Studies on Actin Polymerisation and Deformability in Polymorphonuclear cells and Monocytes

by

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Dedication

To my beloved Kuwait

To my Family

And

To

Jaber

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Abstract

Leukocytes become activated in response to hazards such as bacteria, viruses and particulates, and this activation results in reduced blood flow in disease states. This activation is associated with assembly of the cytoskeleton, including actin polymerisation, in preparation for extravasation and phagocytosis. Cytoskeletal organisation is a major factor determining leukocyte deformability and one of the major causes of leukocyte trapping and reduced microcirculatory flow in disease.

The aim of this thesis is to investigate the alteration in F-actin content and its association with activation and leukocyte deformability. All measurements were performed on whole blood samples of humans and Male Sprague Dawley rats using a simplified method to quantify and visualise F-actin content in leukocytes based on flow cytometric analysis and fluorescence microscopy. The Cardiff Filtrometer was used to study the association between leukocyte transit times, pore-blockers and F-actin content. Full blood counts, F-actin content and plasma viscosity were measured in rat blood after animal instillation of PM10. In addition, *in vitro* measurements were performed to investigate the effects of therapeutic agents that bind peroxisome proliferator-activated receptor (PPAR γ) on the F-actin content of leukocytes.

The results showed an increase in leukocyte F-actin after treating blood with either Phorbol 12-Myristate 13-Acetate (PMA) or Formyl-Methionyl-Leucyl-Phenylalanine (fMLP) and this effect was inhibited by the addition of CTB. These measurements were performed simultaneously with the filtration method, and correlated significantly ($r=0.217$ with $p=0.029$) between the F-actin content of PMNs and leukocyte transit time. Another significant correlation ($r=0.798$ with $p<0.001$)

was also observed between the F-actin content of PMNs and monocytes ($r=0.763$ with $p<0.001$) and the number of pore blockers. There was an apparent increase in leukocyte F-actin content in rats instilled with PM10, although these results were not statistically significant. The treatment of human blood with PPAR γ agonists resulted in a rapid decrease in the F-actin content of both PMNs and monocytes, which may result from the inhibition of early signalling cascades involved in actin polymerisation.

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Chapter One

Chapter One: General Introduction

1.1 Overview

Blood is the major transporting fluid in the body. Blood is composed of cellular elements (erythrocytes, leukocytes, and platelets) and plasma. The overall functions of blood are to transport nutrients (e.g. amino acids, sugars, mineral salts, hormones, enzymes, vitamins) essential for normal cell function, to remove waste products generated by the metabolic activities of tissues and to provide efficient defence against infections and maintain the integrity of circulatory system (Hall & Malia, 1991). By means of the haemoglobin contained in the red blood cells (erythrocytes), blood carries oxygen to the tissues, and also collects the carbon dioxide (CO_2). Blood circulates around the body in blood vessels. The heart pumps blood into the arteries that transport blood to the organs. Blood then percolates, via the microcirculation, into the tissues and completes the circuit by flowing back to the heart in the veins. The microcirculation consists of capillaries as small as 3μm in diameter and with one-cell thick walls. Blood has physiochemical properties such as isotonicity, viscosity and rheological properties all of which permit efficient circulation and function of the blood constituents. There are about 5×10^{12} red blood cells per litre of blood and they constitute about 40% of the total volume of blood. Red cells are bi-concave discs, which have no nucleus and a large membrane area that makes them readily deformable and able to flow with ease through narrow capillaries as small as 3μm in diameter, despite their own resting diameter of about 7μm. The erythrocytes' function is to carry oxygen from the lungs to the tissues around the body and to carry carbon dioxide to the lungs.

White blood cell (leukocytes), which form the first line of defence of the body against invading micro-organisms and dust particles, are one of the main components of blood. White blood cells are three orders of magnitude in number less than red blood cells, about $5x10^9$ white blood cells per litre of blood. There are three major classes of leukocytes in blood: granulocytes, monocytes and lymphocytes (**Figure 1.1**). Three types of granulocytes can be identified according to their morphology: neutrophils, eosinophils and basophils.

The lymphocytes and plasma cells are concerned with antibody production and antigen presentation. However, although polymorphonuclear cells (PMNs) and monocytes have an important role in immunology and host defence, their role is mainly phagocytic.

Granulocytes

The term granulocyte is due to the presence of granules in the cytoplasm of these cells, which are different in the three classes of granulocytes, enabling one to distinguish between them. In fact, these granules have a different affinity towards neutral, acid or basic stains and give the cytoplasm different colours. The shape of their nuclei can also distinguish them. Neutrophils, the most common leukocyte of circulating blood (2.5-7.5 x 10^9/L), have dense nuclei, with between two and five lobes. Eosinophils are similar to neutrophils, except that the cytoplasmic granules are coarser and the nucleus is limited to two or occasionally three lobes. They are less abundant than neutrophils (0.04-0.4 x 10^9/L), and are important in allergic responses. Basophils, the least numerous of the circulating leukocytes (0.01-0.1 x 10^9/L) have lobed nuclei and a granular cytoplasm with many dark granules. Degranulation of basophils is associated with histamine release. Granulocytes circulate in the blood for

4-6 hours before migrating into tissues where they perform their function as phagocytic cells and survive for 4-5 days.

The mononuclear cells are composed of lymphocytes and monocytes.

Lymphocytes

Lymphocytes, the second most frequent leukocyte type in the blood (1.5-3.5 x 10^9/L), are smaller cells than granulocytes with a small amount of cytoplasm surrounding a dense, round nucleus. They are responsible for antibody production. There are two classes of lymphocytes: T-lymphocytes (80% of the total numbers) and B-lymphocytes (20% of the total numbers) that can be differentiated mainly by their immunological characteristics. B-cells proliferate, upon recognition of antigen and mature into plasma cells that secrete antibodies. T-cells are involved in the cell-mediated response and in the regulation of the B-cell response.

Monocytes

The blood monocyte is an intermediate stage in the development of the tissue macrophage. The monocyte is a large cell with a copious cytoplasm, fine granules and a large oval nucleus. There are 0.2-0.8 x 10^9 monocytes in a litre of healthy blood. Monocytes have a full range of functions such as phagocytosis, chemotaxis and are also part of the cellular immune response.

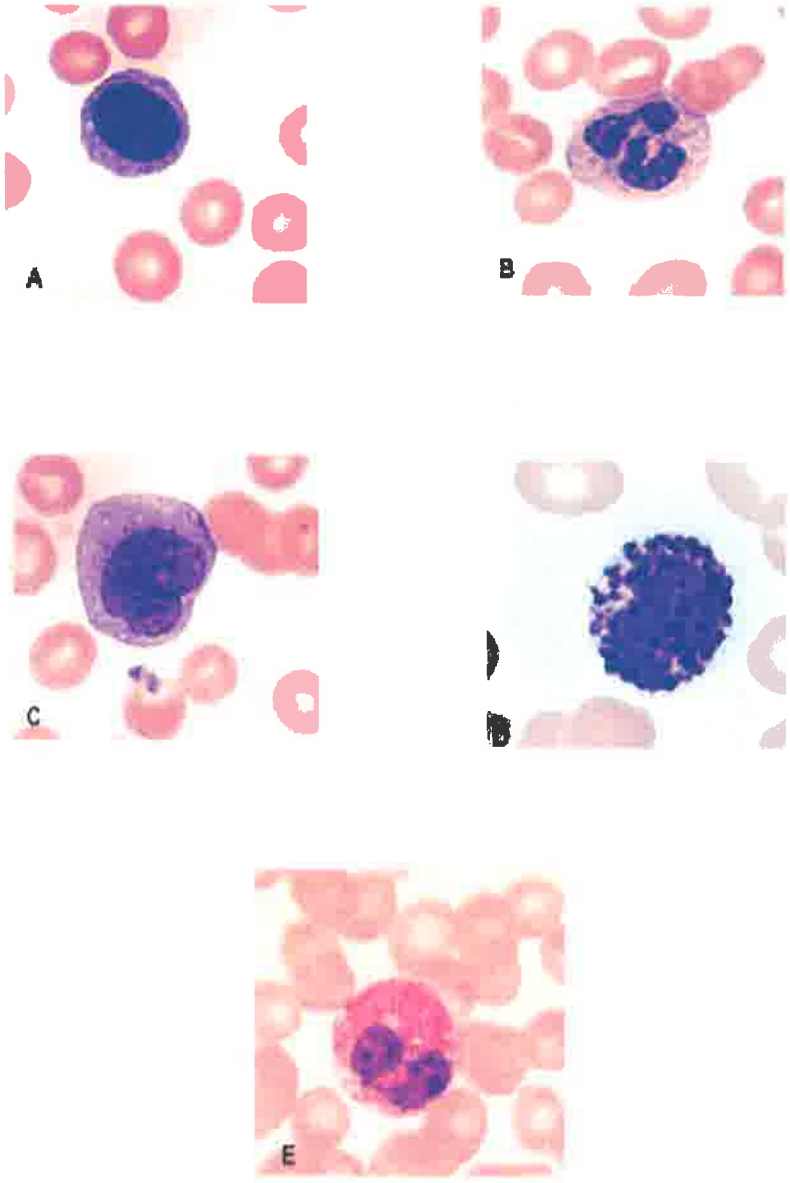


Fig 1.1: Different white blood cells A: lymphocyte, B: Neutrophil, C: monocyte, D: basophil, and E: eosinophil (wright Giemsa stain, x100 oil immersion. Harmening, 1992).

Leukocytes therefore have a much more complex structure than erythrocytes with a nucleus, cytoplasmic organelles and cytoskeleton and are capable of change from a passive to an active state. Leukocytes are an important determinant of flow in the microcirculation because they must deform to flow through capillaries and they become trapped at low perfusion pressure (Braide *et al*, 1984, 1989). Leukocytes become stimulated in response to infection by either bacteria or viruses. This results in morphological alteration in their cellular structure, including changes in the content of filamentous actin (F-actin), the major cytoskeletal component, which can result in leukocytes being trapped in the capillaries. Such changes in leukocyte properties and structure influence their density in the circulation due to resistance to deformation whereas, red cells passively respond to the flow forces upon them and are much more deformable (Warnke & Skalak, 1990). Moreover, white cells can alter vascular resistance by modulating their adhesiveness to endothelial cells (Bagge *et al*, 1986). In conclusion, although white cells are present in small numbers compared to red cells, they can influence microvascular flow and cause intermittent blockage, which can delay the flow of red cells.

1.2 Blood rheology

Blood, the major non-Newtonian transportation fluid of the body, flows through capillaries to deliver oxygen and nutrients to the tissues (discussed earlier). Blood flow in the human circulatory system is a very complex interplay of many factors such as viscosity, geometry of vessels; shear forces and many other factors (Ajmani & Rifkind, 1998). In large vessels, the relative contributions of white and red cells to the rheological behaviour of the blood depend on several factors. First, the number of red cells is higher than of white cells; about 700:1 which determines

that red cells are mainly responsible for blood viscosity. White cells make little direct contribution where the bulk viscosity of the blood is the main rheological factor (Lightman, 1973; Nash, 1992). However, in small vessels, where the diameter of red and white cells is similar to or less than the capillary diameter, the white cells become more important in determining flow.

1.2.1 Factors Governing Blood Rheology

Many factors govern the rheology of blood such as red cell aggregation, red cell deformability, whole blood viscosity and its determinants (haematocrit, fibrinogen, plasma viscosity), white blood cell counts and white cell deformability (Eastham & Slade, 1992; Lowe *et al*, 1993; Ajmani & Rifkind, 1998). All these factors that govern whole blood rheology are of varying significance *in vivo*, depending on the size of the vessels and the variation in shear stress across the diameter of the vessel. Alterations in these factors are associated with a range of disease states and are discussed later in this chapter. A brief review of these factors controlling blood flow maybe useful at this stage.

1.2.1.1 Red cell aggregation

Red cell aggregation plays a pathophysiological role in the microcirculation and is responsible for an increase in whole blood viscosity, especially at low shear rates (Bishop *et al*, 2001a, 2001b). At low shear rates, red cell aggregation results from the action of large plasma proteins, which adhere adjacent red cells, overcoming their mutual response due to negative surface charge (see Lowe, 1987). Further aggregation will form a network reducing the surface tension of the system (Eastham & Slade, 1992; Ehrly, 1990).

Many local haemorheological disturbances in the microvessels are related to intensified red cell aggregation and the subsequent local accumulation. Red cell aggregation is greatly increased by increases in hydrophilic colloids, such as fibrinogen, macroglobulins and IgM, and by the increase in the red cell counts (Ajmani & Rifkind, 1998; Ehrly, 1990).

1.2.1.2 Plasma viscosity

Plasma viscosity is an important contributor to whole blood viscosity. Plasma perfuses all blood vessels, in which it lubricates the passage of individual cells. Plasma viscosity is affected by the concentration of plasma proteins of large molecular size such as fibrinogen, albumin and serum globulins, low-density lipoproteins and very low-density lipoproteins. As these plasma proteins increase in concentration, plasma viscosity increases (Hoffbrand & Phillips, 1995; Ajmani & Rifkind, 1998) and this is seen in a variety of clinical situations such as trauma, surgery and acute infection (see Lowe, 1987).

1.2.1.3 Blood viscosity

Blood viscosity is a function of the concentration and composition of its components. The major determinant of blood viscosity is the haematocrit (Hct) because the red cells occupy the largest volume of blood cells. At low shear rates, whole blood viscosity varies with the plasma concentration of large molecules proteins and red cell aggregation whereas at high shear rates, whole blood viscosity reflects erythrocyte deformability (Eastham & Slade, 1992; Woodward *et al*, 1999; Palanduz *et al*, 1999). Loer *et al*, 1997 studied the relationship between flow resistance and haematocrit taking into account pulmonary blood volume and found

that flow resistance increased with increasing haematocrit due to changes in blood viscosity.

1.2.1.4 Erythrocyte deformability

Erythrocyte deformability is a complex term that reflects many intrinsic factors of the cell, such as internal viscosity, cell geometry, membrane flexibility and fluidity. Erythrocyte membranes are mainly composed of lipid and protein, which determine the plasticity and flexibility of the membrane and influence the membrane function (Brooks & Evans, 1987). Erythrocyte deformability plays an important role in the microcirculation, where the cells change their shape and deform in order to flow (**Figure 1.2**). Erythrocyte deformability increases with rising temperature, where the cells become elongated and irregularly deformed (Eastham & Slade, 1992).

Erythrocyte deformability is an important factor of blood flow in the circulation for several reasons. Firstly, red cell make up about 40% of whole blood volume and any change in the red cell deformability alters the viscosity of whole blood. Secondly, as red cells deform to flow through narrow capillaries, any decrease in red cell deformability increases the resistance to flow through the microcirculation. Finally, erythrocyte deformability affects red cell aggregation and hence viscosity at low shear rates (see Lowe, 1987).

The deformability is necessary for the effective infusion of the microcirculation and also influences the survival of red cells. Decreased deformability is an important risk factor in many disease states such as stroke and other circulatory disorders (Lowe, 1986; Ernst *et al*, 1987; Grau *et al*, 1992; Bradbury *et al*, 1993; Lowe *et al*, 1993). As well as depending on intrinsic factors

such as volume ratio and membrane viscoelasticity, erythrocyte deformability also depends on extrinsic factors such as shear stress acting on the cell and the viscosity of the medium surrounding the red blood cells, (Vaya *et al*, 1996; Palanduz *et al*, 1999).

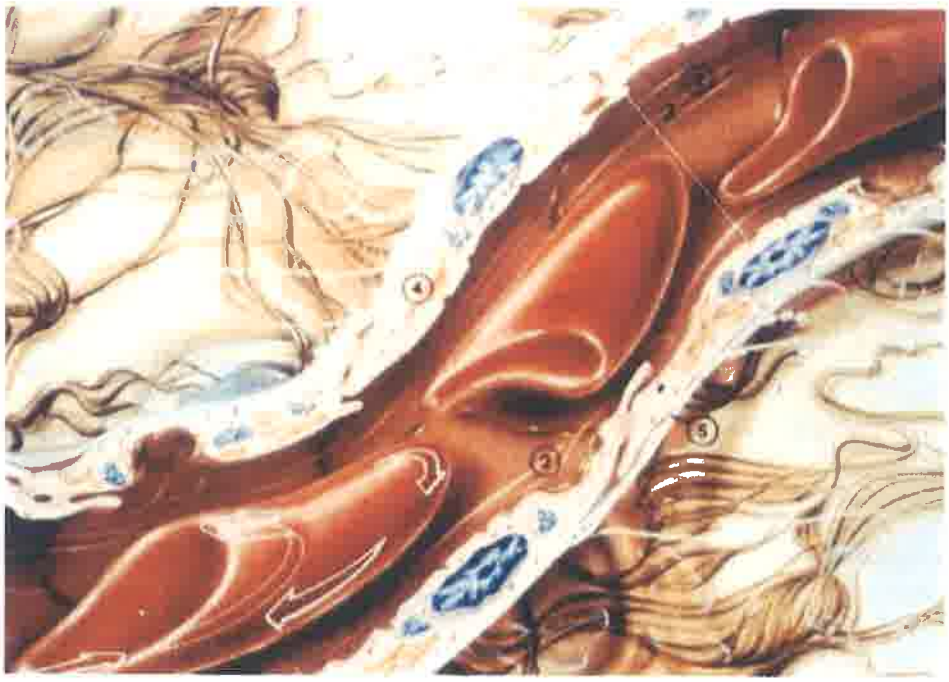


Fig 1.2: Artist's impression of red cells showing the capability of these cells to deform (Courtesy of Hoechst AG).

1. Direction of red cell flow
2. Plasma border
3. Capillary lumen (diameter approximately 4μm)
4. Endothelial cell
5. Basement membrane

1.2.1.5 Leukocyte deformability

Leukocytes, although fewer in number than erythrocytes, are larger with more complex structure and make a significant contribution to capillary flow resistance. Leukocytes are important determinant of flow in the microcirculation (**Figure 1.3**), as they deform approximately 1000 times more slowly than erythrocytes (Jones *et al*, 1994; Kitagawa *et al*, 1997). Their ability to flow is important in determining the overall resistance to flow in the microcirculation (Schmid-Shonbein & Lee, 1995). In healthy blood, the concentration of leukocytes has little effect on the bulk viscosity of blood and blood flow in large vessels (Kitagawa *et al*, 1997).

The normal leukocyte has been described as a viscous or viscoelastic liquid surrounded by a cortical layer that under tension pulls the cell into a spherical shape (Evans & Kukhan, 1984; Dong *et al*, 1988; Frank, 1990; Hochmuth & Needham, 1990). This tension is of a small magnitude and when present in the un-deformed, spherical cell is thought to act as the driving force for the recovery of the geometrically actin rich layer in the cell cortex. This tension is responsible for the increase in the internal viscosity of the cell, so that the deformation of leukocytes is far slower than that of erythrocytes (Schmid-Schonbein *et al*, 1981). Furthermore, the size of leukocytes and the presence of nuclei also affect the ability of the cell to flow. In previous studies, Downey *et al* (1990) showed that neutrophils and monocytes, the largest cell type, were significantly delayed in their pulmonary transit compared with cells of smaller diameter, (lymphocytes and platelets) due to their deformability characteristics as well as their size. It is well accepted that activation of leukocytes, accompanied by cytoskeletal organisation, greatly increases the viscosity

of the cell and decreases cell deformability (Nash & Meiselman, 1986, Pecsvarady *et al*, 1992). Leukocyte activation and the associated cytoskeletal re-organisation will be discussed later.

