a marker enzyme for the plasma membrane of human polymorphonuclear leukocytes, Infection and Immunity, 15, 436-443.

Harlow E. and Lane D. (1988) Antibodies: a laboratory manual, Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

Hartiala K. T., Langlois L., Goldstein I. M. and Rosenbaum J. T. (1985) Endotoxin-induced selective dysfunction of rabbit polymorphonuclear leukocytes in response to endogenous chemotactic factors. Infection and Immunity, 50, 527-533.

Hartwig J. H. and Stossel T. P. (1976) Interactions of actin, myosin and actin-binding protein of rabbit pulmonary macrophages, III: effects of cytochalasin B, Journal of Cell Biology, 71, 295-303.

Hashimoto Y. and Hurd E. R. (1981) Human neutrophil aggregation and increased adherence to human endothelial cells induced by heat-aggregated IgG and immune complexes, Clinical and Experimental Immunology, 44, 538-547.

Haston W. S. (1987) F-actin distribution in polymorphonuclear leukocytes, Journal of Cell Science, 88, 495-501.

Haston W. S. and Shields J. M. (1984) Contraction waves in lymphocyte locomotion, Journal of Cell Science, 68, 227-241.

Haston W. S. and Shields J. M. (1985) Neutrophil leukocyte chemotaxis: a simplified assay for measuring polarising responses to chemotactic factors, Journal of Immunological Methods, 81, 229-237.

Haston W. S. and Shields J. M. (1986) Signal transduction in human neutrophil leukocytes: effects of external Na+ and Ca^{2+} on cell polarity, Journal of Cell Science, 82, 249-261.

Haston W. S. and Wilkinson P. C. (1988) Visual methods for measuring leukocyte locomotion, Methods in Enzymology, 162, 17-38.

Hawkins D. (1971) Biopolymer membrane: a model system for the study of the neutrophilic leukocyte response to immune complexes, The Journal of Immunology, 107, 344-352.

Hayashi H., Yoshinaga M. and Yamamoto S. (1974) The nature of a mediator of leukocyte chemotaxis in inflammation, Antibiotics and Chemotherapy, 19-296-332.

Henson P. M. (1971a) The immunologic release of constituents from neutrophil leukocytes, I: The role of antibody and complement on nonphagocytosable surfaces or phagocytosable particles, The Journal of Immunology, 107, 1535-1546.

Henson P. M. (1971b) The immunologic release of constituents from neutrophil leukocytes, II: mechanisms of release during phagocytosis, and adherence to nonphagocytosable surfaces, The Journal of Immunology, 107, 1547-1557.

Henson P. M., Johnson H. B. and Spiegelberg H. L. (1972) The release of granule enzymes from human neutrophils stimulated by aggregated immunoglobulins of different classes and subclasses. The Journal of Immunology, 109, 1182-1192.

Hopkins N. K., Schaub R. G. and Gorman R. R. (1984) Acetyl glyceryl ether phosphorylcholine (PAF-acether) and leukotriene B_4-mediated neutrophil chemotaxis through an intact endothelial cell monolayer. Biochimica et Biophysica Acta, 805, 30-36.

Howard T. H. and Oresajo C. O. (1985) The kinetics of chemotactic peptide-induced change in F-actin content, F-actin distribution, and the shape of neutrophils, The Journal of Cell Biology, 101, 1078-1085.

Hsu L. S. and Becker E. L. (1975a) Volume decreases of glycerinated polymorphonuclear leukocytes induced by ATP and Ca^{2+}, 1: resemblances to actomyosin contraction. Experimental Cell Research, 91, 469-473.

Hsu L. S. and Becker E. L. (1975b) Volume changes induced in rabbit polymorphonuclear leukocytes by chemotactic factor and cytochalasin B. The American Journal of Pathology, 81, 1-14.

Huey R. and Hugli T. E. (1985) Characterisation of a C5a receptor on human polymorphonuclear leukocytes (PMN). The Journal of Immunology, 135, 2063-2068.

Hugli T. E. and Morgan E. L. (1984) Mechanisms of leukocyte regulation by complement-derived factors. In, Contemporary Topics in Immunobiology, Volume 14: Regulation of Leukocyte Function, Editor, Snyderman R., Plenum Press, New York.

Huizinga T. W. J., van Kemanade F., Koenderman L., Dolman K. M., Von Dem Borne A. E. G. Kr., Tetteroo P. A. T. and Roos D. (1989) The 40 kDa Fc-gamma receptor (FcRII) on human neutrophils is essential for the IgG-induced respiratory burst and IgG-induced phagocytosis. The Journal of Immunology, 142, 2365-2369.

Huizinga T. W. J., Roos D. and Von Dem Borne A. E. G. Kr. (1990) Neutrophil Fc-gamma receptors: a two way bridge in the immune system. Blood, 75, 1211-1214.

Hwang S. B., Lee C. S. C., Cheah M. J. and Shen T. Y. (1983) Specific receptor sites for 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine (platelet activating factor) on rabbit platelet and guinea pig smooth muscle membranes. Biochemistry, 22, 4756-4763.

Jackson A. L. and Warner N. L. (1986) Preparation, staining, and analysis by flow cytometry of peripheral blood leukocytes., in, Manual of Clinical Laboratory

Immunology (third edition), editors; Rose N. R., Friedman H. and Fahey J. L., American Society for Microbiology, Washington, pp 226-235.

Jacob J. (1988) Ca^{2+} ionophores inhibit superoxide generation by chemotactic peptide in rabbit neutrophils and the correlation with intracellular calcium. Molecular and Cellular Biochemistry, 84, 97-103.

Jones D. B. (1970) The morphology of acid mucosubstances in leukocytic sticking to endothelium in acute inflammation. Laboratory Investigation, 23, 606-611.

Junqueira L. C. and Caneiro J. (1983) Basic Histology, fourth edition, pp 264-266, Lange Medical Publications, Los Altos, California.

Kabat E. A. and Mayer M. M. (1961) Experimental Immunochemistry (second edition), pp 165-170, Thomas Books, Springfield, USA.

Kaplan A. P. and Austen K. F. (1975) Activation and control mechanisms of Hageman factor-dependent pathways of coagulation, fibrinolysis, and kinin generation and their contribution to the inflammatory response. Journal of Allergy and Clinical Immunology, 56, 491-506.