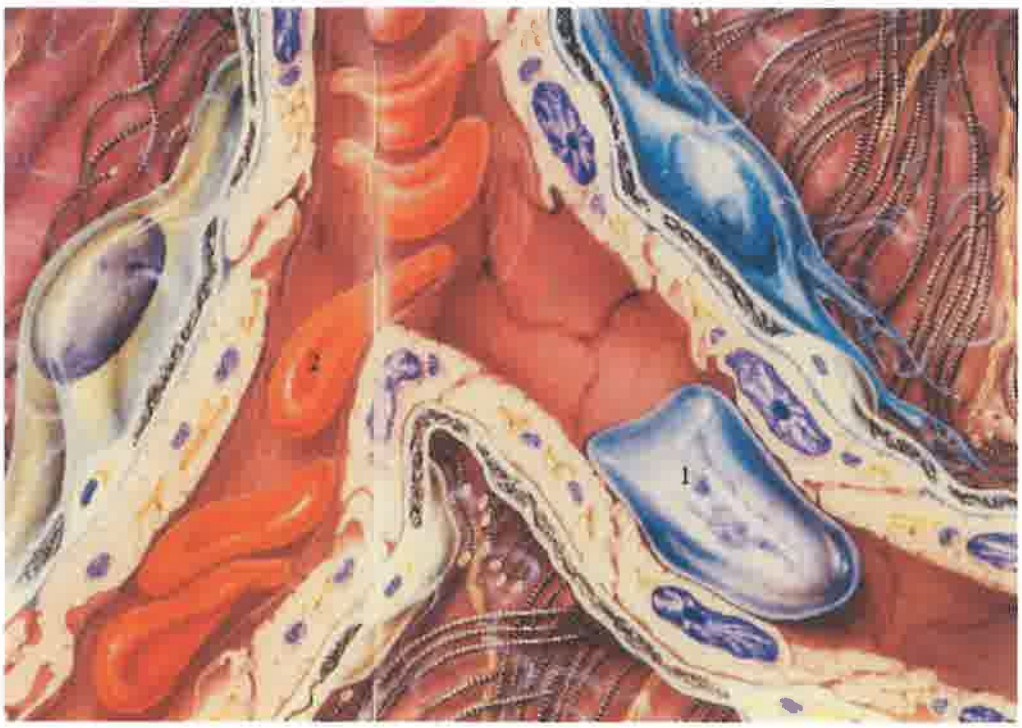


Fig1.3: Artists impression of red and white cells showing the size of white cells and their ability to flow (Courtesy of Hoechst AG).

1. White blood cell
2. Red blood cell

1.3 Leukocyte - endothelial adhesion

In the cardiovascular system, cells are exposed constantly to haemodynamic forces due to the flow of blood. Upon activation, the endothelium begins displaying specific cell surface receptors, such as cell adhesion molecules that bind free flowing neutrophils, slowing their movement and causing them to roll in the direction of flow through labile contact with the vessel wall (Konstantopoulos & McIntire, 1996). This process continues with the cells becoming more firmly adhered, then extravasating through the endothelial cell junctions (see **Figure 1.4**). The major families of adhesion molecules are integrins, selectins and proteins belonging to the immunoglobulin gene super-family.

Adhesion is a pre-requisite for many neutrophil functions. Cell adhesion requires multivalent interaction, and association between the cytoplasmic cytoskeleton and the membrane associated adhesion molecule is important.

The selectins constitutes a family of cell-cell adhesion molecule, involved in leukocyte-endothelial interactions. Selectins mediate various functions such as tethering of flowing leukocytes to the vessel wall and the formation of labile adhesions with the wall that permits leukocyte rolling in the direction of flow (Crockett-Torabi & Fantone, 1995; Springer, 1995; Araki *et al*, 1996; Frenette & Wagner, 1996; Whelan, 1996; Crockett-Torabi, 1998; Hillis & Flapan, 1998). There are three principal members of the selectin family: E, P and L selectin. The first of these, E-selectin, also called endothelial leukocyte adhesion molecule-1 (ELAM-1), is expressed on endothelial cells several hours after stimulation with inflammatory mediators such as bacterial lipopolysaccharide. E-selectin functions as a weak

adhesion receptor that produces a characteristic "rolling" motion of the leukocytes on the endothelial surface (Bevilacqua *et al*, 1987, 1989). Secondly, P-selectin, the granular membrane protein-140 (GMP-140) is also known as platelet activation dependent granule-external membrane protein (PADGEM). They are found in secretory granules in platelets and in the endothelial cells (Kreis & Vale, 1993; Springer, 1995). Expression of P-selectin on the endothelial surface is transient, peaking after 3 minutes and declining to basal levels within 20 minutes (Johnston *et al*, 1989; Larsen *et al*, 1990; Kreis & Vale, 1993). It is involved in the adhesion of platelets to monocytes and neutrophils, playing a central role in neutrophil accumulation within thrombi (Tedder *et al*, 1995). Lastly, L-selectin, first defined as lymphocytes homing receptor is also found on neutrophil surfaces. Neutrophils rapidly downregulate L-selectin by shedding in response to various chemotactic factors and phorbol esters (Kishimoto *et al*, 1989).

Integrins participate in numerous complex biological processes that include cell migration, tissue organisation, cell growth, blood clotting, inflammation, target recognition by lymphocyte and differentiation of many cell types (Schwartz *et al*, 1995). Integrins, as their name suggests, "integrate" the cell with its environment by mediating the adhesion of cells to each other and to the cell membrane. They also provide structural integrity to cells and tissues. In blood, integrins are expressed on platelets as well as white cells and principally interact with members of the immunoglobulin superfamily promoting leukocyte adhesion and infiltration (Hillis & Flapan, 1998). Integrins, transmembrane glycoprotein complexes are heterodimers of a non-covalently linked α and β subunits. Examples of this group are β_2 integrins, They are the most abundant integrin on neutrophils of which CD11b/CD18 and

CD11c/CD18 play a critical role in immune cell adherence and inflammation (Ruoslahti, 1991; Hynes, 1992)

The immunoglobulin superfamily is probably the largest and most diverse adhesion molecule family involved in the adhesion of leukocytes (Hunkapiller & Hood, 1989). They play an important role in the passage of leukocytes through tissues (Hillis & Flapan, 1998). Intracellular adhesion molecule-1 (ICAM-1) is an inducible member of this superfamily that can be expressed on endothelial cells, antigen presenting cells and other cell types. ICAM-1 plays a pivotal role in leukocyte adherence and emigration and important accessory roles in the activation of immune and inflammatory cells.

Thus, these adhesion molecules are relevant in the blood flow because cell adhesion to the endothelium of a vessel reduces the internal diameter of the vessel and hence increases flow resistance. Alterations in adhesion molecule expression and function have been implicated in cardiovascular pathology (Bevilacqua, 1994).

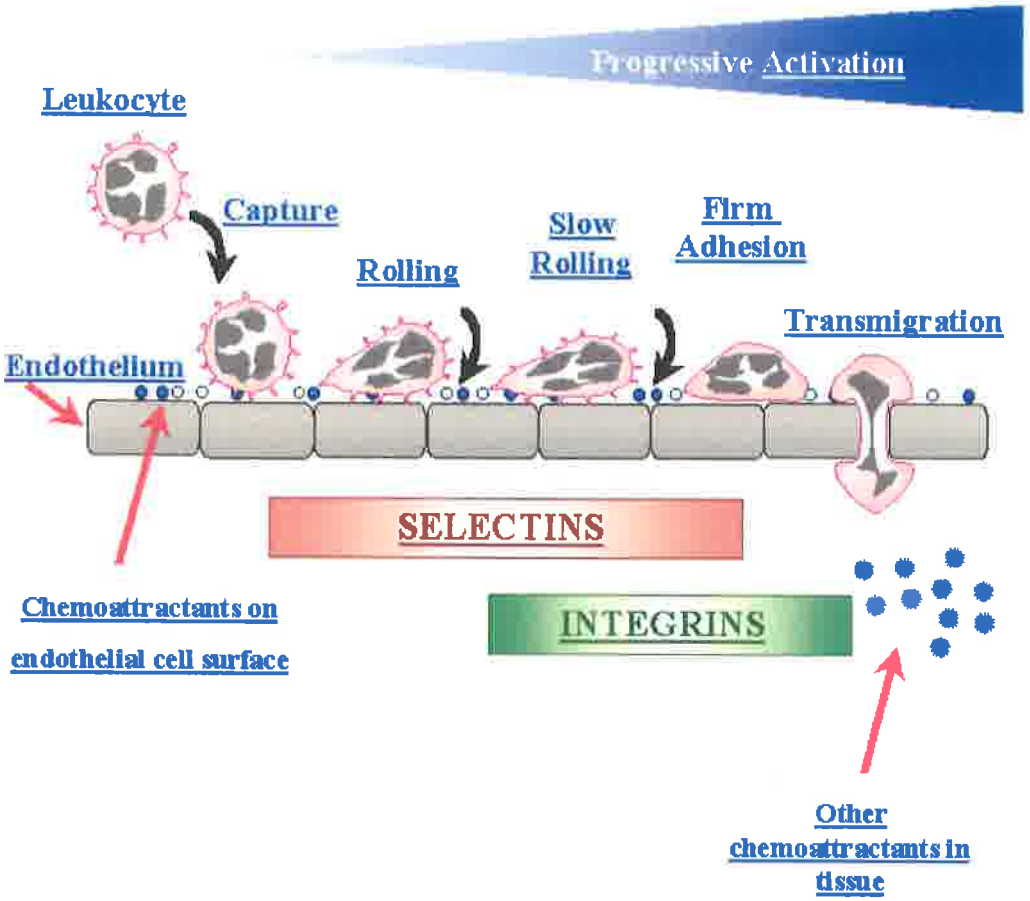


Fig 1.4: Model of the sequential steps in adhesion of leukocytes and the underlying molecular mechanisms (reproduced from Ley *et al*, 1999).

1.4 Cytoskeletal organisation in Eukaryotes

Eukaryotic cells have a complex internal structure (**Figure 1.5**). The larger the cell is, the greater is its need to keep these structures in their proper places and to control their movements. All eukaryotic cells have an internal skeleton, the cytoskeleton that gives the cell its shape, its power to move, and its ability to arrange its internal structures and transport them from one part of the cell to another. The cytoskeleton is composed of a network of protein filaments: actin filaments (F-actin), microtubules and intermediate filaments. Each is formed from a different type of protein: actin for actin filaments, tubulin for microtubules and vimentin or lamin for intermediate filaments (Alberts *et al*, 1994). The main components of the eukaryotic cytoskeleton are essential to many diverse cellular processes, for example, locomotion, mitosis and cytokinesis. (Stossel, 1984; Howard & Oresajo, 1985a, 1985b; Alberts *et al*, 1994). These processes require the precisely organized activity of proteins that regulate actin and tubulin assembly and disassembly together with motor proteins based on microtubules and actin filaments (Cramer *et al*, 1994; Giuliano & Taylor, 1995). Another function of the cytoskeleton is the maintenance of the precise distribution of ions, metabolites, macromolecules and organelles in time and space within the living cell. Actin is one of the most abundant cytoskeletal proteins in humans and is widely expressed in nearly all types of eukaryotic cells (Condeelis, 1993; Stossel, 1994a, 1994b). The cytoskeleton mediates several basic cell functions: chemotaxis, migration, phagocytosis and intracellular signaling (Valerius *et al*, 1982; Ding *et al*, 1993; Stossel, 1993; Hwang and Ding, 1995; Rivero *et al*, 1996; Janmey, 1998; Moller *et al*, 2000). The cytoskeleton network plays a dynamic as well as a structural role in cells.

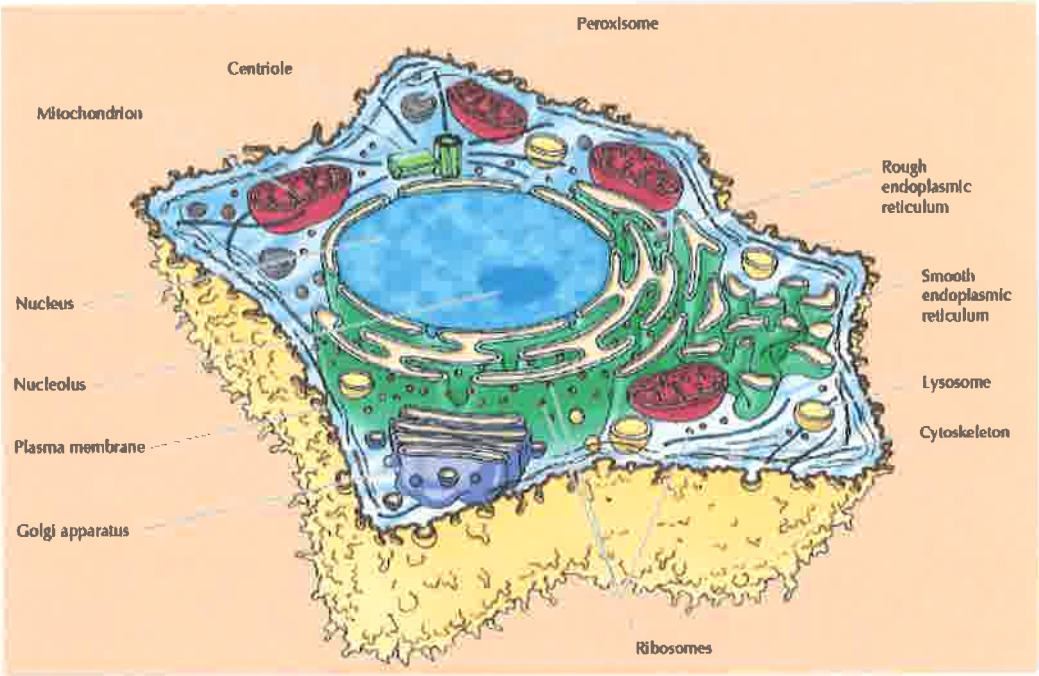


Fig 1.5: A eukaryotic cell surrounded by a plasma membrane and containing a nucleus, many cytoplasmic organells and a cytoskeleton. (Reproduced from Cooper, 2000).

The cytoskeleton of leukocytes is well suited to participate in an array of cellular functions because of its highly regulated and dynamic activity (Giulliano & Taylor, 1995). Dynamic assembly, disassembly and reorganisation of the leukocyte cytoskeleton are required for cellular motile processes such as shape change, chemotaxis, secretion and phagocytosis. The cytoskeleton of leukocytes consists of a complex framework of filamentous actin and associated proteins (Pollard & Cooper, 1986; Watts & Howard, 1993), and leukocyte activation leads to changes in cell content of filamentous actin (F-actin), which is the major cytoskeletal component. In the cytoplasm of leukocytes are a number of actin binding proteins that interact with actin monomer and act to regulate its structure and the mechanical behaviour of the actin network (Frank, 1990; Watts & Howard, 1993).

The actin of the cytoskeleton exists in equilibrium between two forms, a monomeric G-actin form and a polymeric F-actin form. Rapid conversion from G-actin to F-actin in polymerisation and /or F-actin to G-actin in depolymerisation in response to chemotactic factors or cellular activators permits a rapid and reversible cytoskeletal reorganisation to perform required motile functions (Howard & Oresajo, 1985a, 1985b; Cassimeris, 1990; Frank, 1990; Watts & Howard, 1993).

Investigation of cytoskeletal reorganisation in leukocytes may provide diagnostic clues in diseases associated with flow disturbances (Ravel, 1980, Tsai *et al*, 1996, 1998). There now follows a review of leukocyte cytoskeletal reorganisation.

The three components of the cytoskeleton are microtubules, intermediate filaments and filamentous actin (F-actin). The three components differ from each other regarding filament thickness, function and associated protein.

1.4.1 Microtubules

Microtubules, one of the key components of the cytoskeleton, are stiff polymers that extend throughout the cytoplasm and govern the location of membrane-bounded organelles and other cell components. Microtubules are hollow cylinders of about 25nm in diameter with tubulin dimers packed head to tail, with 8nm spacing between dimers to form profilaments running length wise along the microtubule wall (**Figure 1.6**) (Mays *et al*, 1994; Wade & Hayman, 1997; Nedelec *et al*, 1997; Raff *et al*, 1997). Microtubules are formed from tubulin molecules, each of which is a heterodimer consisting of two closely related and tightly linked globular polypeptides called α-tubulin and β–tubulin (Alberts *et al*, 1994; Lodish *et al*, 2000; Cooper, 2000).

Microtubules, highly dynamic structures that can be lengthened and shortened, are polar structures with two ends that are found to be distinctly different during growth and shrinkage: a plus end, which usually points towards the cell periphery that is capable of a rapid growth by adding tubulin molecules by polymerisation events; and a minus end that tends to lose sub-units if not stabilized. The minus ends originate from a specific microtubule organising centre, and are stabilized by embedding them in a structure called a centrosome (Alberts *et al*, 1994; Nedelec *et al*, 1997; Raff *et al*, 1997). Growing microtubules usually have long, curved and sheet-like extensions up to several microns in length, whereas shrinkage involves profilaments curling into tight rings (Wade & Hayman, 1997).

The dynamic instability of microtubules requires GTP hydrolysis to shift the chemical balance between polymerisation and depolymerisation. GTP binds to the β–tubulin subunits and by the addition of tubulin molecules to the end of the mirotubule

GTP is hydrolysed to GDP. The normal role of GTP hydrolysis is to allow the microtubule to depolymerise by weakening the bonds between tubulin subunits in the microtubule (Alberts *et al*, 1994, Lodish *et al*, 2000; Cooper, 2000). The organisation of the microtubule surface has been shown to influence such events as microtubule nucleation and interaction between microtubules and motor proteins (Wade & Hayman, 1997).

Cytoplasmic dyneins and kinesins, motor proteins that generate an intracellular movement in eukaryotic cells, are composed of two heavy chains plus several light chains. Each heavy chain contains a conserved, globular ATP-binding head and tail composed of rod-like domains. These motor proteins move along microtubules carrying membrane-bounded organelles to their desired location in the cell (Alberts *et al*, 1994; Lodish *et al*, 2000; Cooper, 2000). Both motor proteins bind either to actin filaments or a microtubule and use the energy derived from repeated cycles of ATP hydrolysis to move steadily along it. There are different types of motor proteins according to the type of filament they bind to, the direction in which they move along the filaments and the cargo they carry (**Figure 1.7**) (Alberts *et al*, 1994; Lodish *et al*, 2000; Cooper, 2000).

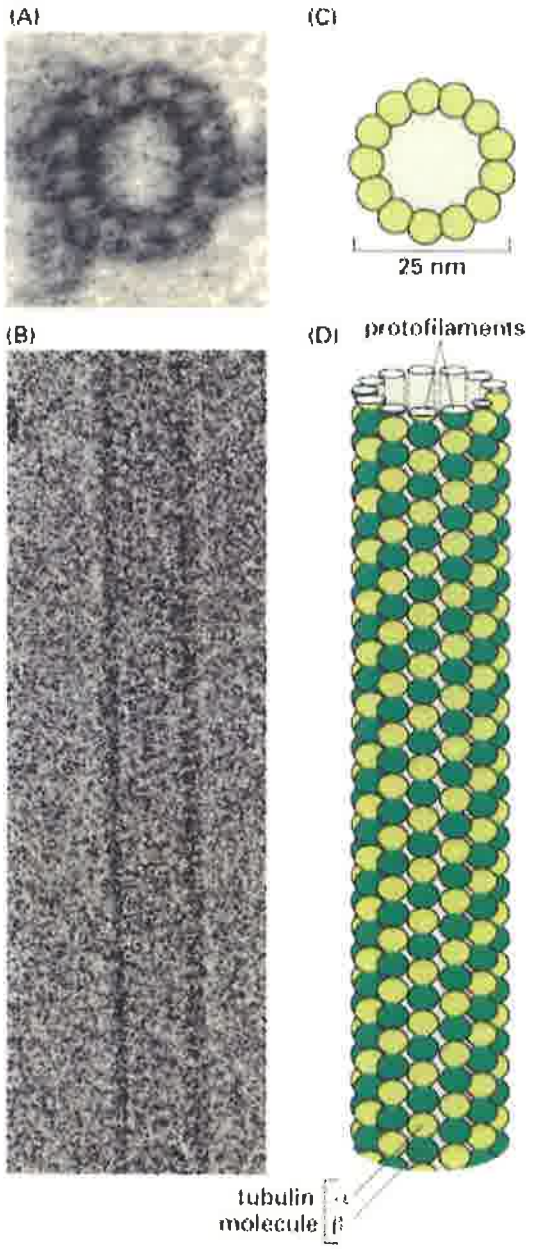


Fig 1.6: Electron micrograph and schematic diagram of microtubule, showing how the tubulin molecules pack together to form the cylindrical wall. Tubulin molecules align into long parallel rows, or protofilaments, each of which is composed of α and β tubulin heterodimer (Reproduced from Alberts *et al*, 1994).

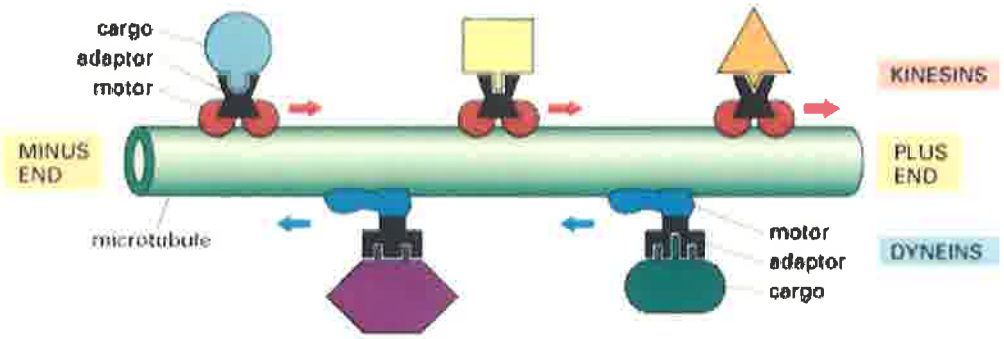


Fig 1.7: Motor proteins that move along microtubules. Kinesins move towards the plus end whereas, dyneins move towards the minus end. Both motor proteins exist in many forms, each of which is thought to transport a different cargo (reproduced from Alberts *et al*, 1994).

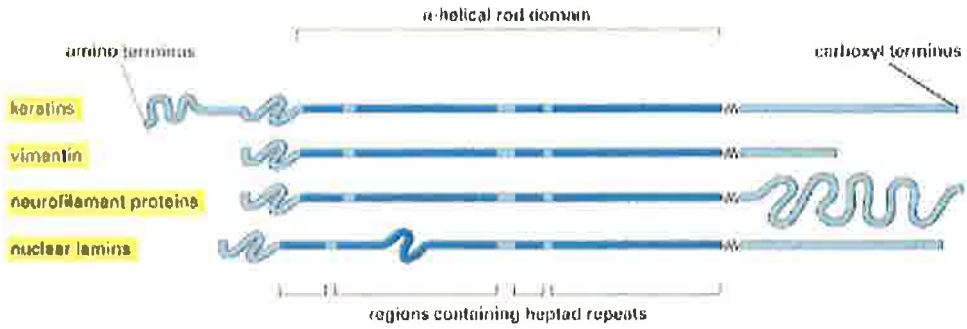
1.4.2 Intermediate filaments

Intermediate filaments are tough, durable, stable and insoluble protein fibers found in the cytoplasm of most animal cells. These filaments are 8-10nm in diameter, and are easily distinguished from microtubules and actin filaments (**Figure 1.8**). The intermediate filament structure occurring in different cells are not always formed by the same protein(s) but by different members of a large multigene family. These proteins are expressed specifically in a given cell type and at distinct states of differentiation (Franke *et al*, 1979 & Osborn & Weber, 1983). In most animal cells, the presence of intermediate filaments surrounding the nucleus and extending out to the cell periphery, where they interact with the plasma membrane, suggests that their principal function is structural (Alberts *et al*, 1994; Lodish *et al*, 2000; Cooper, 2000). Unlike microtubules and microfilaments, which participate in cell motility, the most important function of intermediate filaments is to provide mechanical support for the plasma membrane where it comes into contact with other cells or with the extracellular matrix (Lodish *et al*, 2000).

Intermediate filaments are highly elongated fibrous molecules with an amino-terminal, a carboxyl-terminal tail and a central rod domain that forms an extended coiled structure when the protein dimerizes. The available terminal domains allow each type of filament to associate with specific other component in the cell, so as to position the filament appropriately for a particular cell type. Intermediate filaments are a non-polarized structure where both ends are the same and are symmetrical along its length. A variety of tissue specific forms are known that differ in the type of polypeptide they contain. These forms include keratin filaments of epithelial cells, neuro-filaments of nerve cells, glial filaments of astrocytes and schwann cells,

desmin filaments of muscle cells and vimentin filaments which are expressed in leukocytes, blood vessel endothelial cells, some epithelial cells, and mesenchymal cells such as fibroblasts (Alberts *et al*, 1994; Lodish *et al*, 2000).

(A)



(B)

Fig 1.8: (A) The intermediate filaments in the cytoplasm of a tissue culture cell (Rat Kangaroo epithelial cells). (B) The domain organisation of intermediate filaments. A variety of tissue specific forms share a similar rod domain but differ in the type of polypeptide they contain (Reproduced from

1.4.3 Actin filaments (F-actin)

Actin, the most abundant cytoskeletal protein in many cells, often constitutes 5% or more of the total protein. It constitutes as much as 10% of the total protein in non-muscle cells (Fukui, 1993). Each actin molecule is a single peptide that has a molecule of ATP associated with it (Stossel, 1994a, 1994b). There are three classes of actin: α-, β- and γ-actins. The α-actins are found in various types of muscle cells, whereas, β- and γ-actins are the principal components of non-muscle cell microfilaments. Studies have described differential subcellular localization of γ-actin and isotype specific binding of actin associated proteins (Pardo *et al*, 1983; Otey *et al*, 1986; Sheterline *et al*, 1995).

Actin filaments are about 8nm in diameter (**Figure 1.9**) and are at least 30 times greater than the total length of microtubules which reflects a fundamental difference in the way those cytoskeletal polymers are organized and function in cells (Janmey & Choponnier, 1995; Alberts *et al*, 1994). Actin filaments can form both stable and labile structures in cells. Each structure performs a different function. Stable actin filaments are a crucial component of the contractile apparatus of muscle cells whereas labile actin is involved in many cell movements (Cramer *et al*, 1994).

Changes in cell shape, anchorage and motility are associated with the dynamic reorganisation of the architectural arrays of F-actin that make up the actin cytoskeleton. The different functions in F-actin arrays are linked to the pattern of contacts that a cell develops with its extracellular substrate (Small *et al*, 1999). F-actin assembly is important for cytoskeletal reorganisation and stabilisation and acts as a critical factor in the mechanisms leading to protrusion of lamellapodia and provision of motor function in chemotaxis (Kitagawa *et al*, 1997).

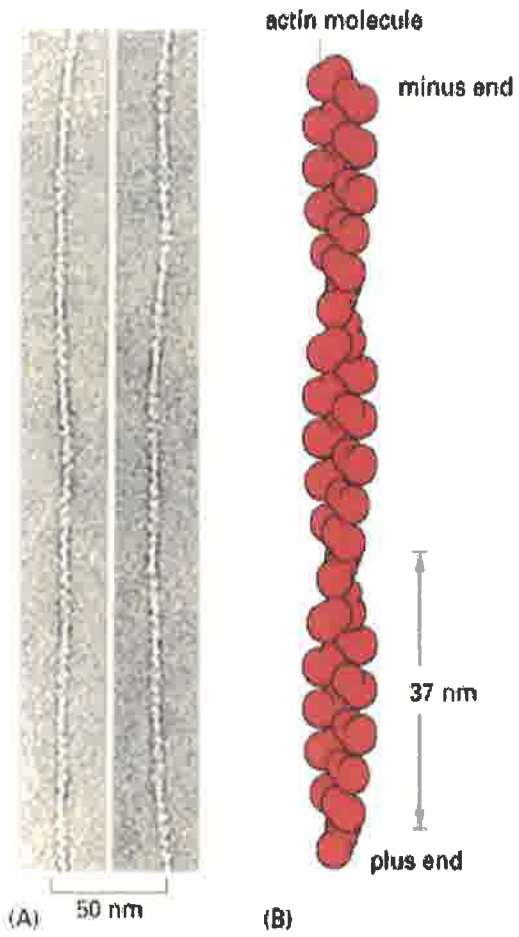


Fig 1.9: (A) Electron micrographs of actin filaments. (B) The helical arrangement of actin molecules in an actin filament. (A, courtesy of Roger Craig.) (Reproduced from Alberts *et al*, 1994).

1.4.4 Actin polymerisation

To form F-actin, monomers of actin (G-actin) assemble into small oligomers, consisting of three or four actin monomers (Coluccio, 1994). It has been found that monomers preferentially add onto pre-existing filament rather than forming new nuclei (Stossel, 1989). Actin polymerises in response to different chemoattractants such as phorbol myristate acetate (PMA) and N-formyl-Met-Leu-Phe (fMLP). Following polymerisation, ATP bound to G-actin is hydrolysed, leaving the resulting ADP trapped in the polymer and leading to conformational changes in the actin structure. Although ATP hydrolysis is not prerequisite for polymerisation, it serves to weaken the bonds in the polymer as in tubulin and thereby, promote depolymerisation (Kabsch & Vandekerckhove, 1992; Mays *et al*, 1994; Welch *et al*, 1997; Alberts *et al*, 1994). When the filaments depolymerise, G-actin with bound ADP is released. This complex has a lower affinity for existing filaments than G-actin with ATP. Therefore, ADP is exchanged for ATP before further polymerisation (Goldschmidt-Clermont & Janmey, 1991; Theriot, 1994). Thus, in cells there exists a dynamic equilibrium between monomers and polymers of actin that is affected by actin binding proteins. Actin filaments display a distinct polarity with a barbed end (plus end) that is much more prone for growth upon addition of monomeric actin. This end differs by an order of magnitude from the pointed (minus end), in the affinity and exchange of G-actin (Carlier, 1991).

For actin polymerisation to occur, a large cellular pool of un-polymerised actin is maintained at a higher concentration well above those needed for actin polymerisation. To maintain this pool, cells employ monomer-binding proteins such as profilin and thymosin B4, which control polymerisation through monomer

availability and barbed end capping protein. Actin polymerisation results in rapid remodelling of the cytoskeleton and a directed movement of the cells towards the chemoattractant (Chen *et al*, 1996; Alberts *et al*, 1994).

1.4.5 Actin depolymerisation

Actin must depolymerise and the subunits be recycled to maintain polymerisation. There are clues that actin depolymerisation is part of the cellular response to chemoattractant and other signalling molecules (Welch *et al*, 1997). Pure F-actin depolymerises by simple dissociation of monomers from free filament ends. Subunits dissociate from pointed ends and associate with barbed ends in a process called turnover and caused by an energy dependent tread milling reaction (**Figure 1.10**). The turnover is much more rapid at the leading edge of F-actin, suggesting the presence of factors that either promote depolymerisation from pointed ends or provide different pathways such as filament severing (Welch *et al*, 1997; Alberts *et al*, 1994).

To allow cytoskeletal organisation and function, actin filaments' depolymerisation must be restricted to appropriate locations, such as the middle and back of leading edges (Rozycki *et al*, 1994; Welch *et al*, 1997).

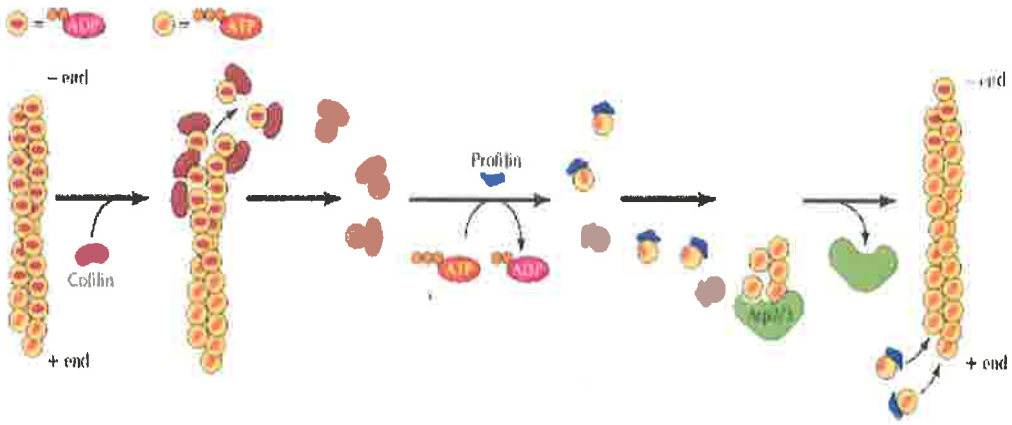


Fig 1.10: Assembly and disassembly of actin filaments by actin binding proteins. Cofilin binding increases the rate of dissociation of actin monomers (bound to ADP) from the minus end. Cofilin remains bound to the ADP-actin monomers, preventing their reassembly into filaments. Profilin can stimulate the exchange of bound ADP for ATP, resulting in the formation of ATP-actin monomers that can be repolymerised into filaments, including new filaments nucleated by the Arp2/3 proteins (Reproduced from Cooper, 2000).

1.4.6 Actin-binding proteins

The fundamental structure of F-actin is the same in every living cell. It is the length of these filaments, their stability and the number and geometry of their attachments that varies in different cytoskeletal assemblies. These properties depend on actin binding proteins, which bind in a special way to F-actin and modulate their properties and functions by cytoskeletal organisation (Alberts *et al*, 1994). Numerous proteins control the assembly of cytoplasmic actin and the organisation of actin filaments into a three-dimensional network. It is the cooperative and competitive interactions among these proteins that prevent the various sets of actin structure from being mixed and from escaping their spatial distributions (Pollard *et al*, 1994).

F-actin can interact with different sets of actin binding proteins at different locations in the cortex. These sets of actin binding proteins act together to generate the movement of cell surface, including cytokinesis, phagocytosis and cell locomotion. These movements are difficult to analyse because of the many components involved, but it is known through genetic mutations that individual actin binding proteins perform specific functions in each process (Alberts *et al*, 1994).

In cells, the precise function of each protein is influenced by competition for the same or adjacent binding sites on actin, by local concentration of a particular protein and by factors such as pH and ionic conditions (Korneeva & Jockusch, 1996). Many proteins display several, varying functions in a cell. Individual actin filaments can associate with several different proteins simultaneously, permitting a diversity of organisational variations (Lauffenburger & Horwitz, 1996).

1.4.6.1 Barbed end-binding proteins

These proteins tend to cap most filament ends in resting cells. Activation results in the dissociation of the capping proteins from the barbed ends. This un-capping occurs just below the plasma membrane at the leading edge of moving cells and provides spatial control of actin assembly for cell motility (Barkalow & Hartwig, 1995). Examples of actin binding proteins in this group are: capping protein, profilin, capG, capZ, tensin and gelsolin. Binding of proteins to actin filaments prevents the addition and loss of G-actin sub-units at the barbed end. These proteins require Ca^{2+} to bind to actin and are inhibited from capping *in vitro* by polyphosphoinositides (Barkalow & Hartwig, 1995). Two classes of proteins are candidates for promoting rapid actin turnover and play an important role in the cellular response to signalling molecules: the gelsolin family and the actin depolymerising factor (ADF)/ cofilin family (Welch *et al*, 1994). Gelsolin, capping protein, can stop further filament growth by capping the barbed end of the filament but because exchange of subunits at the pointed end is slow, capping is not efficient for the depolymerisation process. To achieve rapid depolymerisation, some members of gelsolin family bind to the side of actin filaments, sever bonds holding subunits together which simultaneously shorten them and increase their number providing more ends from which depolymerisation can take place and remain firmly affixed to the severed barbed ends to prevent reannealing of the broken filaments (Stossel, 1994). The second actin depolymerisation protein, ADF/cofilin, nibbles actin monomers out of filaments at places of defects, which also results in an increase in number of shortened filaments. Signals, such as chemoattractants, that induce cellular movement activate an phosphatase, which dephosphorylates ADF/cofilin (**Figure 1.11**). The

dephosphorylated ADF/cofilin becomes activated and translocated to regions of the cell where it promotes filament turnover. These proteins are less efficient than gelsolin in shortening actin filaments but because of their high abundance they could together with gelsolin play an important role in filament shortening (Stossel, 1994a, 1994b).

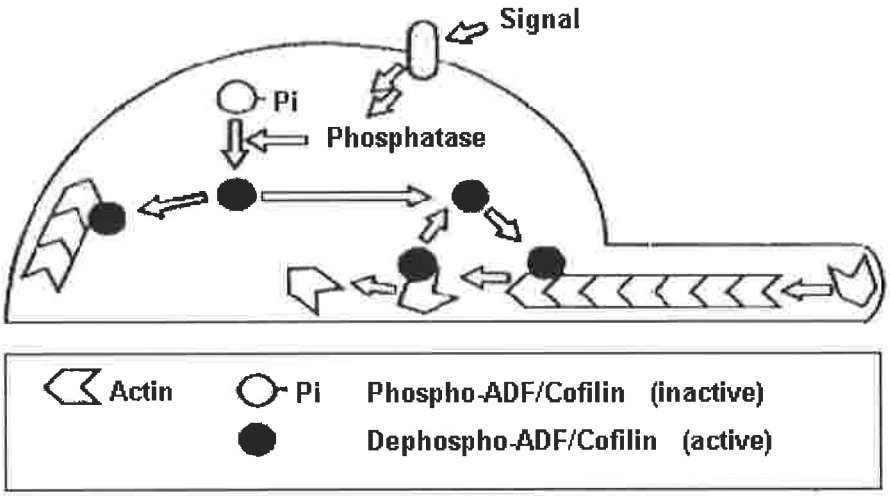


Fig 1.11: Activation and localisation of ADF/cofilin proteins during depolymerisation. A signal(s), such as a chemoattractant that induces cellular movement activates an unknown phosphatase, which dephosphorylates regions of the cell where it promotes filament turnover. At the leading edge of the cell, ADF/cofilin may promote rapid turnover, thus freeing actin monomers for new assembly (Reproduced from Welch *et al*, 1994).

1.4.6.2 Side-binding proteins and cross-linking proteins

Actin filaments stabilised by actin-cross-linking proteins (Matsudaria, 1994; Hartwig & Kwiatkowski, 1991), tend to align in parallel bundles (Stossel, 1993). By connecting filaments together the actin binding proteins exerts a stabilising effect by inhibiting monomer loss. Cross-linking of filaments into structures of higher complexity includes formation of bundles and networks, which gives strength to various protrusions (Aderem, 1992). Examples of actin cross-linking proteins are: villin, α-actinin, filamin-1 and spectrin, and of side-binding proteins are actin-related proteins (Arps2/3) (Machesky & Gould, 1999; Machesky, 1999).

1.4.6.3 Monomer sequestering and severing proteins

A large pool of non-polymerised actin, which exists at a concentration above the critical concentration for polymerisation *in vitro*, is the fuel for actin polymerisation. Maintenance of this pool is provided by employment of different classes of actin-monomer sequestering proteins such as profilin, thymosin β_4 and ADF/cofilin family (Goldschmidt-Clermont & Janmey, 1991; Pollard *et al*, 2000; Haarer & Brown, 1990; Welch, 1997). Severing of actin filaments denotes breaking of non-covalent bonds within a filament. Examples of actin filament severing proteins are gelsolin and villin (Schafer & Cooper, 1995). As the severing proteins dissociate from the filament ends, the proteins will yield new nuclei for actin polymerisation. The best-characterised protein in this group is gelsolin which when activated severs actin filaments and then forms a cap on the exposed plus end (McLaughlin *et al*, 1993).

1.4.6.4 Membrane-associated proteins

Various types of protein attachments are needed for actin filaments to perform their functions at the cell cortex. Actin structures are reversibly anchored to the cytosolic face of the plasma membrane. A class of actin-binding proteins that include myristoylated, alanine-rich C kinase substrate (MARCKS, Thelen *et al*, 1991) facilitates this reversible cycle by binding to the membrane. This binding is regulated by protein kinase C-dependent phosphorylations (Graff *et al*, 1989; Rosen *et al*, 1990). Another class of proteins are thought to function as molecular linkers between actin filaments and the plasma membrane. This class is called ERM proteins (ezrin, radixin and moesin) (Sanchez-Madrid & del-Pozo, 1999).

1.4.7 Role of signal transduction in actin polymerisation

The dynamic cortical meshwork of F-actin rearranges rapidly in response to extracellular signals that impinge on the plasma membrane. These signals bind externally displayed membrane receptors for chemotactic factors and adhesion molecules leading to net actin assembly (Gallin *et al*, 1999).