Kaplan A. P., Kay A. B. and Austen K. F. (1972) A pre-albumin activator of prekallikrein III: appearance of chemotactic activity for human neutrophils by the conversion of human prekallikrein to kallikrein. The Journal of Experimental Medicine, 135, 81-97.

Kaplan A. P., Goetzl E. J. and Austen K. F. (1973) The fibrinolytic pathway of human plasma II: the generation of chemotactic activity by activation of plasminogen proactivator. The Journal of Clinical Investigation, 52, 2591-2595.

Kawamoto S. and Hidaka H. (1984) 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) is a selective inhibitor of protein kinase C in rabbit platelets. Biochemical and Biophysical Research Communications, 125, 258-264.

Keller H. U. (1983) Motility, cell shape and locomotion of neutrophil granulocytes, Cell Motility, 3, 47-60.

Keller H. U. and Cottier H. (1981) Crawling-like movements and polarisation in non-adherent leukocytes, Cell Biology International Reports, 5, 3-7.

Keller H. U. and Zimmermann A. (1987) Shape, movement and function of neutrophil granulocytes, Biomedicine and Pharmacotherapy, 41, 285-289.

Keller H. U., Wilkinson P. C., Abercrombie 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 leukocytes and other cells. Clinical and Experimental Immunology, 27, 377-380.

Keller H. U., Barandun S., Kistler P. and Ploem J. S. (1979a) Locomotion and adhesion of neutrophil granulocytes: effects of albumin, fibrinogen and gamma globulins studied by reflection contrast microscopy, Experimental Cell Research, 122, 351-362.

Keller H. U., Wissler J. H. and Ploem J. (1979b) Chemotaxis is not a special case of haptotaxis, Experientia, 35, 1669-1671.

Keller H. U. and Zimmermann A. and Cottier H. (1983) Crawling-like movements, adhesion to solid substrata and chemokinesis of neutrophils granulocytes, Journal of Cell Science, 64, 89-106.

Keller H. U., Naef A. and Zimmermann A. (1984) Effects of colchicine, vinblastine and nocodazole on polarity, motility, chemotaxis and cAMP levels of human polymorphonuclear leukocytes, Experimental Cell Research, 153, 173-185.

Keller H. U., Niggli V. and Zimmermann A. (1989) Diacylglycerols and PMA induce actin polymerisation and distinct shape changes in lymphocytes: relation to fluid pinocytosis and locomotion, Journal of Cell Science, 93, 457-465.

Keller H. U., Niggli V., Zimmermann A. and Portmann R. (1990a) The protein kinase inhibitor H-7 activates human neutrophils: effect on shape actin polymerisation, fluid pinocytosis and locomotion, Journal of Cell Science, 96, 99-106.

Keller H. U., Zimmermann A. and Niggli V. (1990b) Neutrophil granulocytes: a model for analysing the relationship between shape, function and cytoskeletal structure, NATO ASI series, Vol H42, Biomechanics of Active Movement and Deformation of Cells, Ed. Akkas N., Springer-Verlag, Berlin.

Kemp A. S. and Brown S. (1980) Inhibition of neutrophil migration by aggregated immunoglobulin attached to micropore membranes, Immunology, 39, 529-534.

Kemp A. S., Cripps A. W. and Brown S. (1980) Suppression of leukocyte chemokinesis and chemotaxis by human IgA, Clinical and Experimental Immunology, 40, 388-395.

Khachatrian L., Rubins J. B., Manning E. C., Dexter D., Tauber A. I. and Dickey B. F. (1990) Subcellular distribution and characterisation of GTP-binding proteins in human neutrophils. Biochimica et Biophysica Acta, 1054, 237-245.

Kimberly R. P., Ahlstrom J. W., Click M. E. and Edberg J. C. (1990) The glycosyl phosphatidylinositol-linked Fc gamma $RIII_{PMN}$ mediates transmembrane signaling events distinct from Fc gamma RII. Journal of Experimental Medicine, 171, 1239-1255.

Klebanoff S. J. and Clark R. A. (1978) The Neutrophil: Function and Clinical Disorders. North- Holland Publishing Co., Amsterdam.

Klebanoff S. J. (1988) Phagocytic cells: products of oxygen metabolism. In, Inflammation: Basic Principles and Clinical Correlates, pp 391-444, editors, Gallin J. I., Goldstein I. M. and Snyderman R., Raven Press Ltd., New York.

Korchak H. M., Vienne K., Rutherford L. E., Wilkenfeld C., Finkelstein M. C. and Weissmann G. (1984) Stimulus response coupling in the human neutrophil, II: temporal analysis of changes in cytosolic calcium and calcium efflux, The Journal of Biological Chemistry, 259, 4076-4082.

Kozin F. and Cochrane C. G. (1988) The contact activation system of plasma: biochemistry and pathophysiology. In, Inflammation: Basic Principles and Clinical Correlates, editors, Gallin J. I., Goldstein I. M. and Snyderman R., pp 101-120, Raven Press Ltd., New York.

Kramer I. M., Van Der Bend R. L., Tool A. T. J., Van Blitterswijk W. J., Roos D. and Verhoeven A. J. (1989) 1-O-hexadecyl-2-O-methylglycerol, a novel inhibitor of protein kinase C, inhibits the respiratory burst in human neutrophils. The Journal of Biological Chemistry, 264, 5876-5884.

Krause K-H. and Lew P. D. (1987) Subcellular distribution of Ca^{2+} pumping sites in human neutrophils. Journal of Clinical Investigation, 80, 107-116.

Krause K-H. and Welsh M. J. (1990) Voltage-dependent and Ca^{2+}-activated ion channels in human neutrophils, Journal of Clinical Investigation, 85, 491-498.

Kroegel C., Pleass R., Yukawa T., Chung K. F., Westwick J. and Barnes P. J. (1989) Characterisation of platelet activating factor-induced elevation of cytosolic free calcium concentration in eosinophils. FEBS Letters, 243, 41-46.

Kurlander R. J. and Batker J. (1982) The binding of human immunoglobulin G1 monomer and small, covalently cross-linked polymers of immunoglobulin G1 to human peripheral blood monocytes and polymorphonuclear leukocytes. Journal of Clinical Investigation, 69, 1-8.