Leukocyte chemoattractant receptors have various functions. They direct migration and activate integrin adhesiveness; they also stimulate degranulation, actin polymerisation, shape change and respiratory burst. In neutrophils, polymerisation of actin starts with the binding of chemoattractant receptors to their ligands (**Figure 1.12**). The ligand-receptor complex interacts with G-protein to produce an activated G-protein forming a ligand/receptor/G-protein complex. This complex exchanges GTP for GDP and dissociates to produce α-GTP and βγ-GTP binding subunits (Adams *et al*, 1998). The α- and βγ -subunits of G-protein can affect a multiplicity of

effector enzymes (second messengers) and these effectors can either converge or diverge to trigger the individual functional responses in neutrophils (Cockcroft, 1992). The release of βγ -subunit of G-protein activates two major signalling molecules: phospholipase C (PLC) and phosphoinositide 3-kinase (PI3K), which metabolise phophatidylinositol 4,5-biphosphate (PIP_2). PI3-kinase is activated by small GTP-binding proteins related to to Ras GTPases, which are active regulators of changes in cell shape and neutophils motility (Katanaev, 2001; van Nieuw Amerongen & van Hinsberg, 2001). These small GTPases activate several pathways to activate actin polymerisation (Carpernter, 2000). Upon activation, neutrophil PLC hydrolyses PIP_2 and generates inositol 1,4,5-triphosphate (IP_3) and diacyglycerol (DAG) (Zhelev & Alteraifi, 2002). The precise mechanism of coupling of the signals from the membrane receptors and the processes controlling the rearrangement of the cytoskeleton are not fully understood. Another study suggested that filament uncapping plays a role in actin polymerisation. Using platelets as a model for studying the response of the leading edge of the filament to signal transduction shows that filament uncapping plays a role in actin polymerisation. Filament uncapping in response to thrombin-receptor activation is mediated by small GTP-binding proteins from the Rac family, which cause an increase in the cellular levels of polyphosphoinositides (Hartwig *et al*, 1995; Welch *et al*, 1997). The polyphosphoinositides bind directly to the barbed end capping protein (CapZ) and dissociate it from filament-barbed end, allowing filament elongation to occur (Barkalow *et al*, 1996; Welch *et al*, 1997).

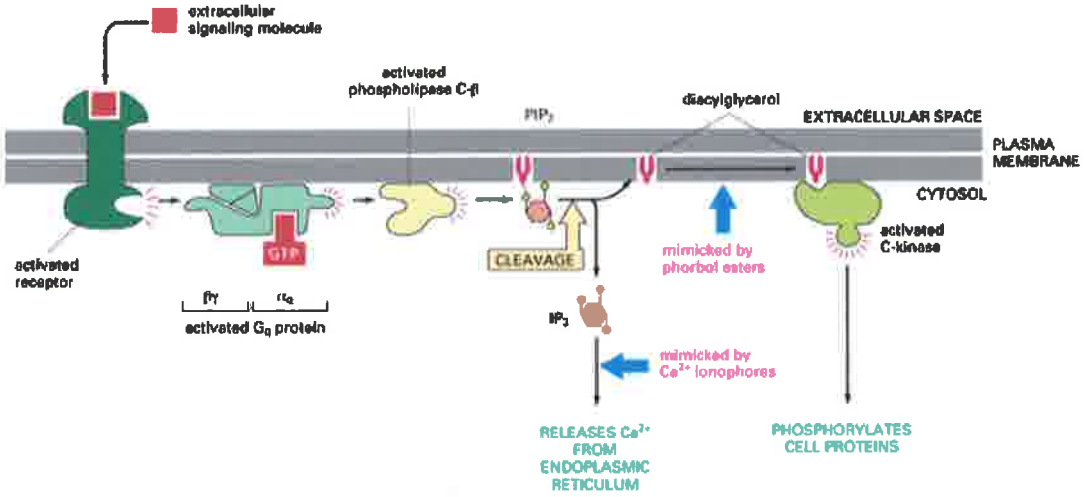


Fig 1.12: Summary of the signalling pathways triggered as a result of binding of chemoattractant receptors to their ligands. (Reproduced from Alberts *et al*, 1994).

1.4.8 Clinical aspects of the actin cytoskeleton

Actin provides stiffness to the cell and an abnormal actin structure or defective regulation can make cells too soft or too stiff to function normally. The actin cytoskeleton is important to control surface motion and structural polarity as emphasised by Leiser & Molitoris (1993). Disruption of the cytoskelton leads to irreversible cellular dysfunction in a highly polarised cell (Janmey & Choponnier, 1995). The actin cytoskeleton is likely to have a pathological role as in many disease states leukocyte activation is implicated. Both monomers and polymers of actin are implicated in many cell functions. These functions of leukocytes include motility, cytokinesis, secretion, mRNA localisation, protein sorting and special ordering of glycolysis and signal transduction. Defects in any of these processes are likely to be related to cytoskeletal alterations in the structure or localisation of actin or actin binding proteins which are directly associated with pathologic events (Janmey & Choponnier, 1995; Giuliano & Taylor, 1995).

A normal actin cytoskeleton is essential for entry propagation of various infectious organisms, and the release of cytoskeletal elements into the extracellular space may contribute to allergies, coagulation defects and cystic fibrosis (Janmey & Choponnier, 1995; Prat *et al*, 1999; Ruf *et al*, 2000; Cantiello, 2001). Abnormal expression of actin and actin-binding proteins are implicated in cell transformation, motility, cancer, fibrotic diseases and scar formation (Janmey & Choponnier, 1995). Melanoma cells lacking actin-binding proteins, ABP, are poorly motile, emphasising that actin gelation is essential for cell motility (Cunningham, 1992). Another study of rat sarcoma cells confirms the hypothesis that the structure of the actin network relates to the malignant cell potential of cell line because it determines their motility

(Pokorna *et al*, 1994). In conclusion, the range of diseases in which abnormal actin expression or organisation occur is broad, and often linked to subtle changes in the regulation of expression of actin and actin-binding proteins (Janmey & Choponnier, 1995).

A normal actin cytoskekleton is also essential for invasion and infection by various bacteria, viruses and other parasites. In bacteria, for example, *salmonella,* actin-mediated membrane ruffling similar to that activated by growth factor is induced. These bacteria managed to do so without the involvement of small G proteins such as Ras and Rho that are required for growth factor mediated cytoskeletal changes (Jones *et al*, 1993; Janmey & Choponnier, 1995). Viruses, for example Measles, also require an actin cytoskeleton because it appears that they express a nucleocaspid protein that associates with actin. Actin association maybe required both for budding of the virus from infected cells and for entry into host cells (Moyer *et al*, 1990; Janmey & Choponnier, 1995). Another example is the HIV virus that uses the actin cytoskeleton of infected lymphocytes to form an actin-rich pseudopod for transfer of the virus into epithelial cells (Pearce-Pratt *et al*, 1994; Janmey & Choponnier, 1995). A drug such as that used to combat schistosomes has been used where the host remodels a parasite's cytoskekleton to kill it. It disrupts the membrane covering actin-rich spikes, thereby exposing them to the actin depolymerising system of mammalian blood, which then destroys the spikes and kills the worm (Linder & Thors, 1992; Janmey & Choponnier, 1995).

1.4.9 Role of the cytoskeleton in activation of adhesion molecules

Upon activation, neutrophils migrate from the blood stream into tissues as part of the inflammatory response. For this migration to occur, the neutrophil must

undergo defined steps: changing from flowing state to rolling via selectin adhesion molecules expressed on stimulated endothelium; activation of neutrophil β_2-integrins which immobilise the cells by binding of β_2-integrins to their ligands, cyclic regulation and actin polymerisation. (Springer, 1994; Sheikh *et al*, 1997; Anderson *et al*, 2000). It has been suggested (Pavalko & Otey, 1994; Sampath *et al*, 1998; Anderson *et al*, 2000) that there is an association between integrin and cytoskeletal function, based on the fact that the cytoplasmic domain of the β_2-integrin is linked to the cytoskeleton through adapter actin-binding proteins including talin and α-actinin (**Figure 1.13**). Activation of neutrophils with chemotactic agents causes actin polymerisation within seconds followed by a net reduction and distribution of F-actin within minutes, which correlate with shape change and migration (Howard & Oresajo, 1985a; Watts *et al*, 1991, Anderson *et al*, 2000).

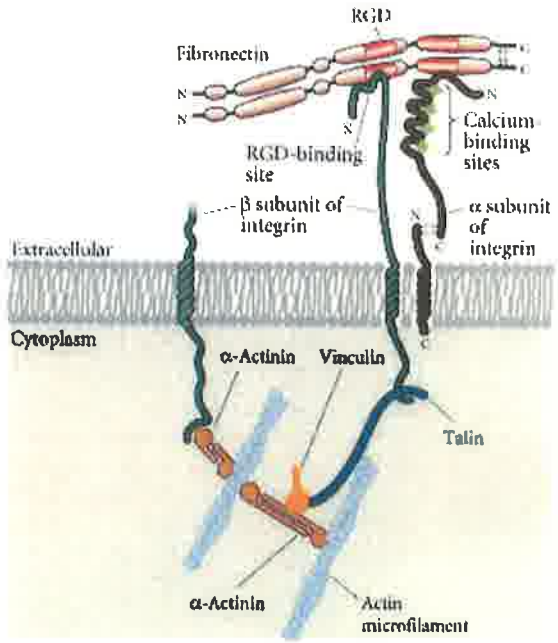


Fig 1.13: Speculative diagram relating the binding of the cytoskeleton to the extracellular matrix through the integrin molecule (Reproduced from Gilbert, 2000).

1.4.10 Role of cytoskeleton in leukocyte deformability

Leukocytes play an important role in the micro-haemodynamic flow pattern and as is known, this role maybe accomplished by means of their altered adhesion to the endothelial wall, their entrapment or their activation. Leukocytes within the circulation are in a dynamic equilibrium with a marginated pool and are thought to reside mainly within the pulmonary capillaries. The size discrepancy between the mean diameters of circulating leukocytes (6-8μm) and that of the pulmonary capillaries ($\approx$5.5μm) forces cells to deform in order to transit the capillary bed (Downey *et al*, 1990).

Assembly and reorganisation of the actin cytoskeleton is involved in activation, chemotaxis and rheology of leukocytes. The actin filaments in circulating leukocytes facilitate their passage through microvenules and capillaries by helping in their deformability. Cytoskeletal changes such as increased actin polymerisation, as observed on activation, reduce cell deformability causing cells to be sequestered in the capillaries, which indicate that cell deformability is influenced by these changes (Drost *et al*, 1993a). Exposure of leukocytes, especially PMNs, to chemoattractants causes cell rigidity that is associated with an alteration in cell shape and an increase in F-actin content (Erzurum *et al*, 1992; Betticher *et al*, 1993; Drost *et al*, 1999). These rheologic changes cause retention of endotoxin-exposed PMN in 6.5μm filter pores, an effect that was distinct from adhesion, because after cell treatment with cytochalsin D, an F-actin disruptor, the deformability behaviour of PMN was reversed (Erzurum *et al*, 1992; Drost *et al*, 1999).

Microtubules in the leukocytes are relatively few in number in comparison to the actin filaments (Anderson, 1982; Tsai, 1998). As the cells become activated with

colchicines, microtubule depolymerising agents, the alteration of the cytoskeletal microtubules does not seem to affect the mechanical properties of neutrophils as F-actin which has dramatic changes in cellular deformability (Tsai, 1998).

1.5 The neutrophil, a double edge sword

Neutrophils play an important role in host defence against micro-organisms and all classes of infectious agents through their microbicidal processes which consist of the formation of a combination of reactive oxygen species and various hydrolytic enzymes and anti microbial polypeptides (Smith, 1994). Paradoxically, neutrophils are involved in the pathology of various inflammatory conditions.

1.5.1 Neutrophil activation

Human neutrophils, which represent 50 to 60% of the proportion of the leukocytes found in systemic blood, have been the subject of great experimental attention. This is because they play a crucial role of host defence against microbes and so have the potential of producing strong bioactive substances capable of the tissue damage observed in pathophysiologic state. They migrate up chemotactic gradients to infection sources where they kill invading micro-organisms by the production of reactive oxygen intermediates (ROIs) and lysosomal enzymes. The synthesis of highly toxic ROIs is initially catalysed by the membrane bound enzyme system NADPH oxidase (respiratory burst oxidase) that adds an electron to oxygen present in the extracellular fluid, yielding the superoxide anion (O_2^-) (Babior, 1999). The NADPH oxidase system is composed of several components, which comprises a short electron transport chain that facilitates the production of O_2^-. In resting neutrophils, the NADPH oxidase components are dissociated, with some embedded

in the plasma membrane where others are stored in the cytosol. Upon activation, the cytosolic units become phosphorylated, initiating them to translocate to the plasma membrane where the functional NADPH oxidase system assembles (Roitt *et al*, 1993; Smith, 1994). Generation of further reactive oxygen species is catalysed by Superoxide dismutase (SOD), which adds another electron to O_2^- to produce hydrogen peroxide (H_2O_2). Further oxidation of H_2O_2 can occur following the reactions catalysed by myeloperoxidase (MPO), an active enzyme contained within phagocyte primary granules, which can add further electrons to H_2O_2 producing hydroxyl radicals (OH^-) (Mathews & van Holde, 1990). MPO can also convert H_2O_2 in the presence of halides into hypohalous acid (HOX), involved in which is usually Cl^- due to its high concentration at most host sites, yielding hypochlorous acid (HOCl), an efficient microbicide produced by an activated phagocyte causing severe tissue damage (Weiss, 1989). The different oxidation steps are shown below.

$$2O_2 + NADPH \longrightarrow \textit{NADPH oxidase} \longrightarrow 2O_2^- + NADP^+ + H^+$$

$$O_2^- + O_2^- + 2\,H^+ \longrightarrow \textit{Superoxide dismutase} \longrightarrow H_2O_2 + O_2$$

$$H_2O_2 + X^- + H^+ \longrightarrow \textit{Myeloperoxidase} \longrightarrow HOX + H_2O$$

$$(X^- = Cl^-, Br^-, I^-, SCN^-)$$

Either particulate agents or soluble factors such as phorbol myristate acetate (PMA) can activate neutrophils (**Figure 1.14**). Neutrophils can also be activated by different stimuli, such as endotoxin, chemotactic factors, smoking and ischaemia. Activation causes stiffening of cells and promotes neutrophil adhesion by switching between rolling to the stationary state in seconds at the same time as transformation of G-actin to F-actin (Howard & Oresajo, 1985a, 1985b; Frank, 1990; Neumann *et al*, 1990; Sheikh *et al*, 1997; Nash *et al*, 2001; Suwa *et al*, 2001). Thus activation of

neutrophils or priming of their responses to stimuli increases their potential to cause flow obstruction through their adhesion to endothelium and cell-cell aggregation as well as changes in cell rigidity (Nash, 1992).

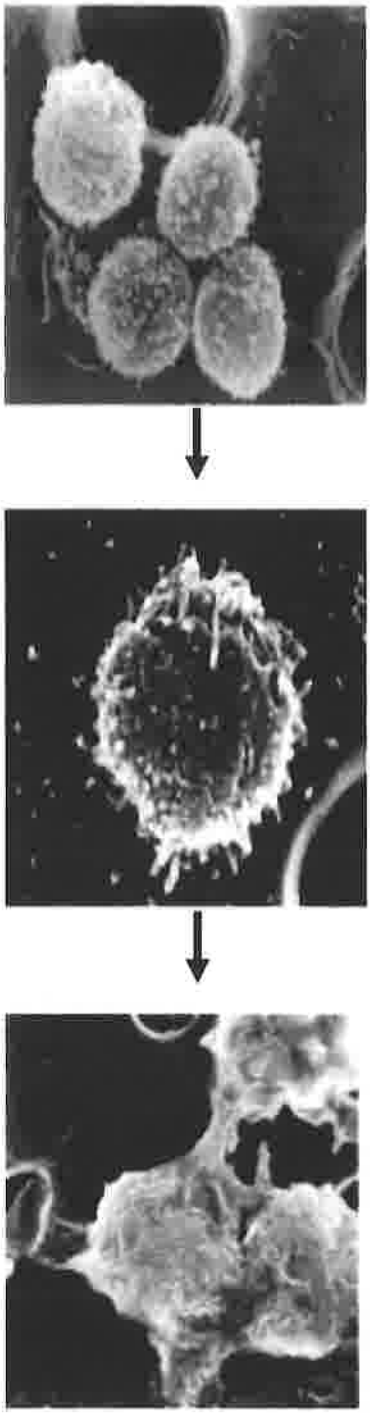


Fig 1.14: Electron microscopy images showing the effect of activation of cells on cell shape, which is associated with changes in the content of F-actin. As cells become activated, projections are formed and in advance activation cells can aggregate to form a meshwork, which makes it difficult for cells to pass through capillaries. Magnification A: x3200, B and C: x6400 (Supplied by Dr. Shelley-Ann Evans).

1.5.2 Diseases associated with leukocyte activation

The interest in studying the effects of leukocyte activation exists because many clinical disorders involve abnormalities in one or more of the factors affecting blood rheology (**Figure 1.15**). As agents or stimuli activate leukocytes, this activation results in altering the normal leukocyte rheology through morphological alteration in their cytoskeletal components, which results in decreasing leukocyte deformability and leukocytes being trapped in the capillaries. Neutrophils (PMN) are the most sensitive leukocyte and their activation contributes to different disease states (Ravel, 1980, Tsai *et al*, 1996, 1998). Although a comprehensive study of clinical disorders is beyond the scope of this thesis, a brief review of clinical conditions associated with abnormal blood rheology, actin polymerisation and cytoskeletal reorganisation will be useful here, to reinforce the significance of this study.

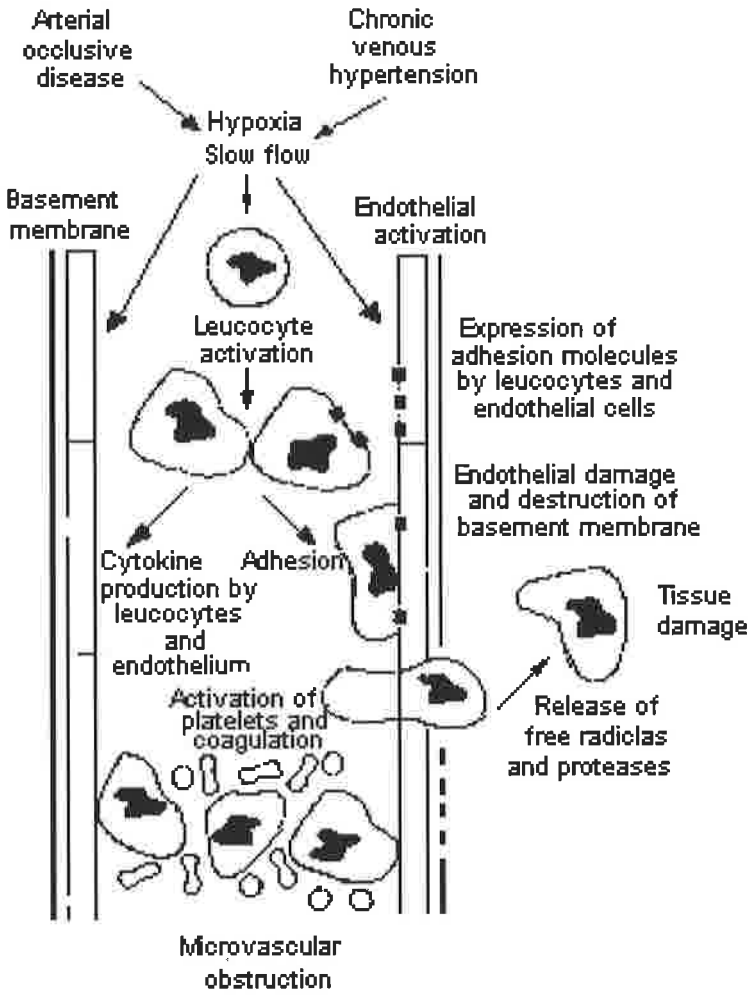


Fig 1.15: White blood cell trapping and activation in venous and arterial disease (Reproduced from Bradbury *et al*, 1993).

1.5.2.1 Ischaemia

Ischaemia is an insufficient supply of blood to an organ. During cerebral ischaemia, microvascular permeability barriers are lost, the microvascular endothelium responds by the sequential expression of leukocyte adhesion receptors, the basal lamina and extracellular matrix undergo progressive loss of component antigens and cell matrix adhesion interaction within microvessels are altered (del Zoppo & Garcia, 1994; del Zoppo *et al*, 1998).

Leukocytes contribute to this tissue injury by the adhesion of PMNs to specific endothelial cell receptors which contributes to loss of microvascular potency, initiates diapedesis and transit into parenchyma and thereby adds to tissue injury (del Zopp *et al*, 1998).

Alterations in rheological factors such as viscosity, haematocrit, fibrinogen and activated leukocytes may worsen ischaemia by promoting atherosclerosis, thrombosis or obstruction to microcirculatory flow distal to atherosclerotic stenosis (Lowe *et al*, 1993). During ischaemia, leukocytes become activated causing the following effects: Firstly, during leukocyte transmigration, the respiratory burst generates free radicals and release of granule proteolytic substances contributes to blood brain barrier, basal lamina and perivascular tissue degradation (Hinshaw, 1996). Cleavage products of laminin are potent chemoattractants for leukocytes (del Zoppo *et al*, 1998). Secondly, the presence of activated complement generated during ischaemia may also contribute to leukocyte activation. In other words, ischaemia reperfusion injury is associated with an influx of neutrophils into the affected tissue and subsequent activation, which may trigger superoxide generation. Superoxide also signals recruitment of additional neutrophils to the affected site and

ischaemia may be sustained by plugging of capillaries with aggregates of activated neutrophils (Bradbury *et al*, 1993; Smith, 1994).

1.5.2.2 Stroke

Stroke is one of the leading causes of death and disabilities. It is the severe restriction or complete cessation of blood flow to the brain as a result of any cerebrovascular disease or neurological brain injury (Hademenos & Massoud, 1997).

Leukocytes, PMNs in particular, are known to contribute to such brain tissue damage. Wang *et al* (1993) and Pozzilli *et al* (1985) demonstrated that leukocyte accumulation occurred in the early stages of stroke and persisted for more than a month. It is likely that different factors affect or initiate PMN recruitment from the circulatory bed. These factors include complement activation, expression of adhesion molecules and release of chemotactic factors such as platelet activating factor (PAF) (Akopov *et al*, 1996). All these factors contribute to leukocyte activation leading to leukocyte trapping in the capillaries due to an increase in actin polymerisation.

1.5.2.3 Venous ulceration

Venous ulceration results from sustained venous hypertension, which in turn arises from incompetence of valves in the deep and/or superficial veins. These macrovascular abnormalities lead to disturbances of the microvasculature and a wide variety of abnormalities have been described in the tissue surrounding venous ulcer (Smith *et al*, 1988).

In recent studies, the role of leukocytes in venous disease has been discovered in patients with chronic venous insufficiency where the number of trapped

leukocytes in these patients is more than that seen in normal controls. This led to the proposal that these trapped and activated leukocytes are poorly deformable, and adhere to the activated and damaged endothelium releasing proteolytic enzymes and free radicals that are harmful to tissues by causing tissue damage (Bradbury *et al*, 1993). The repeated accumulation of these cells in capillaries and venules of the foot may contribute to trophic changes in the skin and lead to ulceration (Smith *et al*, 1988; Thomas *et al*, 1988).

1.5.2.4 Myocardial Infarction (MI)

MI is a sudden death of part of the heart muscle and is popularly known as a heart attack. Leukocytes play an important part in the development and prognosis of acute myocardial infarction (AMI). Different experimental methods suggested that PMNs contribute to the pathophysiological course of AMI (Bauersachs *et al*, 1997). Potential mechanisms include local activation of PMN, initiation and promotion of myocardial necrosis by generation of free radicals and release of proteolytic enzymes from PMN granules (Anderson *et al*, 1991).

Bauersachs *et al* (1997) studied the behaviour of PMN on the development and prognosis of AMI and found that after infarction, there is an increase in white blood cell counts with no accumulation of stiff PMN in the circulating blood in the early stages of AMI but these findings did not accord with earlier studies that showed increased flow resistance and a high degree of activation after AMI (Nash *et al*, 1989). The authors suggested that "*This discrepancy can be resolved if there is an increase in cell turnover with a left-shifted PMN population that is not yet stiffened by activators washed out from the myocardium or that is less likely to be activated. This detection of a low level of activation could also be caused by trapping of highly*

activated cells in lungs or heart" (Bauersachs *et al*, 1997). Trapping due to the loss of deformability seems like a valid explanation because actin provides stiffness to the cell and an abnormal actin structure can make cells too stiff to function normally.

1.5.2.5 Septic shock/ sepsis

Septic shock is a highly dangerous condition in which there is tissue damage and a dramatic drop in blood pressure as a result of bacterial multiplication and the presence of bacterial toxins in the blood. Leukocytes have both direct and indirect effects on septic shock. The microcirculation undergoes massive alterations during septic shock. These alterations include slowing of capillary blood flow due to depressed perfusion as a result of systemic pressure reduction and local arteriolar constriction. There is therefore decreased capillary passage of cellular elements such as erythrocytes and neutrophils. The presence of white blood cells maybe responsible for decreases in red cell deformability through changes in red cell membrane visocity, and they may also be responsible for conformational changes in the membrane proteins of red cell induced by endotoxin as emphasised by Todd *et al* (1993) and Bellary *et al* (1995). Conformational changes, such as oxidative damage to the membrane are likely to be associated with the increase in oxygen free radicals originating from activated leukocytes (Machiedo *et al*, 1989; Powell *et al*, 1991; Baskurt *et al*, 1998). Todd & Mollitt (1994) studied red cell size and cell membrane fluidity during septic shock and observed significant increases in red cell membrane lipid viscosity and concluded that red cell deformability changes were due primarily to an increase in membrane viscosity and were not related to cell surface area/volume ratio.

Septic shock is characterised by alteration in leukocyte rheology, which may contribute to vascular obstruction as has been observed by Lavkan *et al* (1998). Increases in neutrophil aggregation, decreased deformability and increased neutrophil adhesion are features of septic shock (Venzio *et al*, 1982; Yodice *et al*, 1997; Lavkan *et al*, 1998).

Sepsis is an infection of wound or body tissues with bacteria that leads to the formation of *pus* or to the multiplication of the bacteria in the blood. Various studies have shown that mediators (IL-8, C5a, PAF, TNF-α and fMLP) released during sepsis from the inflammatory response activate endothelium and neutrophils resulting in accumulation of neutrophils at the site of infection (Astiz *et al*, 1991; Astiz *et al*, 1995; Skoutelis *et al*, 2000). Exposure of PMN to these mediators *in vitro* causes a further increase in PMN rigidity associated with an alteration in the cell content of F-actin as described previously, and the associated decrease in cell deformability. Neutrophils of patients with sepsis are more rigid than neutrophils from normal subjects (Hinshaw *et al*, 1997; Yodice *et al*, 1997; Drost *et al*, 1999; Skoutelis *et al*, 2000). Attenuation of increased rigidity and decrease in deformability of neutrophils in sepsis cause changes in cytoplasm viscoelasticity due to dysfunction of the cytoskeletal architecture and leads to neutrophils being trapped in capillaries. These changes are most likely owing to the observed increase in F-actin content within the sub membrane region (Worthen *et al*, 1989; Erzurum *et al*, 1992; Doerschuk, 1999).

1.5.2.6 Respiratory Distress Syndrome (RDS)

RDS, a lung disorder that causes difficulty in breathing, results in a life threatening deficiency of oxygen in the blood. RDS in both adults and neonates is a

good example of the neutrophil paradox. Neutrophils have been implicated in the pathology of RDS because of the large influx of neutrophils into the lung and the associated tissue damage caused by oxidants, arachidonic acid and metabolites and hydrolytic enzymes released from activated neutrophils (Smith, 1994; Brus *et al*, 1997; Gando *et al*, 1997). Neutrophils are also responsible for acute RDS where they play an important role in the pathogenesis of the lung injury by sequestering in the microvessels of the lung (Hogg, 1994; Downey *et al*, 1995; Hogg & Doerschuk, 1995; Sato *et al*, 1999). Factors that have been proposed by Carlos & Harlan (1994) to be responsible for PMN sequestration in the lung are the size and deformability of PMN as well as the adhesiveness of PMN to the endothelium. The decrease in deformability is mediated by a rapid assembly of F-actin from G-actin at the cell periphery which increases cell rigidity and viscosity of PMN, which further increase PMN transit through the lung (Worthen *et al*, 1989; Frank, 1990; Inano *et al*, 1992; Sato *et al*, 1999).

Neutrophils have been shown to play a central role in the pathogenesis of RDS. Gando *et a*l (1997) studied the serial changes of plasma neutrophil elastase and found that there is a marked increase in this in both the early and sub-acute phases after injury in patients with RDS. Brus *et al* (1997) studied whether number and activation of circulating platelets and PMN are associated with neonatal RDS and showed that decreased PMN and platelet counts and increased elastase are correlated with increased RDS severity.

1.5.2.7 Diabetes mellitus

Diabetes is a disorder caused by insufficient or absent production of the hormone insulin by the pancreas. There are two types of diabetes mellitus: Insulin-

dependent (type I) diabetes and non-insulin dependent (type II) diabetes. Diabetes is considered to be one of the major factors implicated in cardiovascular diseases. It is known that several properties of blood are altered in diabetes and various rheological abnormalities have been widely reported but it remains unclear which blood components affect blood flow at the level of the microcirculation (McDonagh *et al*, 1997; Miyamoto *et al*, 1997). For this reason a large number of studies on diabetic patients have been performed to differentiate which blood component results in microcirculatory disturbances seen in diabetes. Miyamoto's investigations, based on the new cell-flow microchannel method, found that the fibrinogen, a plasma protein with high viscosity, was significantly higher in diabetic blood compared with the controls. This increase in plasma protein level contributes to an increase in whole blood and plasma viscosity. However, measurement of red cell deformability showed no significant differences in the erythrocyte suspension transit time between the diabetic and controls in the study by Miyamoto *et al* (1997).

Leukocytes also play a potential role in diabetic microcirculatory disturbances (Miyamoto *et al*, 1997). Vermes *et al* (1987) reported that by using a filtration method, white blood cells have shown a reduced filterability in diabetic patients and Kelly *et al* (1993) found that mononuclear leukocytes of diabetic cats were less deformable than those of normal cats. Moreover, PMNs were reported to be less deformable in diabetic patients (Pecsvarady *et al*, 1994). These rheological abnormalities in leukocytes were reported because in diabetes leukocytes tend to be more active resulting in increasing the rigidity of cell membrane (Schroder *et al*, 1991).

In addition, diabetic monocytes have intense procoagulant activity (Jude *et al*, 1989) and have increased adhesiveness to fibronectin, one of the plasma proteins as a result of increased numbers of fibronectin receptors. These interactions occurring in diabetes would lead to reduced deformability of leukocytes, which in turn might lead to leukocytes being trapped in the microcirculation (Setiadi *et al*, 1987; McDonagh *et al*, 1997).

Diabetes may also induce functional abnormalities in cytoskeletal elements due to reduced levels of insulin, as has been suggested to occur in skeletal muscle (Shimoni *et al*, 1999). Some studies made in this area will be discussed later in chapter six.

1.5.2.8 Atherosclerosis

Atherosclerosis is a disease of the vessel wall in which the inner layers thicken, causing narrowing of the vessel which in turn causes loss of the smooth lining of the blood vessels and encourages thrombus formation and thus impairing blood flow. Leukocytes have been implicated in the pathology of this disorder through monocyte activation. Monocyte activation, as assessed by tissue factor expression, has been observed in various diseases with vascular complications (Jude *et al*, 1994; Wautier *et al*, 1999).

Overall, as has been mentioned in the literature there is increasing evidence of leukocytes being implicated in many vascular disorders. Neutrophils appear to be the most frequent cell involved in acute clinical situations such as septic shock and ischaemia whereas, monocytes exhibit crucial activities in chronic conditions such as atherosclerosis and thrombosis.

1.6 Environmental influences on blood rheology

In addition to the clinical conditions described above, various environmental influences are also thought to affect leukocyte deformability. Two of these are discussed below:

1.6.1 Smoking

Cigarette smoking is associated with an excess of deaths from cardiovascular disease. There is a clear relationship between the degree and duration of exposure to cigarettes and the incidence of cardiovascular disease events. Furthermore, a decline in risk follows quitting, although residual effects may persist longer (Witteman *et al*, 1993; Fitzgerlad, 1997).

Smoking has an effect on blood rheology as studied by Lowe *et al* (1988), Lowe (1994) and confirmed by Campisi *et al* (1998). Smokers had higher values of blood viscosity associated with an increase in haematocrit, plasma viscosity and fibrinogen and lower whole blood filterability (Ajmani & Rifkind, 1998; Woodward *et al*, 1999). Exposure of neutrophils to cigarette smoke *in vitro* induced a rapid loss of filterability through 5μm pores (Buttrum *et al*, 1994). Smokers also had higher white blood cell counts but lower red cell aggregation, possibly with decreased red cell membrane deformability (Lowe *et al*, 1992). In addition, exposure of neutophils to cigarette smoke caused a marked change in cell shape with the formation of pseudopods (Buttrum *et al*, 1994). Pseudopods, which blocked the pipette tip of micropipettes, consist of rigidified actin with localised expression of adhesion proteins, which leads to an enhanced probability for microvascular trapping of leukocytes (Schmid-Schonbein & Lee, 1995).

Cigarette smoke damages endothelial cells because it contains a large number of oxidants and recent observations described a role of oxygen-derived free radicals in mediating endothelial dysfunction (Campisi *et al*, 1998). Smoke activates platelets and leukocytes, which in turn release growth factors and proinflammatory cytokines and enhances the ability of leukocytes to adhere to the endothelium. This adherence is an early event in the atherosclerosis process (FitzGerald, 1997).

1.6.2 Particulate environmental pollution

Epidemiological studies have identified an association between particulate air pollution, especially particles with a diameter less than 10μm (PM10) and increased respiratory and cardiovascular morbidity and mortality (Pope *et al*, 1995; Pope *et al*, 1999; Schwartz, 1999). The biological mechanisms responsible for these increases are still unclear. Seaton (1995) postulated that the inhalation of these fine particles provokes an inflammatory response in the lung that aggravates lung disease and a change in blood coagulability that increased pulmonary and cardiovascular deaths. Terashima *et al* (1997) demonstrated that subjects who performed outdoor training have a leukocytosis that returned to normal after the pollution cleared. These findings suggest that acute exposure to particulate air pollution induces a systemic inflammatory response that includes bone marrow stimulation. Goldsmith *et al* (1998) suggested that alveolar macrophages (AM) are the most likely link between the inflammatory response in the lung and the systemic response because AM are the cells responsible for ingesting and clearing inhaled particles. Van Eeden *et al*, (2001) and Mukae *et al* (2001) also studied this link by exposing AM to particles and finding that AM produced a broad range of cytokines, and these cytokines were present in the circulation of subjects during the episode of acute air pollution.

1.7 Aims

Disturbances in blood flow are associated with a range of diseases including cardiovascular disease and Type II diabetes. Changes in intracellular actin polymerisation may cause changes in leukocyte rheology. Blood rheology may be modified by exposure to a range of stimuli including particulate air pollution and by Type II diabetes. Pharmacological intervention to reduce actin polymerisation may normalise leukocyte rheology. A new group of drugs, the PPAR-gamma agonists, used to treat Type II diabetes, may modify actin polymerisation.

The overall aims of this thesis are, therefore, to determine the effect of actin polymerisation on leukocyte rheology, to determine the effect of a range of stimuli on actin polymerisation, and to investigate pharmacological intervention to reduce actin polymerisation. The detailed aims are:

1. To develop and fully evaluate a whole blood method for determination of intracellular actin polymerisation in PMNs and monocytes.
2. To evaluate whole blood flow cytometric determination of the F-actin content of leukocytes as a measure of leukocyte rheological changes.
3. To use the above method to measure clinically relevant changes in blood rheology.
4. To use a rat model to determine the effect of exposure to particulate air pollution on blood rheology.
5. To determine the effect of PPAR-gamma agonists and a specific PPAR-gamma antagonist on actin polymerisation.

Chapter Two

Chapter Two: Optimisation of Assays for determination of intracellular actin polymerisation

2.1 Introduction

The development of various fluorescent probes has opened new perspectives to study actin dynamics, and fluorescently labelled actin is an important tool for investigating actin dynamics *in vivo* (Shimada *et al*, 1997). Such labels interact by adsorption onto or non-covalent binding to a protein or other macromolecule or incorporation into a non-polar region of a membrane (Rost, 1991). Several fluorescently labelled derivatives of phallotoxins such as phalloidin and phallacidin selectively bind and label F-actin, and are used in most applications. These substances are bi-cyclic heptapeptides derived from the green-capped mushroom *Amanita phalloides*, which bind specifically and with a high affinity to filamentous actin (F-actin) but not to G-actin (Cooper, 1987; Katanaev & Wymann, 1998). The binding of these phallotoxins does not significantly change the properties of other actin binding proteins, but inhibits non-specific dissociation of subunits from the filament ends (Cooper, 1987; Watts & Howard, 1993). Phallotoxins have the same binding affinity to large and small filaments in a ratio of about one phallotoxin molecule per actin subunit in muscle and non-muscle cells from many different species of plants and animals. Labelled phallotoxins have several advantages over other labels: they do not bind to monomeric G-actin, their binding properties do not change with actin from different species including animals and plants, and their non-specific staining is negligible, thus the contrast between stained and un-stained areas is high (Faulstich *et al*, 1988; Adams & Pringle, 1991; De La & Pollard, 1994). Phalloidin is water-soluble and acts to induce the assembly of F-actin of the cytoskeletal protein as well as stabilising existing actin filaments. Phalloidin also

influences the nucleation phase of actin polymerisation by reducing the critical concentration of actin (concentration below which actin exists only as a monomer), hence, induces actin polymerisation to occur (Alexander *et al*, 1988; Cooper, 1987; Korthuis *et al*, 1991; Philips *et al*, 1989). Phalloidin is required in low concentrations for staining as stated by supplier (Molecular Probes, Haugland, 2001), thus providing convenient labels for identifying and quantifying actin in tissue sections, cell suspensions or cell free preparations (Amato & Taylor, 1986; Kellog *et al*, 1988).

There are various examples of the labelling of phallotoxins, which include Fluorescein Isothiocyanate (FITC), Nitrobenzoxadiasole (NBD), Rhodamine, BODIPY, and Oregon green® 514 that have been used in different studies. Many studies have used NBD-phalloidin to look at actin polymerisation in leukocytes. For example, Mineshita *et al*, (1997) used (NBD)-phallacidin to stain fixed neutrophils. Howard & Oresajo (1985a) and Watts & Howard (1993) used this probe to quantify and study the mechanism of actin reorganisation in stimulated cells using a FACS sorter. Klut *et al* (1997) also used NBD-phallacidin to study the effect of activation with zymogen-activated plasma (ZAP) on F-actin content in bone marrow compared to that of peripheral blood. Despite its widespread use, NBD-phalloidin has several drawbacks: it requires an incubation time of 30 minutes at room temperature (Watts *et al*, 1994), and stained cells need to be filtered through 50μm nylon mesh filter to remove large aggregates (Chen *et al*, 1996; Naik *et al*, 1990). Belloc *et al* (1990) used Rhodamine phalloidin to quantify the rate of actin polymerisation. The disadvantage of using this probe is that it needs a long incubation time of approximately 1 hour and needs further washing cycles after incubation (Katanev & Wymann, 1998; Belloc *et al*, 1990; Mills *et al*, 2000; Rotsch & Radmacher, 2000).

BODIPY FL-phallacidin also needs a lengthy incubation time (45-60) minutes, as illustrated by Tsai *et al* (1998). Oregon green® 514 phalloidin is a new probe used for labelling F-actin in fixed and permeabilised cells with more photo stability compared to other probes. It has been used successfully for staining F-actin in mammalian fibroblasts via secondary antibodies (Sells *et al*, 1999), and in the determination of sacromere organisation (Yue *et al*, 2000). FITC-phallodin has also been used by Fernandezsegura *et al* (1995) to quantify F-actin and to study shape and morphological changes in activated human neutrophils.

Two types of probe were studied here: FITC-phalloidin and Oregon Green® 514 phalloidin phallotoxins. FITC-phalloidin is stable and it is potentially toxic with toxicity data of: LD50: 2mg/kg but is not a significant hazard in quantities used in this study. Oregon green is also potentially toxic with toxicity data of: LD50: 2mg/kg, but is used here at nanomolar concentrations. It is extremely water soluble, thus providing a convenient probe for labelling. Although FITC and Oregon green have both been used in other studies, each probe has its own advantages and disadvantages. Although FITC-phalloidin is a popular probe known for its stability in labelling F-actin in fixed and permeabilised cells based on long-term usage, it photo bleaches so rapidly that image capture is difficult. Oregon green® 514 phalloidin is a new probe with unknown stability but is one of the most photo stable probes, thus making it easy to visualize capture images. One disadvantage of Oregon green® 514 is that it is an expensive probe to buy compared with FITC-phalloidin, although both probes are easy to use.

In order to gain a more complete understanding of the influence of different phalloidin probes (FITC-phalloidin and Oregon Green 514® phalloidin) on the

measurement of F-actin polymerisation, flow cytometric analysis and fluorescent microscopy were used for comparison.