Kvarstein B. (1969) Effects of proteins and inorganic ions on the adhesiveness of human leukocytes to glass beads, Scandanavian Journal of Clinical and Laboratory Investigation, 24, 41-48.

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. Proceedings of the National Academy of Sciences USA, 82, 869-873.

Larsen C. G., Anderson A. O., Appella E., Oppenheim J. J. and Matsushima K. (1989) The neutrophil-activating protein (NAP-1) is also chemotactic for T lymphocytes. Science, 243, 1464-1466.

Larsen G. L. and Henson P. M. (1983) Mediators of inflammation. Annual Review of Immunology, 1, 335-359

Laster C. E. and Gleich G. J. (1971) Chemotaxis of eosinophils and neutrophils by aggregated immunoglobulins, Journal of Allergy and Clinical Immunology, 48, 297-304.

Lauffenburger D., Farrel B., Tranquillo R., Kistler A. and Zigmond S. (1987) Gradient perception by neutrophil leukocytes, continued. Journal of Cell Science, 88, 415-416.

Lawrence D. A., Weigle W. O. and Spiegelberg H. L. (1975) Immunoglobulins cytophilic for human lymphocytes, monocytes and neutrophils. The Journal of Clinical Investigation, 55, 368-376.

Leikin S. and Bessman S. P. (1956) The effects of various EDTA complexes on coagulation, Blood, 11, 916-923.

Lew D. P., Andersson T., Hed J., Di Virgilio F., Pozzan T. and Stendahl O. (1985) Ca^{2+}-dependent and Ca^{2+}-independent phagocytosis in human neutrophils. Nature, 315, 509-511.

Lew P. D. (1990) Receptors and intracellular signalling in neutrophils, American Review of Respiratory Disease, 141, S127-S131.

Lew P. D., Wollheim C. B., Waldvogel F. A. and Pozzan T. (1984) Modulation of cytosolic-free calcium transients by changes in intracellular calcium buffering capacity: correlation with exocytosis and O_2^- production in human neutrophils, The Journal of Cell Biology, 99, 1212-1220.

Lewis W. H. (1934) On the locomotion of the polymorphonuclear neutrophiles of the rat in autoplasma cultures, Bulletin of the John Hopkins Hospital, 55, 273-279.

Lewis R. A., Austen K. F. and Soberman R. J. (1990) Leukotrienes and other products of the 5-lipoxygenase pathway. The New England Journal of Medicine, 323, 645-655.

Lichtman M. A., Santillo P. A., Kearney E. A., Roberts G. W. and Weed R. I. (1976) The shape and surface morphology of human leukocytes in vitro: Effect of temperature, metabolic inhibitors and agents that influence membrane structure, Blood Cells, 2, 507-531.

Lin A. H., Morton D. R. and Gorman R. R. (1982) Acetyl glyceryl ether phosphorylcholine stimulates leukotriene B_4 synthesis in human polymorphonuclear leukocytes. Journal of Clinical Investigation, 70, 1058-1065.

Lin A. H., Ruppel P. L. and Gorman R. R. (1984) Leukotriene B_4 binding to human neutrophils. Prostaglandins, 28, 837-849.

Lo S. K., Lai L., Cooper J. A. and Malik A. B. (1988) Thrombin-induced generation of neutrophil activating factors in blood. American Journal of Pathology, 130, 22-32.

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) Recombinant human granulocyte-macrophage colony-stimulating factor stimulates in vitro mature human neutrophil and eosinophil function, surface receptor expression, and survival, Journal of Clinical Investigation, 78, 1220-1228.

Lord R. A. and Roath S. (1990) Evaluation and comparison of neutrophil bipolar shape formation with a migration assay. Journal of Clinical Pathology, 43, 342-345.

Low M. G. (1989) Glycosyl-phosphatidylinositol: a versatile anchor for cell surface proteins. The FASEB Journal, 3, 1600-1608.

Malawista S. E. and Bensch K. G. (1967) Human polymorphonuclear leukocytes: demonstration of microtubules and effect of colchicine, Science, 156, 521-522.

Malech H. L. (1988) Phagocytic cells: egress from marrow and diapedesis. In, Inflammation: Basic Principles and Clinical Correlates, pp 297-308, editors, Gallin J. I., Goldstein I. M. and Snyderman R., Raven Press Ltd., New York.

Marasco W. A., Becker E. L. and Oliver J. M. (1980) The ionic basis of chemotaxis, American Journal of Pathology, 98,749-764.

Marcus A. J. (1988) Eicosanoids: transcellular metabolism. In, Inflammation: Basic Principles and Clinical Correlates, pp 129-137, editors, Gallin J. I., Goldstein I. M. and Snyderman R., Raven Press Ltd., New York.

Marks P. W. and Maxfield F. R. (1990a) Local and global changes in cytosolic free calcium in neutrophils during chemotaxis and phagocytosis, Cell Calcium, 11, 181-190.

Marks P. W. and Maxfield F, R. (1990b) Transient increases in cytosolic free calcium appear to be required for the migration of adherent human neutrophils, The Journal of Cell Biology, 110, 43-52.

Marshall R. W. (1976) Calcium, Phosphate and Magnesium Metabolism: Clinical Physiology and Diagnostic Procedures. Ed. Nordin B. E. C., Churchill Livingstone, Edinburgh.

Meshulam T., Proto P., Diamond R. D. and Melnick D. A. (1986) Calcium modulation and chemotactic response: divergent stimulation of neutrophil chemotaxis and cytosolic calcium response by the chemotactic peptide receptor, The Journal of Immunology, 137, 1954-1960.

Metcalf D. (1988) Haemopoietic growth factors. The Medical Journal of Australia, 148, 516-519.

Metchnikoff E. (1891) Lectures on the comparative pathology of inflammation. Reprinted in 1968 by Dover Press, New York.

McColl S. R. (1987) Factors affecting the regulation of leukotriene production by neutrophils. Doctorate thesis, University of Adelaide, South Australia, p 83.

McNeil P. L., Kennedy A. L., Waggoner A. S., Taylor D. L. and Murphy P. F. (1985) Light scattering changes during chemotactic stimulation of human neutrophils: kinetics followed by flow cytometry, Cytometry, 6, 7-12.

Ming W. J., Bersani L. and Mantovani A. (1987) Tumor necrosis factor is chemotactic for monocytes and polymorphonuclear leukocytes. The Journal of Immunology, 138, 1469-1474.

Molski T. F. P. and Sha'afi R. I. (1987) Intracellular acidification, guanine-nucleotide binding proteins, and cytoskeletal actin. Cell Motility and the Cytoskeleton, 8, 1-6.

Morgan B. P. (1989) Complement membrane attack on nucleated cells: resistence, recovery and non-lethal effects. Biochemical Journal, 264, 1-14.

Movat H. Z. (1983) Leukocyte emigration-a brief historical review: 1843-1982. Survey and Synthesis of Pathology Research, 1, 120-124.

Moy J. N., Nelson R. D., Richards K. L. and Hostetter M. K. (1990) Identification of an IgA inhibitor of neutrophil chemotaxis and it's membrane target for the metabolic burst. Immunology, 69, 257-263.

Müller-Eberhard H. J. (1988) Complement: chemistry and pathways. In, Inflammation: Basic Principles and Clinical Correlates, pp 21-53, editors, Gallin J. I., Goldstein I. M. and Snyderman R., Raven Press Ltd., New York.

Murphy E., Freudenrich C. C., Levy L. A., London R. E. and Lieberman M. (1989) Monitoring cytosolic free magnesium in cultured chicken heart cells by use of the fluorescent indicator Furaptra. Proceedings of the National Academy of Sciences USA, 86, 2981-2984.

Murphy P. (1976) The Neutrophil. Plenum Publishing Corporation, New York.

Naccache P. H., Showell H. J., Becker E. L. and Sha'afi R. I. (1977a) Transport of sodium, potassium and calcium across rabbit polymorphonuclear leukocyte membranes: Effect of chemotactic factor. Journal of Cell Biology, 73, 428-444.

Naccache P. H., Showell H. J., Becker E. L. and Sha'afi R. I. (1977b) Changes in ionic movements across rabbit polymorphonuclear leukocyte membranes during lysosomal enzyme release. Possible ionic basis for lysosomal enzyme release. Journal of Cell Biology, 75, 635-649.

Naccache P. H., Showell H. J., Becker E. L. and Sha'afi R. I. (1979) Involvement of membrane calcium in the response of rabbit neutrophils to chemotactic factors as evidenced by the fluorescence of chlortetracycline, Cell Biology, 83, 179-186.

Naccache P. H., Sha'afi R. I. and Borgeat P. (1989) Mobilization, metabolism, and biological effects of eicosanoids in polymorphonuclear leukocytes. In, The Neutrophil: Cellular Biochemistry and Physiology, Ed. Hallett M. B., pp113-139, CRC Press Inc, Florida.

Nathan C. F. (1987) Secretory products of macrophage. Journal of Clinical Investigation, 79, 319-326.

Nathan C. and Sporn M. (1991) Cytokines in context, The Journal of Cell Biology, 113, 981-986.

Norgauer J., Eberle M., Fay S. P., Lemke H. D. and Sklar L. A. (1991) Kinetics of N-formyl peptide receptor up-regulation during stimulation in human neutrophils. The Journal of Immunology, 146, 975-980.

O'Flaherty J. T., Kreutzer D. L., and Ward P. A. (1977) Neutrophil aggregation and swelling induced by chemotactic agents. The Journal of Immunology, 119, 232-239.

O'Flaherty J. T., Redman J. F. and Jacobson D. P. (1986) Protein kinase C regulates leukotriene B_4 receptors in human neutrophils. FEBS Letters, 206, 279-282.

Oliver J. M., Krawiec J. A. and Becker E. L. (1978) The distribution of actin during chemotaxis in rabbit neutrophils, Journal of the Reticuloendothelial Society, 24, 697-704.

Omann G. M., Allen R. A., Bokoch G. M., Painter R. G., Traynor A. E. and Sklar L. A. (1987) Signal transduction and cytoskeletal activation in the neutrophil. Physiological Reviews, 67, 285-322.

O'Reilly R. A. (1985) Anticoagulant, antithrombotic, and thrombolytic drugs. In, The Pharmacological Basis of Therapeutics, seventh edition, editors; Gilman A. G, Goodman L. S., Rall T. W. and Murad F. Macmillan Publishing Co., New York.

Pangburn M. K. and Müller-Eberhard H. J. (1985) The alternative pathway of complement. In, Complement, Editors, Muller-Eberhard H. J. and Miescher P. A., Springer-Verlag, Berlin.

Pedersen J. O., Hassing L., Grunnet N., Jersild C. (1988) Real-time scanning and image analysis. A fast method for the determination of neutrophil orientation under agarose. Journal of Immunological Methods, 109, 131-137.

Perez H. D., Elfman F., Marder S., Lobo E. and Ives H. E. (1989) Formyl peptide induced chemotaxis of human polymorphonuclear leukocytes does not require either marked changes in cytosolic calcium or specific granule discharge, Journal of Clinical Investigation, 83, 1963-1970.

Perussia B., Dayton E. T., Lazarus R., Fanning V. and Trinchieri G. (1983) Immune interferon induces the receptor for monomeric IgG1 on human monocytic and myeloid cells. Journal of Experimental Medicine, 158, 1092-1113.

Petroni K. C., Shen L. and Guyre P. M. (1988) Modulation of human polymorphonuclear leukocyte IgG Fc receptors and Fc receptor-mediated

functions by IFN-gamma and glucocorticoids. The Journal of Immunology, 140, 3467-3472.

Petty H. R., Francis J. W. and Anderson C. L. (1989) Cell surface distribution of Fc receptors II and III on living human neutrophils before and during antibody dependent cellular cytotoxicity. Journal of Cellular Physiology, 141, 598-605.

Peveri P., Walz A., Dewald B. and Baggiolini M. (1988) A novel neutrophil-activating factor produced by human mononuclear phagocytes. Journal of Experimental Medicine, 167, 1547-1560.

Pfister R. R., Haddox J. L. and Snyder T. L. (1988) The effect of citrate on the adherence of neutrophils to nylon fibers in vitro. Investigative Ophthalmology and Visual Science, 29, 869-875.