2.1.1 Flow Cytometry

Flow cytometry is a powerful tool that enables one to measure multiple parameters on individual cells at high speed (Epling *et al*, 1992). The flow cytometer is an apparatus that detects and counts individual cells passing in a stream through a laser beam. The principle of flow cytometry is that cells or cellular suspensions are passed through a sensing region where optical or electrical signals are generated and measured. A flow cytometer equipped to separate the identified cells is called a fluorescence-activated cell sorter (FACS). Flow cytometry is therefore used here as a sensitive and reliable method for assessing actin dynamics which allows the identification of cell subpopulations (Chen *et al*, 1996; van Eeden *et al*, 1999).

A blood cell suspension (whole blood and isolated sub-populations), which is fixed, permeabilised and labelled, is forced through a nozzle, creating a fine stream of liquid containing cells spaced singly at intervals. As each cell passes through a laser beam it scatters the laser light and the dye molecules bound to the cell will be excited and fluoresce. Photomultiplier tubes detect the scattering of light and fluorescence emissions giving information on the size and granularity of the cell, and on the binding of the label by each cell type respectively. This information is then analysed using a computer.

2.1.2 Fluorescence Microscopy

Fluorescence microscopy is a technique of microscopy whereby fluorescent stained structures or fluorescent substances are examined. Because many substances

are fluorescent or can be made so by chemical reaction with another substance which is itself also non-fluorescent, fluorescence microscopy is widely applicable. Fluorescence microscopy has a number of advantages over other forms of microscopy, offering high sensitivity and specificity. Particular constituents of the specimen can be seen even in extremely small amounts because fluorescence is observed as luminosity on a dark background (Rost, 1992).

In fluorescence microscopy, the specimen is illuminated with light of a short wavelength such as ultraviolet or blue. Part of this light is absorbed by the specimen and re-emitted as fluorescence with a longer wavelength. Weak fluorescence is difficult to see despite the strong illumination. To overcome this problem, a secondary filter placed between the specimen and the eye filters out the light used for excitation. This filter should be fully opaque at the wavelength used for excitation and fully transparent for longer wavelengths so as to transmit the fluorescence (Rost, 1992).

2.1.3 Aims

The aims of the experiments performed in this chapter were to establish the appropriate gating of PMNs and monocytes using density gradient centrifugation to isolate these cells. Two different phalloidin probes (FITC and Oregon green 514® phalloidin) were studied and characterised, based on F-actin measurement and cell shape change to define the appropriate probe to be used for further investigations.

2.2 Materials and Methods

2.2.1 Chemicals required for the study

All chemicals were from Sigma-Aldrich Company Ltd unless stated otherwise.

Preparation of Phosphate buffered saline (PBS/EDTA)

The phosphate buffered saline was prepared and it contained EDTA (1.5mg/L), 0.02M Na_2HPO_4, 0.12M NaCl, and 0.005M KH_2PO_4 (Bessis & Mohandas, 1975). All chemicals were mixed and made up to one litre with distilled water. The pH was adjusted to 7.4. PBS was filtered through 0.45μm membranes (Millex-HA; Millipore SA) immediately before use to avoid any bacterial or debris contaminations.

Preparation Oregon Green® 514 Phalloidin

A working solution was prepared by dissolving 0.013mg (300Units) of product in 1.5ml methanol to yield a final concentration of 200units/ml, which is equivalent to approximately 6.6μM. Stock was prepared and kept frozen at –20°C until used. One unit is the amount of stain used in staining cells and is equivalent to 5μl of stock solution (as recommended by supplier).

Preparation of FITC-Phalloidin

FITC-phalloidin (Molecular Probes, F432, 300U) stock ($80x10^{-5}\mu M$) was prepared by dissolving 0.1mg (300 units) of product in 1ml water. Stock was prepared and kept frozen at -20°C until used.

Cell Permeabilisation Kit

A Cell Permeabilsation Kit (CPK from Harlan SERA-LAB Ltd, Loughborough, UK) was used. This kit contains two reagents: Fixation medium (Reagent A) which contains formaldehyde and permeabilisation medium (Reagent B) that contains sodium Azide. Using the procedure provided by CPK gives fluorochrome access to intracellular structures and leaves the morphological characteristics of cells intact. Specific formulations reduce background staining and allow simultaneous addition of permeabilisation medium and fluorochrome as specified by the supplier. Reagents were stored at room temperature.

2.2.2 Blood samples

Blood was collected from healthy volunteers by venepuncture of the ante-cubital vein, and collected into vacutainers containing sufficient EDTA i.e dipottasium Ethylenediamine tetra acetic acid to give a final concentration of 1.5mg/ml (Becton & Dickinson UK. Ltd, between Towns Road, Cowley, Oxford OX4 3LY). All blood samples were stored at room temperature during laboratory procedures and used within six hours of the venepunture. Ethical approval was obtained for the study from the local ethics committee.

2.2.2.1 Blood Counts

Following venepuncture, red cell and partial differential white cell were performed using Coulter® MicroDiff[18] apparatus (Coulter Electronics Ltd, Luton, Beds, UK) Isolated leukocytes were counted in order to assess the efficacy of isolation protocols and also to optimally adjust leukocyte concentration ($5x10^6$/ml) prior to further investigations.

2.2.2.2 Isolation of Leukocytes

Cells were isolated by an improved density centrifugation method described by (Lennie *et al*, 1987) to yield reasonably pure (~95%) suspensions of polymorphonuclear (PMNs) cells and monocytes. Two solutions of different densities were prepared. Solution I: (Ficoll-Hypaque with a density gradient of $1.077gml^{-1}$) consists of 3.2g Ficoll and 5.25g Hypaque (Sodium dia trizoate) made up to 50ml with distilled water. Solution II: (Ficoll-Hypaque with a density gradient of $1.114gml^{-1}$) consists of 8.182g Ficoll and 15.452g Hypaque that are mixed together and made up to 100ml with distilled water.

The two solutions of different densities were used as follows: 8ml of 1.114 specific gravity followed by 2ml of 1.077 specific gravity Ficoll-Hypaque solutions were layered within 25ml Universal tubes followed by a layer of approximately 8ml test blood. All tubes were then centrifuged at 450g for 35 minutes. After centrifugation, different bands were observed containing the different populations of leukocytes. Plasma was removed and discarded, as was the denser, erythrocyte band. Mononuclear (MN) and PMN leukocytes were transferred to clean tubes following which they were re-suspended in approximately 10ml phosphate buffer saline (PBS/EDTA). Suspensions were then washed twice with PBS/EDTA to achieve a final concentration of $5x10^6$ cells/ml. The total number of cells following the separation of each 8ml blood sample was determined by counting cell numbers using Coulter® MicroDiff[18] to achieve a yield of 90% or more of each cell type (PMN and MN). The differential count was used to provide an assessment of cell purity. Cells were used immediately and kept at room temperature during analysis.

2.2.3 Preparation of blood cell suspensions and staining of F-actin.

2.2.3.1 Red Cell Lysis, Partial Fixation and Permeabilisation of Cells

For each sample to be analysed, 50μl of blood was incubated with 100μl CPK reagent A (fixation medium) for 15 minutes at room temperature to fix leukocytes. After incubation, the cells were washed with 5ml PBS and centrifuged at 300g for 5 minutes. After centrifugation, the supernatant was removed. The erythrocytes were lysed and the remaining leukocytes were then permeabilised using cell lysis and permeabilisation reagent from CPK kit (reagent B) for probe accessibility. Simultaneously, the blood sample was stained with each labelled phalloidin (2 μl for FITC-phalloidin, 5 μl for Oregon green). The mixture was vortexed at a low speed for 1-2 seconds. The sample was incubated in the dark for another 15 minutes at room temperature. Cells were washed again after incubation with probes. The supernatant was removed and the remaining leukocytes were resuspended in 0.5ml PBS before analysis.

2.2.3.2 Effect of FITC-phalloidin and Oregon green phalloidin Concentration

Blood was stained with various concentrations of phalloidin (FITC, Oregon Green 514) to monitor the effect of changing the concentration on measurement of F-actin polymerisation (F-actin content) with each probe and to study the optimum concentration to be used in further investigations. Various concentrations of the probes were used: 0.05, 0.1, 0.5, 1.0, 1.6, 2.0 and 2.5 μM for FITC-phalloidin and 0.013, 0.025, 0.033, 0.04, 0.046, and 0.053 μM for Oregon green 514 phalloidin.

2.2.3.3 Flow cytometry

Cell suspensions of whole blood samples were prepared as described earlier, using polystyrene tubes as recommended by the manufacturer, and analysed on a Beckton Dickinson FACScan (Becton Dickinson, San Jose, CA, USA) and a Cell Quest™ software (V 3.1) installed on a power Macintosh (Apple Computers, Inc. CA, USA). Blood suspensions of fixed, permeabilised and phalloidin stained cells were analysed according to their size and cellular granularity by measuring their forward and side scattered light, respectively (Fernandezsegura *et al*, 1995). Results were stored and displayed as histograms. Histograms of cell number versus log fluorescence intensity were recorded for five thousand events per sample (Carulli *et al*, 1997). Fluorescence gain and photomultiplier voltage were identical for all samples and F-actin content was expressed as the mean fluorescence intensity (MFI). Cells were analysed immediately or within 24 hours.

2.2.3.4 Assignment of flow cytometric gates for leukocyte sub-populations

Using the combination of forward angle light scatter and side light scatter, lymphocytes, remaining red blood cells and debris were excluded from analysis by appropriate gating (Mineshita *et al*, 1997). Leukocyte sub-populations were selected by assignment of gates normally associated with PMNs and monocytes.

2.2.3.5 Fluorescent Microscopy

Formaldehyde fixed, permeabilised and FITC-labelled cell suspensions (the same samples as used for flow cytometry) were mounted on slides and coverslips and cell shape changes and distribution of F-actin content were determined using fluorescence microscopy. The cells were viewed on the x100 oil immersion

objective. Cells were visualised in an Olympus microscope (Olympus Optical Co. LTD, Japan) with a digital driver electronics unit (DDE, UltraPix 2000), which is connected to a two part apparatus composed of a camera and software. The Qimaging Retiga 1300 digital camera (Quantitative Imaging Corporation, Canada, **Figure 2.1**) from Improvision (Improvision, University of Warwick Science Park, Coventry, UK) is a high resolution and high performance camera designed for quantitative applications and connected to a Machintosh PC (Apple Computers, Inc. CA, USA) with Openlab software installed (V 3.1.2). Openlab software is a modular imaging program designed specifically for multi-user scientific imaging. It consists of a core program and an extensive range of modules. This software enables images to be captured and transferred on the computer screen where images are saved. Images can be captured individually into an experimental file, and displays all images as thumbnails. Images can be viewed in pseudo-colour or monochrome or combined to composite colour images on the high-resolution colour graphics display. In these investigations a pseudo-colour was used.

Fig 2.1: Improvision camera used with fluorescent microscope.

2.2.3.6 Statistical Analysis

All experiments were performed in at least five times. Results are reported as mean ± standard error (SEM). Differences were analysed using one-way analysis of variance (ANOVA) using Minitab® software (Minitab Inc., State college, PA).

2.3 Results

2.3.1 Assignment of gates

Gates were adjusted so that the percentage of cells were identical to that identified using a coulter® MicoDiff[18] apparatus. Lymphocytes, red cells and debris were excluded from defined gates. Leukocytes sub-populations were selected by assignment of gates normally associated with PMNs and monocytes (**Figure 2.2**).

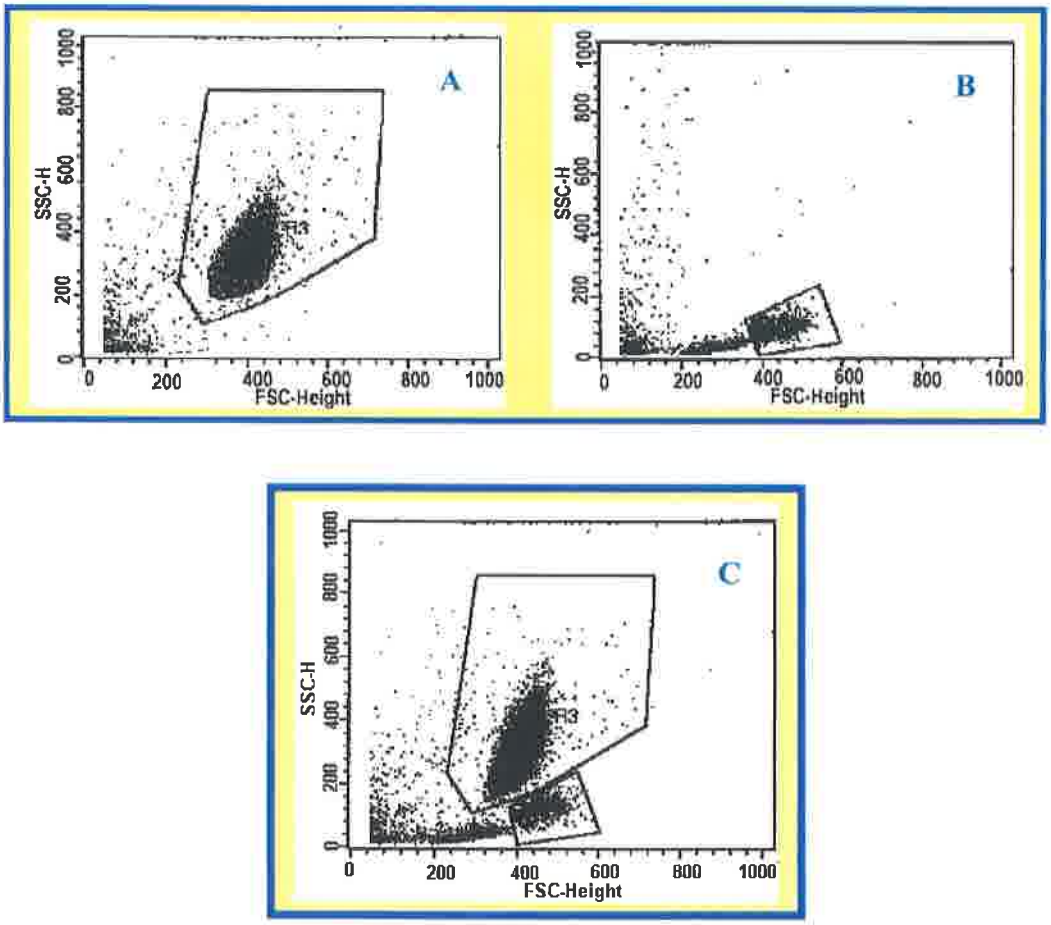


Fig 2.2: Gating of leukocytes applied using flow cytometry analysis of isolated cells (A: PMNs, B: Monocytes) and whole blood (C) suspensions.

2.3.2 Effect of FITC-phalloidin concentration on MFI

The mean fluorescence intensity of un-stimulated, gated PMNs and monocytes in whole blood after staining with various concentrations (0.05-2.5 μM) of FITC-phalloidin is shown in **Figure 2.3**. The MFI rose steeply as FITC-phalloidin concentrations were raised from 0 to 1.6 μM and plateaued at concentrations above that. This increase was observed in PMNs ($p<0.001$) and monocytes ($p<0.001$) as determined by ANOVA, although the MFI in monocytes was consistently higher than that in PMNs.

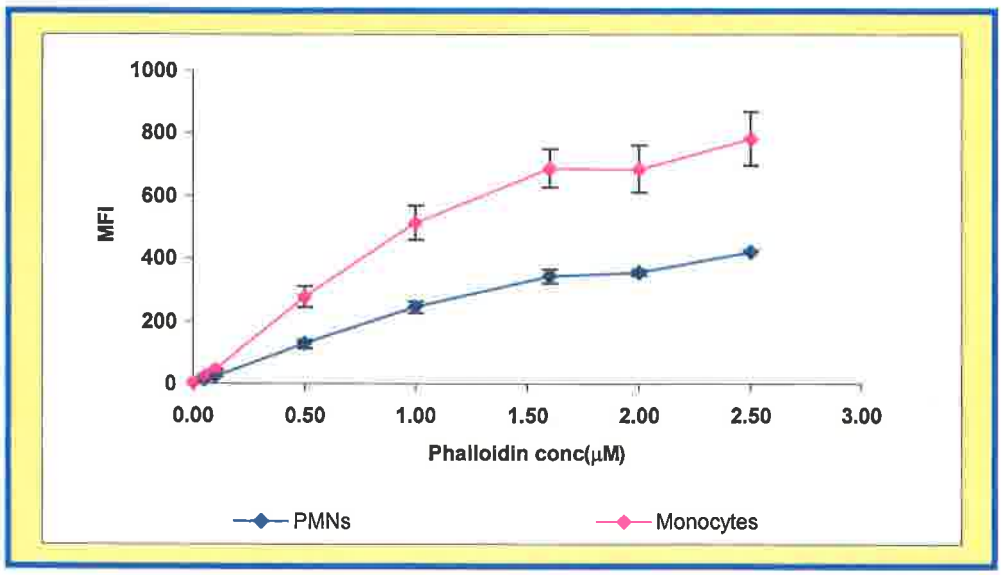


Fig 2.3: Effect of FITC-phalloidin concentration on MFI of PMN ($p<0.001$) and monocytes ($p<0.001$) in whole blood samples. The points represent the means ± SEM at n=5. The p-values were determined by ANOVA.

2.3.3 Effect of Oregon green® 514 phalloidin concentration on MFI

Flow cytometric analysis showed that the mean fluorescence intensity of un-stimulated, gated PMNs and monocytes in whole blood after staining with various concentrations (0.013-0.053 μM) of Oregon green® 514 phalloidin increased with increasing concentrations of Oregon green (**Figure 2.4**). This increase was observed in both PMNs ($p<0.001$) and monocytes ($p<0.001$), as determined by ANOVA.

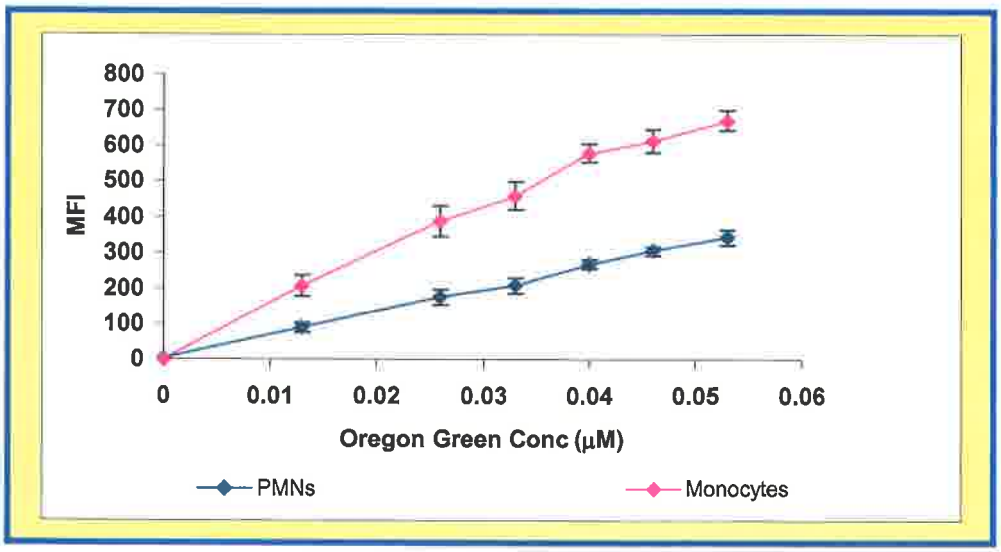


Fig 2.4: Effect of Oregon green® 514 phalloidin concentration on MFI of PMN ($p<0.001$) and monocytes ($p<0.001$) in whole blood samples. The points represent the means ± SEM at n=5. The p-values were determined by ANOVA.

2.3.4 Microscopic examinations

The morphology of un-stimulated cells in whole blood suspensions was investigated using fluorescence microscopy. Microscopic examination using FITC-phalloidin showed that cells were rounded in shape and weak in fluorescence. The fluorescence appears to slightly increase as the concentration of FITC-phalloidin increases, and the F-actin seen is diffusely distributed throughout the cell but with accumulation of F-actin towards the periphery as the concentration increases (**Figure 2.5**). These observations were clear at concentrations 0.05-2 μM of FITC-phalloidin. However, at the higher concentration (2.5μM) there were alterations seen in the cellular morphology, with focal accumulation of F-actin in lamellipodium. In contrast, Oregon green® 514 phalloidin labelled cells showed high fluorescence with a majority of cells showing morphological changes in the form of small blebs (lamellipodium), and this observation was true even at low concentrations of Oregon green. In these cells, F-actin is concentrated in these blebs, where fluorescence was much higher at peripheral regions than other parts of the cell (**Figure 2.6**).