Pike M. C. (1990) Chemoattractant receptors as regulators of phagocytic cell function. Current Topics in Membranes and Transport, 35, 19-43.

Pinckard R. N., Ludwig J. C. and McManus L. M. (1988) Platelet-activating factors. In, Inflammation: Basic Principles and Clinical Correlates, pp 139-167, editors, Gallin J. I., Goldstein I. M. and Snyderman R., Raven Press Ltd., New York.

Pinkerton P. H., Robinson J. B. and Senn J. S. (1978) Lazy leukocyte syndrome-disorder of the granulocyte membrane? Journal of Clinical Pathology, 31, 300-308.

Platten E., Gallin J. I., Clark R. A. and Kimball H. R. (1973) Effects of cell concentration and various anticoagulants on neutrophil migration, Blood, 41, 711-719.

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. The Journal of Biological Chemistry, 261, 8309-8313.

Postlethwaite A. E. and Kang A. H. (1988) Fibroblasts. In, Inflammation: Basic Principles and Clinical Correlates, pp 577-597, editors, Gallin J. I., Goldstein I. M. and Snyderman R., Raven Press Ltd., New York.

Raetz C. R. H. (1990) Biochemistry of endotoxins. Annual Review of Biochemistry, 59, 129-170.

Ramsey W. S. (1972a) Analysis of individual leukocyte behaviour during chemotaxis, Experimental Cell Research, 70, 129-139.

Ramsey W. S. (1972b) Locomotion of human polymorphonuclear leukocytes, Experimental Cell Research, 72, 489-501.

Reibman J., Haines K. and Weissmann G. (1990) Alterations in cyclic nucleotides and the activation of neutrophils. Current Topics in Membranes and Transport, 35, 399-424.

Remick D. G., Kunkel R. G., Larrick J. W. and Kunkel S. L. (1987) Acute in vivo effects of human recombinant tumor necrosis factor. Laboratory Investigation, 56, 583-590.

Ribeiro R. A., Cunha F. Q. and Ferreira S. H. (1990) Recombinant gamma interferon causes neutrophil migration by the release of a macrophage neutrophil chemotactic factor. International Journal of Experimental Pathology, 71, 717-725.

Robbins S. L., Cotran R. S. and Kumar V. (1984) Pathologic Basis of Disease, (third edition), W. B. Saunders Co., Philadelphia, p 53.

Rogers S. D. and Bignold L. P. (1990) Which method for the assessment of spreading of polymorphonuclear leukocytes? Journal of Immunological Methods, 135, 281-284.

Roitt I. M., Brostoff J. and Male D. K. (1985) Immunology. Gower Medical Publishing, London.

Rolleston H. (1934) The history of haematology. Proceedings of the Royal Society of Medicine, 27, 1161-1178.

Roos F. J., Zimmermann A. and Keller H. U. (1987) Effect of phorbol myristate acetate and the chemotactic peptide fNLPNTL on shape and movement of human neutrophils, Journal of Cell Science, 88, 399-406.

Ruddle N. H. (1987) Tumour necrosis factor and related cytotoxins. Immunology Today, 8, 129-130.

Ruddy S. (1986) Complement. In, Manual of Clinical Laboratory Immunology (third edition), Editors, Rose N. R., Friedman H. and Fahey J. L., American Society for Microbiology, Washington, D. C.

Sandborg R. R. and Smolen J. E. (1988) Biology of disease: early biochemical events in leukocyte activation, Laboratory Investigation, 59, 300-320.

Sawyer D. W., Sullivan J. A. and Mandell G. L. (1985) Intracellular free calcium localisation in neutrophils during phagocytosis, Science, 230, 663.

Schapira M., Despland E., Scott C. F., Boxer L. A. and Colman R. W. (1982) Purified human plasma kallikrein aggregates human blood neutrophils. The Journal of Clinical Investigation, 69, 1199-1202.

Schiffmann E., Showell H. V., Corcoran B. A., Ward P. A., Smith E. and Becker E. L. (1975) The isolation and partial characterisation of neutrophil chemotactic factors from Escherichia coli, The Journal of Immunology, 114, 1831-1837.

Schmid-Schonbein G. W., Shih Y. Y. and Chien S. (1980) Morphometry of human leukocytes. Blood, 56, 866-875.

Schroder J. -M. and Christophers E. (1983) Role of filter-bound IgA in inhibiting polymorphonuclear leukocyte chemotaxis. Immunobiology, 164, 127-135.

Schroeder T. E. (1978) Cytochalasin B, cytokinesis and the contractile ring. In, Cytochalasins, Biochemical and Cell Biological Aspects, p 91, Ed., Tanenbaum S. W., North-Holland, Amsterdam.

Sha'afi R. I., Molski T. F. P., Huang C. -K. and Naccache P. H. (1986) The inhibition of neutrophil responsiveness caused by phorbol esters is blocked by the protein kinase C inhibitor H7. Biochemical and Biophysical Research Communications, 137, 50-60.

Shaw J. O., Pinkard 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). The Journal of Immunology, 127, 1250-1255.

Shen L., Guyre P. M. and Fanger M. W. (1987) Polymorphonuclear leukocyte function triggered through the high affinity Fc receptor for monomeric IgG. The Journal of Immunology, 139, 534-538.

Sheterline P. and Rickard J. E. (1989) The cortical actin filament network of neutrophil leukocytes during phagocytosis and chemotaxis. In, The Neutrophil: Cellular Biochemistry and Physiology, Ed., Hallett M. B., CRC Press Inc., Florida.

Shields J. M. and Haston W. S. (1985) Behaviour of neutrophil leukocytes in uniform concentrations of chemotactic factors: contraction waves, cell polarity and persistence, Journal of Cell Science, 74, 75-93.

Shotton D. (1989) Confocal microscopy: the way ahead. Microscopy and Analysis, July, 1989, 7-12.

Showell H. J., Freer R. J., Zigmond S. H., Schiffmann E., Aswanikumar S., Corcoran B. A. and Becker E. L. (1976) The structure activity relations of synthetic peptides as chemotactic factors and inducers of lysosomal enzyme secretion for neutrophils. Journal of Experimental Medicine, 143, 1154-1169.

Simchowitz L., Spilberg I. and De Weer P. (1982) Sodium and potassium fluxes and membrane potential of human neutrophils. Journal of General Physiology, 79, 453-479.

Simchowitz L. and Cragoe E. J., Jr. (1986) Regulation of human neutrophil chemotaxis by intracellular pH. Journal of Biological Chemistry, 261, 6492-6500.

Singer S. J. and Kupfer A. (1986) The directed migration of eukaryotic cells. Annual Review of Cell Biology, 2, 337-365.

Siraganian R. P. (1988) Mast cells and basophils. In, Inflammation: Basic Principles and Clinical Correlates, pp 513-542, editors, Gallin J. I., Goldstein I. M. and Snyderman R., Raven Press Ltd., New York.

Skogen W. F., Senior R. M., Griffin G. L. and Wilner G. D. (1988) Fibrinogen-derived peptide ß1-42 is a multidomained neutrophil chemoattractant. Blood, 71, 1475-1479.

Skubitz K. M. and Lakatua D. A. (1990) Identification of the major glycosyl-phosphatidylinositol anchored proteins on the surface of human neutrophils. Journal of Leukocyte Biology, 48, 50-59.

Smolen J. E. and Weissman G. (1981) Stimuli which provoke secretion of azurophil enzymes from human neutrophils induce increments in adenosine cyclic 3'-5'-monophosphate. Biochimica Biophysica Acta, 672, 197-206.

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, Biochimica et Biophysica Acta, 677, 512-520.

Smith C. W., Hollers J. C., Patrick R. A. and Hassett C. (1979) Motility and adhesiveness of human neutrophils: effect of chemotactic factors, Journal of Clinical Investigation, 63, 221-229.

Smith C. W. and Hollers J. C. (1980) Motility and adhesiveness in human neutrophils: redistribution of chemotactic factor-induced adhesion sites, Journal of Clinical Investigation, 65, 804-812.

Smith R. J., Speziale S. C., Ulrich R. G. and Bowman B. J. (1986) Characteristics of aggregated immunoglobulin G as an immunologic phagocytic stimulus for granule enzyme release from human neutrophils. Inflammation, 10, 131-143.

Smith W. B., Gamble J. R., Clark-Lewis I. and Vadas M. A. (1991) Interleukin-8 induces neutrophil transendothelial migration, Immunology, 72, 65-72.

Smith W. L. (1989) The eicosanoids and their biochemical mechanism of action, Biochemistry Journal, 259, 315-324.

Snyder F. (1989) Biochemistry of platelet-activating factor: a unique class of biologically active phospholipids (42839), Proceedings of the Society for Experimental Biology and Medicine, 190, 125-149.

Snyderman R. and Uhing R. J. (1988) Phagocytic cells: stimulus-response coupling mechanisms., in, Inflammation: Basic Principles and Clinical Correlates, pp 309-323, editors, Gallin J. I., Goldstein I. M. and Snyderman R., Raven Press Ltd., New York.

Southwick F. S., Dabiri G. A., Paschetto M. and Zigmond S. H. (1989) Polymorphonuclear leukocyte adherence induces actin polymerization by a transduction pathway which differs from that used by chemoattractants. The Journal of Cell Biology, 109, 1561-1569.

Spiegelberg H. L. (1988) Immunoglobulins. In, Inflammation: Basic Principles and Clinical Correlates, editors, Gallin J. I., Goldstein I. M. and Snyderman R., pp 11-19, Raven Press Ltd., New York.

Stecher V. J., Sorkin A. and Ryan G. B. (1971) Relation between blood coagulation and chemotaxis of leukocytes. Nature, 233, 95-96.

Stecher V. J. and Sorkin A. (1974) The chemotactic activity of leukocytes related to blood coagulation and fibrinolysis. Antibiotics and Chemotherapy, 19, 362-368.

Stossel T. P. (1982) The structure of cortical cytoplasm. Philosophical Transactions of the Royal Society of London, B, 299, 275-289.

Stossel T. P. (1988) The mechanical responses of white blood cells, in, Inflammation: Basic Principles and Clinical Correlates, pp 325-342, editors, Gallin J. I., Goldstein I. M. and Snyderman R., Raven Press Ltd., New York.

Stossel T. P. (1989) From signal to pseudopod: how cells control cytoplasmic actin assembly. The Journal of Biological Chemistry, 264, 18261-18264.

Stossel T. P. and Pollard T. D. (1973) Myosin in polymorphonuclear leukocytes, The Journal of Biological Chemistry, 248, 8288-8294.

Sullivan S. J., Daukas G. and Zigmond S. H. (1984) Asymmetric distribution of the chemotactic peptide receptor on polymorphonuclear leukocytes, Journal of Cell Biology, 99, 1461-1467.

Sundsmo J. S. and Fair D. S. (1985) Relationships among the complement, kinin, coagulation, and fibrinolytic systems. In, Complement, Editors Muller-Eberhard H. J. and Miescher P. A., pp 379-406, Springer-Verlag, Berlin.

Swallow C. J., Grinstein S. and Rotstein O. D. (1990) Regulation and functional significance of cytoplasmic pH in phagocytic cells. Current Topics in Membranes and Transport, 35, 227-247.

Ternowitz T., Herlin T. and Fogh K. H. (1987) Human monocyte and polymorphonuclear leukocyte chemotactic and chemokinetic responses to leukotriene B4 and FMLP. Acta Pathologica Microbiologica Scandanavica, 95, 47-54.

Thelen M., Peveri P., Kernen P., Von Tscharner V., Walz A. and Baggiolini M. (1989) Mechanism of neutrophil activation by NAF, a novel monocyte-derived peptide agonist. The FASEB Journal, 2, 2702-2706.

Tonetti M., Budnick M. and Niederman R. (1990) Receptor-stimulated actin polymerisation requires cytoplasmic acidification in human neutrophils. Biochimica et Biophysica Acta, 1054, 154-158.

Tonnesen M. G., Anderson D. C., Springer T. A., Knedler A., Avdi N. and Henson P. M. (1989) Adherence of neutrophils to cultured human microvascular endothelial cells. Journal of Clinical Investigation, 83, 637-646.

Tosi M. F. and Berger M. (1988) Functional differences between the 40 kDa and 50 to 70 kDa IgG Fc receptors on human neutrophils revealed by elastase treatment and anti-receptor antibodies. The Journal of Immunology, 141, 2097-2103.