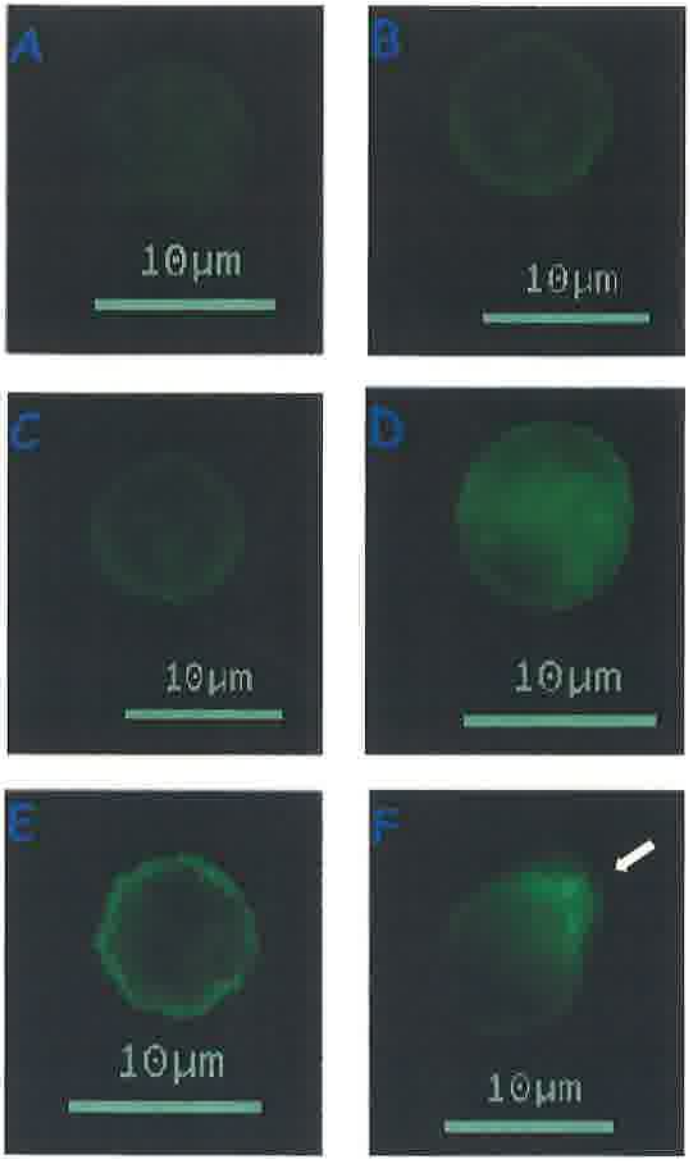


Fig 2.5: Fluorescence microscopy of whole blood samples stained for F-actin content with increasing concentration of FITC-phalloidin. (A-E are concentrations: 0.1, 0.5, 1.0, 1.6, and 2.0 µM respectively. At concentration 2.5µM (F) cells start to change shape and F-actin is accumulated in lamellipodium (arrow).

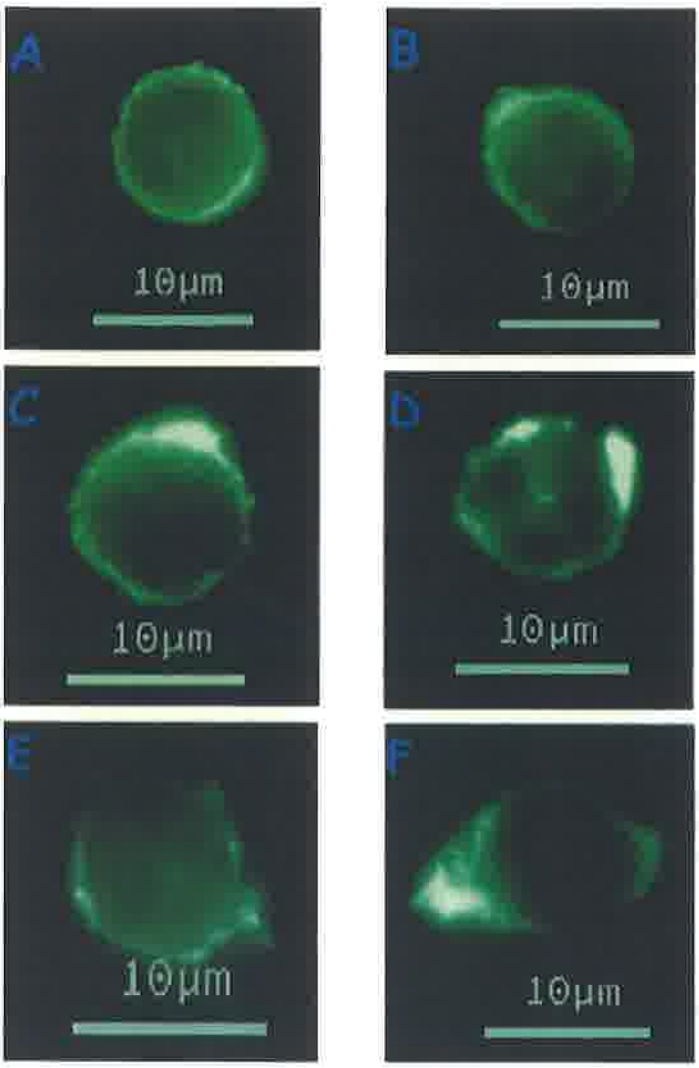


Fig 2.6: Fluorescence microscopy images of whole blood samples stained for F-actin content with increasing concentrations of Oregon® Green 514-phalloidin (A-F are concentrations 0.013-0.053 µM, respectively).

2.4 Discussion

In this study a direct comparison between two probes, which have been known for their binding to F-actin was presented. These probes, FITC-phalloidin and Oregon green® 514 phalloidin were used in flow cytometric and fluorescence microscope evaluations of leukocyte F-actin content determined in whole blood. Flow cytometric evaluation was initially based on a 15 minutes exposure to increasing concentrations of FITC and Oregon green® 514 phalloidin. Although flow cytometric data showed an increase in fluorescence intensity as concentrations increased for both phalloidin probes used to perform this experiment, FITC-phalloidin showed more stability in MFI as concentration increased (0.1-2.0 μM) for both PMNs and monocytes. Oregon green® 514 phalloidin showed a continuous increase in fluorescence intensity as concentration increased (0.013-0.053 μM). It has been shown that stabilisation of actin filaments by phalloidin derivatives (FITC and Oregon green® 514) does not inhibit the shift of G-actin to F-actin, which means that it does not inhibit actin polymerisation but accentuates the effect (Watts & Howard, 1993). Stability in FITC-phalloidin labelled cells was more noticeable at concentrations of 1.6μM-2.0 μM, which showed that the optimum concentration required for measurement of actin polymerisation was between these concentrations, therefore, 1.6 μM FITC-phalloidin was chosen as the optimum concentration in further investigations. This concentration was reasonably low to decrease the non-specific binding, to obtain a high staining intensity, to reduce cost and decrease the degree of toxicity.

Although flow cytometric data showed similar results for each phalloidin probe used, morphological analysis using fluorescence microscope indicated a

difference in the effect on cell structure. It is possible that morphological changes observed on phalloidin stained cells may be due to the partial fixation in preparation. Phalloidin is a large molecule thus not able to enter the cell. Coluccio & Tilney (1984) and Rotsch & Radmacher (2000) studied the effect of phalloidin on cytoskeletal structure and mechanics in fibroblast, and concluded that phalloidin could have affected their cells even though the cells were not permeabilised, hence causing changes in cell elasticity. Since phalloidin may have an effect on cell elasticity after permeabilisation, it may have an effect on cell shape as well. In these experiments cells were permeabilised before staining to enable phalloidin to enter the cell and bind to filamentous actin. Permeabilisation here therefore may have had an effect on cell shape especially after the fluorescence stain has entered the cell, although this shape change is only apparent with Oregon green. FITC-phalloidin labelled cells were smooth, round with weak and diffuse fluorescence throughout the cell. F-actin of FITC labelled cells present on the periphery of cells and almost no evidence of surface protrusions or irregularities at concentrations up to 2.0 μM. At concentration higher than 2.0 μM cells showed membrane blebs that were present on almost all cells.

Lots of difficulties were faced in setting the compatibility between fluorescence intensity measurement of flow cytometry and fluorescence microscope observations of Oregon green® 514 phalloidin labelled cells. Here, the fluorescence microscopic images showed strong fluorescence even at low concentrations of label. In addition to this, Oregon green® 514 phalloidin labelled cells showed an irregular shape and formation of blebs at all concentrations which were present on the majority of cells. Although Oregon green® 514 phalloidin concentrations were ten fold lower than that of FITC-phalloidin, this strong fluorescence made it difficult to

visualize and select images. To overcome this problem of brightness, the exposure time was decreased, but this has the disadvantage of using this probe as it necessitates constant changing of the fluorescent microscope settings.

In summary FITC-phalloidin was the appropriate probe to be used in this assay for further investigations. Oregon green® 514 phalloidin is a new probe with many limitations that may need further studies to evaluate. Due to the detrimental changes seen in cell morphology, this probe was excluded from further use here. The optimum concentration of FITC-phalloidin was $1.6x10^{-6}$ μM and will be used as a control concentration in un-stimulated cells in further investigations. This low concentration thus decreases the risk of toxicity or exposure to fluorescence materials. Although FITC-phalloidin photo bleaches rapidly, speed in 'grabbing' images make it possible to photograph the best images. Thus, this decision to use FITC-phalloidin for all further studies was based on a variety of factors such as stability of results, compatibility between fluorescence intensity of flow cytometric measurements and fluorescence microscope images, best image resolution and cost.

Chapter Three

Chapter Three: Actin reorganisation in response to chemotactic activation

3.1 Introduction

Phagocytic leukocytes such as monocytes and neutrophils are of major importance for the inflammatory response of the body. The binding of chemoattractants to leukocyte receptors initiates via a signal transduction cascade a coordinated series of responses, including shape change, projection of membrane ruffles and pseudopodia, cell movement and phagocytosis. These responses require the assembly and reorganisation of cytoskeletal microfilaments, which are composed primarily of actin, as detailed in the main introduction to this thesis (Stossel, 1989, Downey *et al,* 1992). This binding also stimulates secretory functions, for example, reactive oxygen intermediate generation and lysosomal enzyme release (Lepidi *et al*, 1995).

Leukocytes are stimulated by phorbol esters such as phorbol myristate acetate (PMA) and chemotactic factors such as n-formyl-Met-Leu-Phe (fMLP). The most potent stimulants of PMNs are bacterial peptides containing formylmethionine as their N-terminal amino acid (Lepidi *et al*, 1995). The bacterial peptide, fMLP, (**Figure 3.1**) interacts with its specific plasma membrane receptor, formyl peptide receptor (FPR). It has been extensively studied because this peptide stimulates a variety of biochemical and physiological responses in addition to chemotaxis (Fernandez-Segura *et al*, 1994). Co-ordinated responses are induced by fMLP at the biochemical and cellular levels. These responses are activation of pertussis toxin-sensitive G protein and increased activity of phospholipase C, which induces the formation of inositol 1,4,5-triphosphate and diacyglycerol. Inositol 1,4,5-

triphosphate increases intracellular free calcium and diacyglycerol activates protein kinase C (PKC) (Fernandez-Segura *et al*, 1994; Omman *et al*, 2002).

The effects of fMLP on rolling neutrophils have been studied by Sheikh *et al* (1997). They found that neutrophils treated with fMLP were transformed from rolling to stationary through activation of constitutive $\beta 2$-integrin and prolonged attachment through *de novo* expression of CD11b/CD18 (Sheikh & Nash, 1996). Hence, stimulation with fMLP results in an increase in CD11b/CD18 expression, a respiratory burst and cell locomotion which are associated with changes in filamentous actin. As mentioned previously, shape changes are a prerequisite for cellular migration (Zhang *et al*, 1995). It is known that actin assembly induced by fMLP and leukotriene B_4 is transduced through GTP-binding regulatory proteins (G proteins) (Downey, 1992).

PMA (**Figure 3.2**) is a protein kinase C (PKC) activator that mimics diacyglycerol (DAG). Protein kinase C stimulation follows activation of G-protein in response to various extracellular signals by the receptor-ligand complex (**Figure 1.11**); it binds to the cytoplasmic face of the plasma membrane, where phosphatidylserine is concentrated and requires this negatively charged phospholipid in order to act (Alberts, *et al*, 1994). It is known that exposure of cells to phorbol esters or DAG results in alterations in the content of and organisation of filamentous actin in a variety of cell types (Roos *et al*, 1987, Grant & Aunis, 1990, Downey, 1992). In leukocytes, exposure to PMA induces membrane ruffling, which is known to be dependent on changes and redistribution of F-actin (Howard & Wang, 1987, Roos *et al*, 1987, Downey, 1992). Stimulation with PMA leads to more intense activation, marked by prolonged cellular aggregation, CD18 phosphorylation,

exocytosis and phagocytosis (Jaconi *et al*, 1991, Hughes *et al*, 1992 and Zhang *et al*, 1995). It also leads to changes in F-actin organisation, where actin was distributed at the cell margin, at sites of adherence and throughout the cytoplasm (Pryzwansky & Merricks, 1998).

As the actin network is supposed to play a major role in determining mechanical properties of living cells, the importance of actin polymerisation and depolymerisation can be understood by studying the effects of drugs such as cytochalasins. Cytochalasins are a family of fungal metabolites excreted by various molds. The principle of the cytochalasins' action is to bind to the fast growing ends (barbed) of actin filaments, sever F-actin, inhibit interaction between filaments and prevent the addition and loss of actin monomers at this end (Bray, 1979; Cooper 1987; Sampath and Pollard, 1991; Sheikh and Nash, 1998; Rotsch and Radmacher, 2000). This binding leads to depolymersation of the filaments due to the net loss of actin monomers at their minus ends. Many studies have investigated the relationship between F-actin content and cell rigidity, using cytochalasins to inhibit F-actin polymerisation (Frank, 1990; Pecsvarady *et al*, 1992; Tsai *et al*, 1994; Buttrum *et al*, 1994, Sheikh and Nash, 1998). There are different types of cytochalasins but the most commonly used ones, which were used in this study, were cytochalasin B (CTB, **Figure 3.3**) and cytochalasin D (CTD, **Figure 3.4**).

3.1.1 Aims

The experiments described in this chapter were performed on whole blood samples with the following aims:

1. To establish methods for the measurable manipulation of cellular F-actin content, by selection of the most appropriate stimulant under optimum experimental conditions.
2. To investigate and to compare the effect of cytochalasins (B&D), F-actin disruptors, as part of these investigations into the mechanisms by which actin polymerisation occurs.

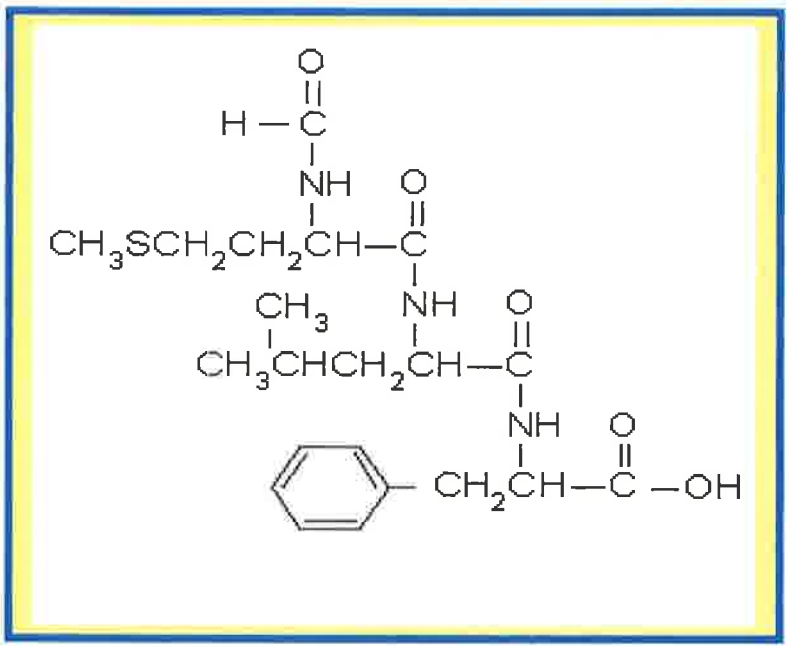


Fig 3.1: The structure of fMLP, a bacterial peptide, known to activate Leukocytes.

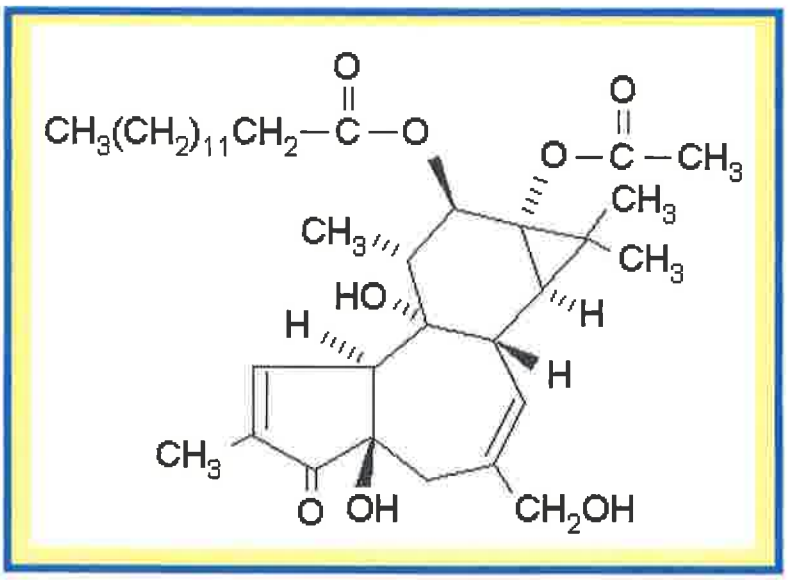


Fig 3.2: The structure of phorbol esters. The molecule acts as an analog to Diacylglycerol.

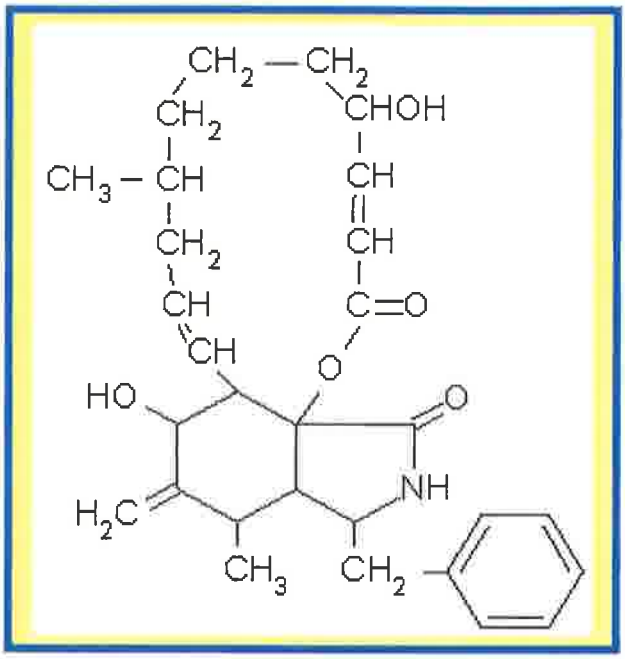


Fig 3.3: The structure of CTB, a fungal metabolite, known to disrupt F-actin.

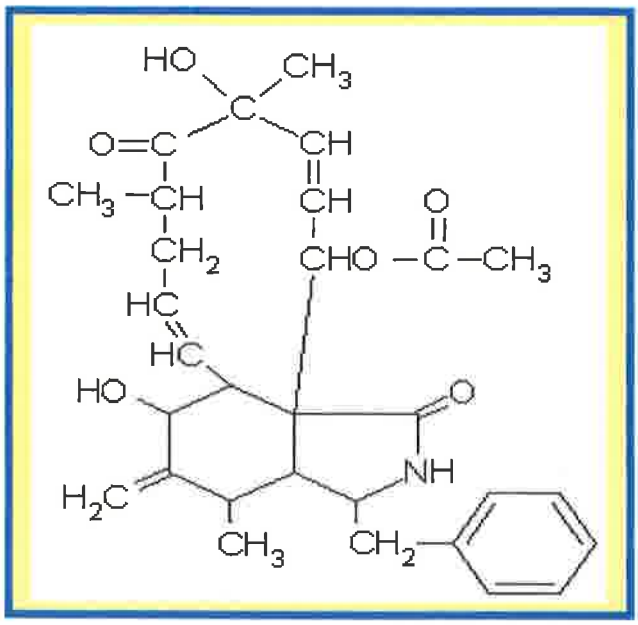


Fig 3.4: The structure of CTD, a fungal metabolite, known to disrupt F- actin.