Traynor-Kaplan A. E. (1990) Phosphoinositide metabolism during phagocytic cell activation. Current Topics in Membranes and Transport, 35, 303-332.

Tsien R. Y., Pozzan T. and Rink T. J. (1982) Calcium homeostasis in intact lymphocytes: cytoplasmic free calcium monitored with a new, intracellularly trapped fluorescent indicator, The Journal of Cell Biology, 94, 325-334.

Unkeless J. C. (1989) Function and heterogeneity of human Fc receptors for immunoglobulin G. Journal of Clinical Investigation, 83, 355-361.

Valone F. H. and Goetzl E. J. (1983) Specific binding by human polymorphonuclear leukocytes of the immunological mediator 1-O-hexa-decyl/octadecyl-2-acetyl-sn-glycero-3-phosphorylcholine. Immunology, 48, 141-149.

Van de Winkel J. G. J. and Anderson C. L. (1990) Biological functioning of human IgG Fc receptors. Research in Immunology, 141, 64-67.

Van Damme J., Van Beeumen J., Opdenakker G. and Billiau A. (1988) A novel, NH_2-terminal sequence-characterized human monokine possessing neutrophil chemotactic, skin-reactive, and granulocytosis promoting activity. Journal of Experimental Medicine, 167, 1364-1376.

Van Epps D. E. and Williams R. C. (1976) Suppression of leukocyte chemotaxis by human IgA myeloma components. The Journal of Experimental Medicine, 144, 1227-1242.

Wachtfogel Y. T., Kucich U., James H. L., Scott C. F., Schapira M., Zimmerman M., Cohen A. B. and Colman R. W. (1983) Human plasma kallikrein releases neutrophil elastase during blood coagulation. Journal of Clinical Investigation, 72, 1672-1677.

Wachtfogel Y. T., Pixley R. A., Kucich U., Abrams W., Weinbaum G., Schapira M. and Colman R. W. (1986) Purified plasma factor XIIa aggregates human neutrophils and causes degranulation. Blood, 67, 1731-1737.

Wallace P. J., Wersto R. P., Packman C. H. and Lichtman M. A. (1984) Chemotactic peptide-induced changes in neutrophil actin conformation. The Journal of Cell Biology, 99, 1060-1065.

Walsh G. M. and Kay A. B. (1986) Binding of immunoglobulin classes and subclasses to human neutrophils and eosinophils. Clinical and Experimental Immunology, 63, 466-472.

Wang D., Berry K. and Howard T. H. (1985) Kinetic analysis of chemotactic peptide-induced actin polymerisation in neutrophils. Cell Motility and the Cytoskeleton, 16, 80-87.

Wang J. M., Colella S., Allavena P. and Mantovani A. (1987) Chemotactic activity of human recombinant granulocyte-macrophage colony-stimulating factor. Immunology, 60, 439-444.

Ward P. A. and Becker E. L. (1968) The deactivation of rabbit neutrophils by chemotactic factor and the nature of the activatable esterase. Journal of Experimental Medicine, 127, 693-709.

Warkentin T. E. and Kelton J. G. (1990) Heparin and platelets. Hematology/ Oncology Clinics of North America, Vol. 4, p 245, W. B. Saunders Co., Philadelphia.

Weiss N. A. and Hassett M. J. (1987) Introductory Statistics, second edition, pp 463-470, Addison-Wesley Publishing Co., Reading, Massachusetts.

Weiss P. (1961) Guiding principles in cell locomotion and cell aggregation. Experimental Cell Research, Supplement, 8, 260-281.

West M. A. (1990) Role of cytokines in leukocyte activation: phagocytic cells. Current Topics in Membranes and Transport, 35, 537-570.

Westwick J. and Poll C. (1986) Mechanisms of calcium homeostasis in the polymorphonuclear leukocyte, Agents and Actions, 19, 80-86.

White M. V. and Kaliner M. A. (1988) Histamine. In, Inflammation: Basic Principles and Clinical Correlates, pp 169-193, editors, Gallin J. I., Goldstein I. M. and Snyderman R., Raven Press Ltd., New York.

White J. R., Huang C. -K. Hill J. M., Naccache P. H., Becker E. L. and Sha'afi R. I. (1984) Effect of phorbol 12-myristate 13-acetate and it's analogue 4-alpha-phorbol 12, 13-didecanoate on protein phosphorylation and lysosomal enzyme release in rabbit neutrophils. Journal of Biological Chemistry, 259, 8605-8611.

Wiggins R. C., Giclas P. C., Henson P. M. (1981) Chemotactic activity generated from the fifth component of complement by plasma kallikrein of rabbit. Journal of Experimental Medicine, 153, 1391-1404.

Wilkinson P. C. (1973) Recognition of protein structure in leukocyte chemotaxis, Nature (London), 244, 512.

Wilkinson P. C. (1982) Chemotaxis and Inflammation (second edition), Churchill Livingstone, Edinburgh.

Wilkinson P. C. (1975) Leukocyte locomotion and chemotaxis: the influence of divalent cations and cation ionophores, Experimental Cell Research, 93, 420-426.

Wilkinson P. C. (1980) Effects of human IgG on locomotion of human neutrophils related to IgG binding of a hydrophobic probe, Immunology, 41, 457-466.

Wilkinson P. C. and Lackie J. M. (1983) The influence of contact guidance on chemotaxis of human neutrophil leukocytes. Experimental Cell Research, 145, 255-264.

Wilkinson P. C. and McKay I. C. (1971) The chemotactic activity of native and denatured serum albumin, International Archives of Allergy and Applied Immunology, 41, 237-247.

Wilkinson P. C., Borel J. F., Stecher-Levin V. J. and Sorkin E. (1969) Macrophage and neutrophil specific chemotactic factors in serum, Nature, 222, 244-247.

Wilkinson P. C., Shields J. M. and Haston W. S. (1982) Contact guidance of human neutrophil leukocytes. Experimental Cell Research, 140, 55-62.

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, The Journal of Cell Biology, 99, 1761-1768.

Williams L. T., Snyderman R., Pike M. C. and Lefkowitz R. J. (1977) Specific receptor sites for chemotactic peptides on human polymorphonuclear leukocytes. Proceedings of the National Academy of Sciences USA, 74, 1204-1208.

Wright D. G. (1981) Activation and deactivation of neutrophils., in, Infection: The Physiological and Metabolic Response of the Host., editors, Powanda M. C and Canonico P. G., Amsterdam, Elsevier, North-Holland, pp1-30.

Wymann M. P., Kernen P., Deranleau D. A. and Baggiolini M. (1989) Respiratory burst oscillations in human neutrophils and their correlation with fluctuations in apparent cell shape, The Journal of Biological Chemistry, 264, 15829-15834.

Yamamoto S., Nishiura M., and Hayashi H. (1973) The natural mediator for PMN emigration in inflammation, V: The site of structural change in the chemotactic generation of immunoglobulin G by inflammatory SH-dependent protease, Immunology, 24, 791-801.

Yoshimura T., Matsushima K., Tanaka S., Robinson E. A., Appella E., Oppenheim J. J. and Leonard E. J. (1987) Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defence cytokines. Proceedings of the National Academy of Science USA, 84, 9233-9237.

Yuli I. and Snyderman R. (1984) Rapid changes in light scattering from human polymorphonuclear leukocytes exposed to chemoattractants. Discrete responses correlated with chemotactic and secretory functions. Journal of Clinical Investigation, 73, 1408-1417.

Zigmond S. H. (1977) Ability of polymorphnuclear leukocytes to orient in gradients of chemotactic factors, Journal of Cell Biology, 75, 606-616.

Zigmond S. H. and Hirsch J. G. (1973) Leukocyte locomotion and chemotaxis: new methods for evaluation, and demonstration of a cell derived chemotactic factor. The Journal of Experimental Medicine, 137, 387-410.

Zigmond S. H. and Sullivan S. J. (1979) Sensory adaptation of leukocytes to chemotactic peptides, Journal of Cell Biology, 82, 517-527.

Zigmond S. H., Levitsky H. I. and Kreel B. J. (1981) Cell polarity: an examination of it's behavioural expression and it's consequences for polymorphonuclear leukocyte chemotaxis, Journal of Cell Biology, 89, 585-592.

Zigmond S. H., Slonczewski J. L., Wilde M. W. and Carson M. (1988) Polymorphonuclear leukocyte locomotion is insensitive to lowered cytoplasmic calcium levels, Cell Motility and the Cytoskeleton, 9, 184-189.

Zimmermann A. and Keller H. U. (1987) Locomotion of tumor cells as an element of invasion and metastasis, Biomedicine and Pharmacotherapy, 41, 337-344.

Zimmermann G. A., McIntyre T. M. and Prescott S. M. (1987) Naturally occurring lipids influence the interaction of human endothelial cells and neutrophils. In,

Leukocyte Emigration and It's Sequelae, Ed. Movat H. Z., pp 105-118, Karger, Basel.

Zimmermann A., Gehr P. and Keller H. U. (1988a) Diacylglycerol-induced shape changes, movements and altered F-actin distribution in human neutrophils. Journal of Cell Science, 90, 657-666.

Zimmermann A., Keller H. U. and Cottier H. (1988b) Heavy water (D_2O)-induced shape changes, movements and F-actin redistribution in human neutrophil granulocytes. European Journal of Cell Biology, 47,320-326.

ERRATA

Page 4, line 7: replace "10^{14}" with "10^{11}".

Page 4, line 8: replace " to almost 10^{18}" with " four to five fold".

Page 145, line 17: delete "(Fig. 6. 4.)".

Page 146, line 19: replace "(Fig. 6. 5. a.)" with "(Fig. 6. 4. a.)".

Page 147, line 11: replace "(Fig. 6. 5. b.)" with "(Fig. 6. 4. b.)".

Page 148, line 1: replace "(Fig. 6. 5. c.)" with "(Fig. 6. 4. c.)".

Page 149, line 12: replace "(Fig. 6. 6. a.)" with "(Fig. 6. 5. a.)".

Page 150, line 3: replace "(Fig. 6. 6. b.)" with "(Fig. 6. 5. b.)".

Page 150, line 20: replace "(Fig. 6. 6. c.)" with "(Fig. 6. 5. c.)".

Page 152, line 1: replace "(Fig. 6. 7.)" with "(Fig. 6. 6.)".

Page 152, line 5: delete "(Fig. 6. 8.)".

Page 161, line 17: delete "(Fig. 7. 4.)".

Page 162, line 18: replace "(Fig. 7. 5. a.)" with "(Fig. 7. 4. a.)".

Page 163, line 14: replace "(Fig. 7. 5. b.)" with "(Fig. 7. 4. b.)".

Page 164, line 3: replace "(Fig. 7. 5. c.)" with "(Fig. 7. 4. c.)".

Page 164, line 22: replace "(Fig. 7. 5. d.)" with "(Fig. 7. 4. d.)".

Page 166, line 4: replace "(Fig. 7. 6. a.)" with "(Fig. 7. 5. a.)".

Page 166, line 21: replace "(Fig. 7. 6. b.)" with "(Fig. 7. 5. b.)".

Page 167, line 13: replace "(Fig. 7. 6. c.)" with "(Fig. 7. 5. c.)".

Page 168, line 3: replace "(Fig. 7. 6. d.)" with "(Fig. 7. 5. d.)".

Page 169, line 8: replace "(Fig. 7. 7.)" with "(Fig. 7. 6.)".

Page 169, line 12: delete "(Fig. 7. 8.)".

Page, 202, line 14: insert "Harris E. D. (1988) Pathogenesis of rheumatoid arthritis: a disorder associated with dysfunctional immunoregulation. In, Inflammation: Basic Principles and Clinical Correlates, pp 751-773, editors, Gallin J. I., Goldstein I. M. and Snyderman R., Raven Press Ltd., New York.".

Page 217, line 23: insert "Simon R. H. and Ward P. A. (1988) Adult respiratory distress syndrome. In, Inflammation: Basic Principles and Clinical Correlates, pp 815-827, editors, Gallin J. I., Goldstein I. M. and Snyderman R., Raven Press Ltd., New York.".

Page 220, line 7: insert "Tauber A. I., Karnad A. B. and Ginis I. (1990) The role of phosphorylation in phagocyte activation. Current Topics in Membranes and Transport, 35, 469-494.".