3.2 Materials and Methods

All chemicals are from Sigma-Aldrich Company Ltd, unless stated otherwise.

3.2.1 Chemicals required for the study

Preparation of Phorbol 12-Myristate 13-Acetate

Phorbol 12-Myristate 13-Acetate (PMA) stock (0.32 mM) was prepared by dissolving 2 mg of PMA in 1 ml of DMSO and storing frozen in the dark at –20° C until use. A working solution of desired concentration was made by adjustment with PBS immediately before use to yield concentrations of 50, 100, 150, 200, and 250nM.

Preparation of n-formyl-Met-Leu-Phe

N-formyl-Met-Leu-Phe (fMLP) stock (9.14 mM) was prepared by dissolving 4 mg of fMLP in 1ml of DMSO and stored frozen as aliquots at –20° C until used. A working solution of desired concentrations was made by adjustment with PBS immediately before use to yield concentrations of 50, 100, 150, and 200 nM.

Preparation of Cytochalasin B

Cytochalasin B (1mg) was dissolved in 1ml Ethanol to obtain a stock solution of a concentration of 2.1mM and was kept frozen at -20° C.

Preparation of cytochalasin D

Cytochalasin D (1mg) was dissolved in 1ml Ethanol into 1.97mM stock solution and was kept frozen in adequate at –20° C.

Anticoagulated blood (EDTA) was collected from healthy volunteers (section 2.2.2). All blood samples were stored at room temperature during assays and used within six hours of venepuncture.

3.2.2 Measurement of F-actin in PMA-activated cells

Blood (0.5 ml) from normal volunteers was incubated with various concentrations of PMA (50, 100, 150 and 200 nM) for 15 minutes in the dark at 37° C. Reactions were terminated by fixation, and the erythrocytes lysed by incubating 50 μl of activated whole blood with 100 μl of reagent A, a fixation medium of Cell Permeabilising Kit for 15 minutes in the dark at room temperature (section 2.2.3.1). Cells were washed using PBS and centrifuged at 300g for 5 minutes. After centrifugation, the supernatant containing the lysed erythrocytes was removed and discarded and 50 μl of pellet was incubated with 100 μl of reagent B, permeabilsation medium (section 2.2.3.1) of CPK and stained in the same step with 1.6μM with FITC-phalloidin. A second cell wash was performed after 15 minutes of incubation with reagent B and FITC-phalloidin in the dark at room temperature. PBS (0.5 ml) was added to stimulated cells for flow cytometric analysis. Controls were incubated with the equivalent amount of DMSO.

3.2.3 PMA time course

Whole blood samples were incubated with 100 nM PMA, the optimum concentration for cell response obtained from section 3.2.2. Measurements were made from 60 seconds incubation time up to 900 seconds at various time intervals, and the effect on actin polymerisation at each time was determined by flow cytometric analysis.

3.2.4 Measurement of F-actin in fMLP-activated cells

The previous steps (section 3.2.2) were repeated using fMLP at various concentrations (50-200 nM). Cells were treated with fMLP in the dark for 15 minutes at 37° C before being fixed, lysed, permeabilised and labelled with FITC-phalloidin. Whole blood suspensions were ready for flow cytometric analysis and fluorescent microscopic observations after adding 0.5 ml of PBS.

3.2.5 fMLP time course

Incubation of cells with fMLP for 15 minutes as a starting point to study the effect on actin polymerisation showed low F-actin content of both PMNs and monocytes compared to controls, therefore a time course was necessary. Incubation times were 5-1800 seconds at various time intervals. At each time point cells were fixed, permeabilised and stained as previously described for flow cytometric analysis.

3.2.6 Dose response of CTB and CTD

In order to select the optimum conditions for further study, whole blood (0.5 ml) was incubated with different concentrations (0.1, 2, 6, 10, 15 and 20 μM) of CTB or CTD. After incubation for 5 minutes at 37° C, cells were fixed, permeabilised and labelled as described previously. Controls were incubated with the equivalent volume of ethanol.

3.2.7 CTB Time course

Whole blood was treated with CTB (10 μM) for different incubation periods (30,180, 300, 600, 900, 1200, 1800 seconds) before fixation, to establish the

optimum time required for disrupting F-actin. After incubation at 37° C, cells were fixed, permeabilised and labelled as described previously. Controls were incubated with the equivalent volume of ethanol.

3.2.8 Effect of CTB on F-actin content of fMLP stimulated cells

To study the effect of cytochalasin B on the F-actin content of PMNs and monocytes, whole blood cells were incubated simultaneously with fMLP (5 nM) and CTB (10 μM) in the dark for 5 minutes at 37° C. After incubation cells were fixed, permeabilised and stained for flow cytometric analysis.

3.2.9 Flow cytometric analysis

Cell suspensions were fixed, permeabilised and labelled with FITC-phalloidin, as described in section 2.2.3 analysed according to their size and granularity by measuring their forward and side scattered light, respectively. (Section 2.2.3.3) (Fernandezsegura *et al*, 1995).

3.2.10 Fluorescence microscopy

The same blood suspensions used for flow cytometric analysis were used in fluorescent microscopy by mounting a small volume of a suspension on a slide and coverslips to determine cell shape change and distribution of F-actin. The same microscope mentioned earlier was used (section 2.2.3.5).

3.2.11 Statistical analysis

ANOVA was used to determine the effect of concentration and incubation time with stimulants (PMA & fMLP) on F-actin content of PMNs and monocytes.

ANOVA was also used to study the same effects (concentration and incubation time) of cytochalasins (CTD& CTB) on F-actin content.

3.3 Results

3.3.1 Effect of PMA on F-actin content

Dose response

F-actin content was evaluated in whole blood samples (gated for PMNs and monocytes), with and without PMA stimulation. When blood was incubated with PMA (50-250 nM) at 37°C for 15 minutes, PMA induced actin polymerisation in both types of cells. The mean fluorescence intensity of the activated cells (gated PMNs and monocytes), with various concentrations of PMA (50-250 nM) is shown in **Figure 3.5**. The F-actin content significantly increased in both PMNs ($p<0.001$) and monocytes ($p<0.001$) as determined by ANOVA, after 15 minutes of incubation with PMA. This increase was noticeable at a concentration as low as 50 nM and this activation remained stable at higher concentrations.

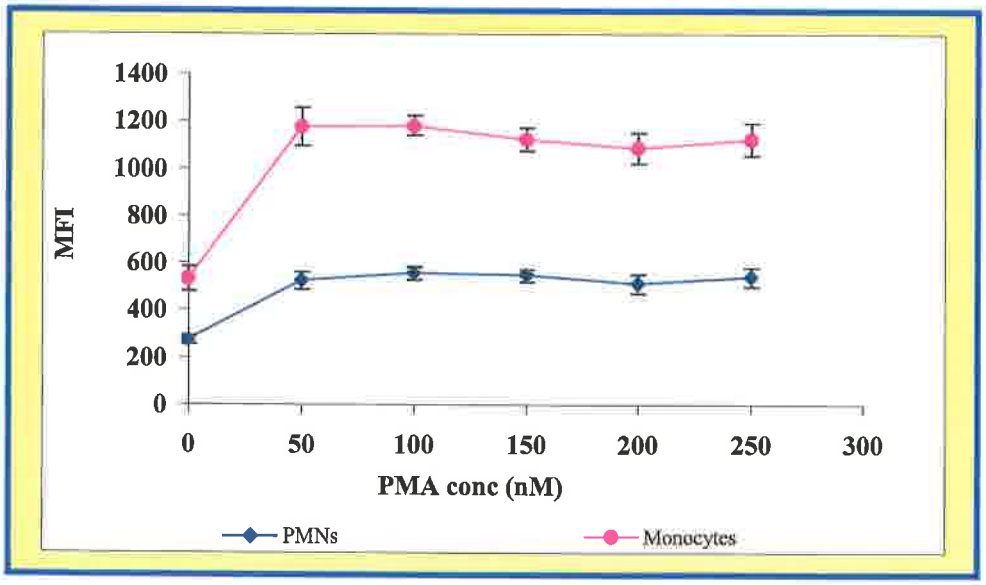


Fig 3.5: Effect of PMA concentration on F-actin content (MFI) in PMNs ($p<0.001$) and monocytes ($p<0.001$) as measured by phalloidin-stained cells. Each value represents the mean ± SEM of n=10. The p-values were determined by ANOVA.

Time course with PMA

The effect of incubation time with PMA was also tested in whole blood samples. Blood treated with PMA (100nM) at different times (60-900sec) at 37°C showed an increase in F-actin content of both PMNs and monocytes up to 600 seconds (**Figure 3.6**). This increase was statistically significant in PMNs ($p<0.001$) and monocytes ($p<0.001$) as determined by ANOVA.

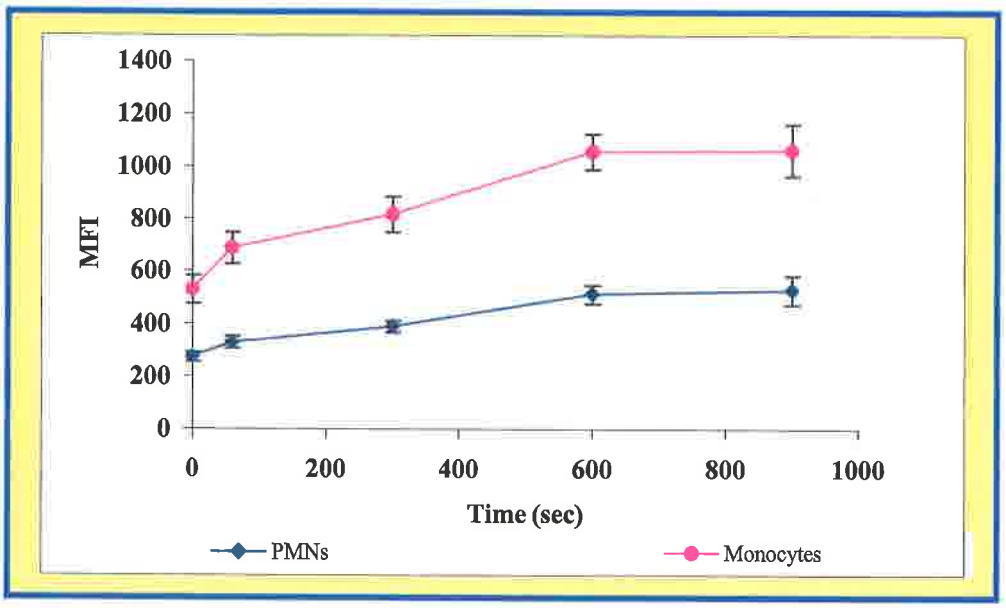


Fig 3.6: The effect of incubation time with PMA on F-actin content in PMNs ($p<0.001$) and monocytes ($p<0.001$) measured by FITC-phalloidin staining of fixed and permeabilised cells at specific time points after addition of stimulus (PMA 100nM). Flow cytometric data are expressed as MFI. Each value represents mean ± SEM of n=10. The p-values were determined by ANOVA.

3.3.2 Effect of fMLP on F-actin content

Dose response

F-actin content in PMNs and monocytes was evaluated in whole blood, with and without fMLP stimulation. Flow cytometric analysis showed a statistically significant increase in F-actin content of PMNs ($p<0.001$) and monocytes ($p<0.001$) as determined by ANOVA, after stimulation with various concentrations of fMLP (50-200nM) at 37°C for 15 minutes. The p-values were determined by ANOVA. The data, shown in **Figure 3.7**, illustrate that this increase was maximal at 50nM fMLP, and then declined at higher concentrations. The F-actin content in PMNs, and monocytes showed a continued slight decline at the same concentrations.

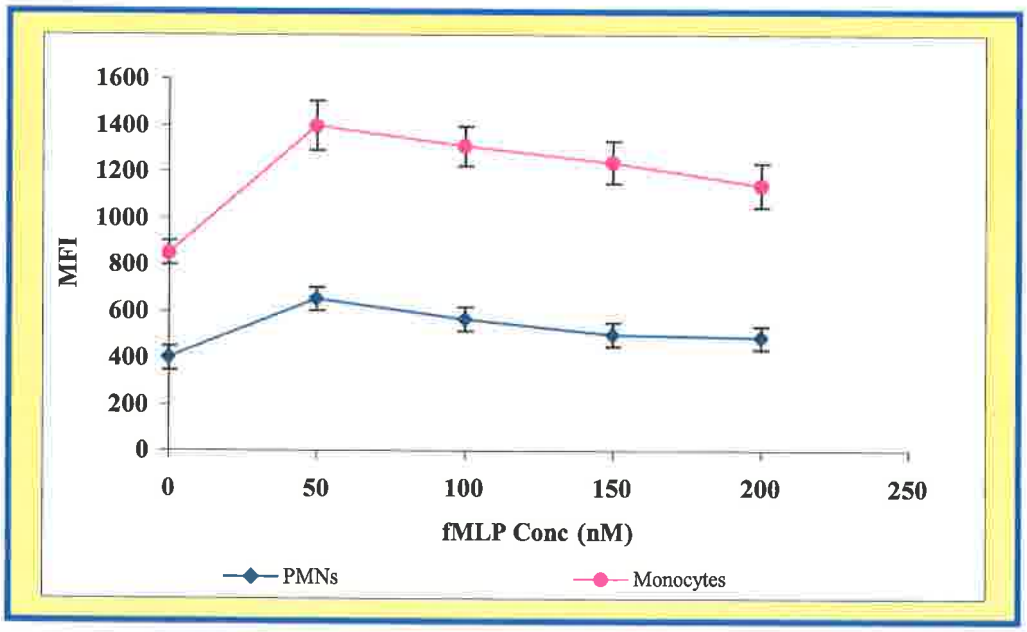


Fig 3.7: Effect of fMLP concentration on F-actin content (MFI) in PMNs ($p<0.001$) and monocytes ($p<0.001$). Each value represents the mean ± SEM of n=10.The p-values were determined by ANOVA.

Time course with fMLP

Activation of PMNs with fMLP (100nM at 37°C) caused an increase in F-actin content to a maximal level at 60 seconds, followed by depolymerisation to basal level at 1800 seconds ($p<0.001$, **Figure 3.8**). Activation of monocytes with the same concentration of fMLP again showed an increase in F-actin content to a maximal level at 60 seconds ($p<0.001$), but it decreased slightly after that time and plateaus. The p-values were determined by ANOVA.

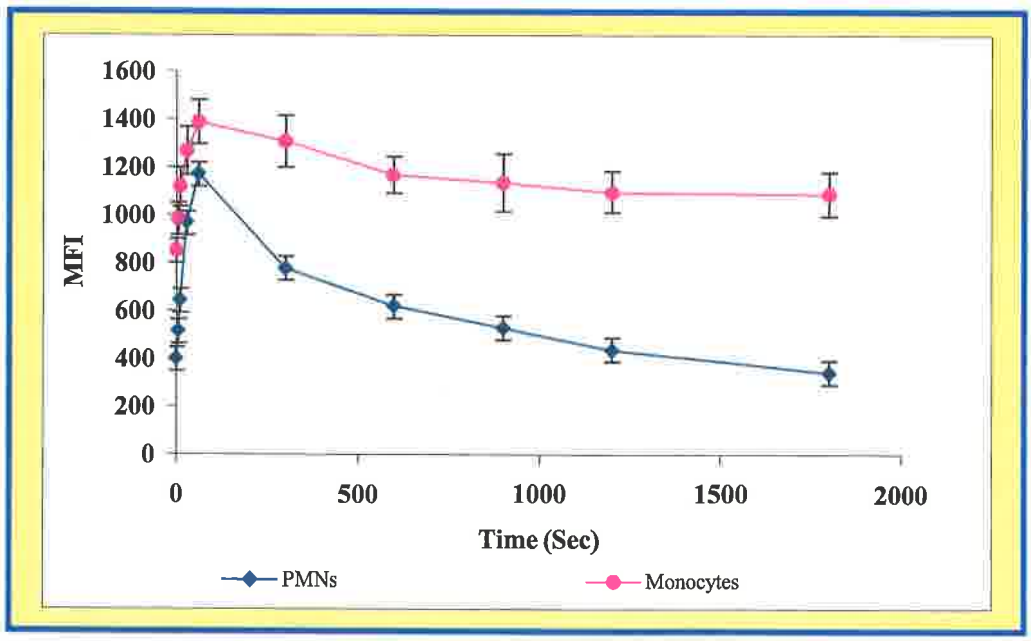


Fig 3.8: The effect of incubation time with fMLP on F-actin content was measured in PMNs ($p<0.001$) and monocytes ($p<0.001$) by FITC- phalloidin staining of fixed and pemeabilised cells at specific time points (5-1800 seconds) at 37°C after addition of stimulus (fMLP 100nM). Each value represents mean ± SEM of n=10. The p-values were determined by ANOVA.

3.3.3 Effects of CTB and CTD on F-actin content

A five-minute exposure to different concentrations of CTB and CTD showed that both PMNs and monocytes responded differently to each cytochalasin. Cells treated with CTB (**Figure 3.9**) showed a decrease in F-actin content compared to controls in both PMNs ($p=0.177$) and monocytes ($p=0.031$) as determined by ANOVA, and this reduction was clear at a concentration as low as 0.1 μM. F-actin content at higher concentrations showed a stable reduction which was dose-independent. Treatment of blood with CTD (**Figure 3.10**) resulted in changes in F-actin content of PMNs ($p<0.001$) and monocytes ($p<0.001$) as determined by ANOVA. At concentrations of 0.1-6μM, F-actin content decreased but at higher concentrations, 10-20μM, F-actin content increased. At concentrations higher than 6μM, CTD produced a dose-dependent increase in F-actin content. Accordingly, CTB was chosen for use in further investigation, as F-actin disruption is more reproducibly measured. Hence, the rest of this results section refers only to CTB.

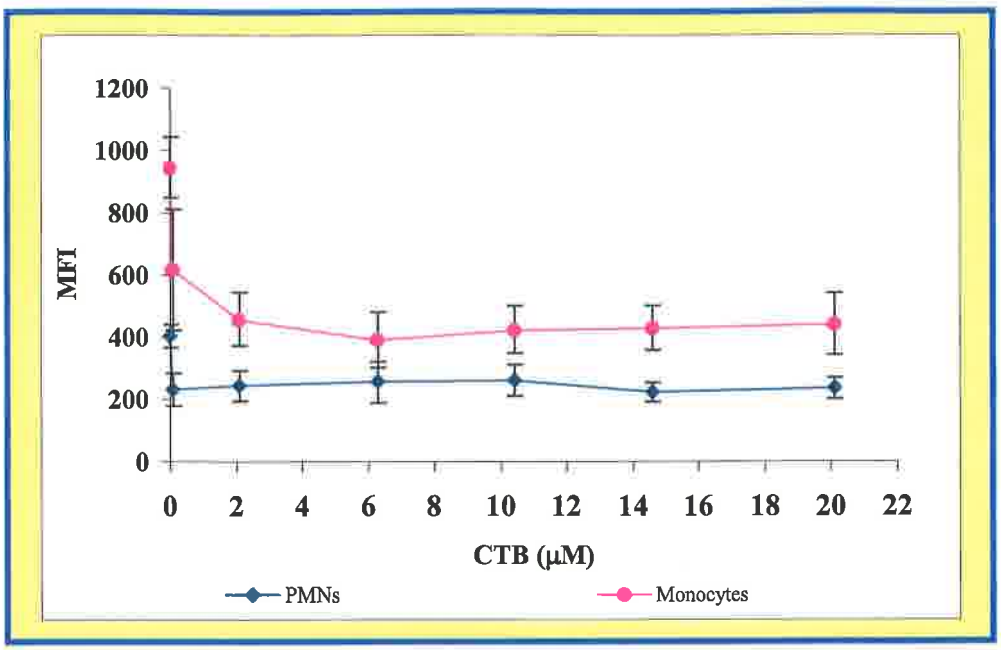


Fig 3.9: Summary of the effect of CTB on F-actin content (MFI) in PMNs ($p=0.177$) and monocytes ($p=0.031$) as measured by flow cytometry. Each point represents mean± SEM of n=3. The p-value values were determined by ANOVA.

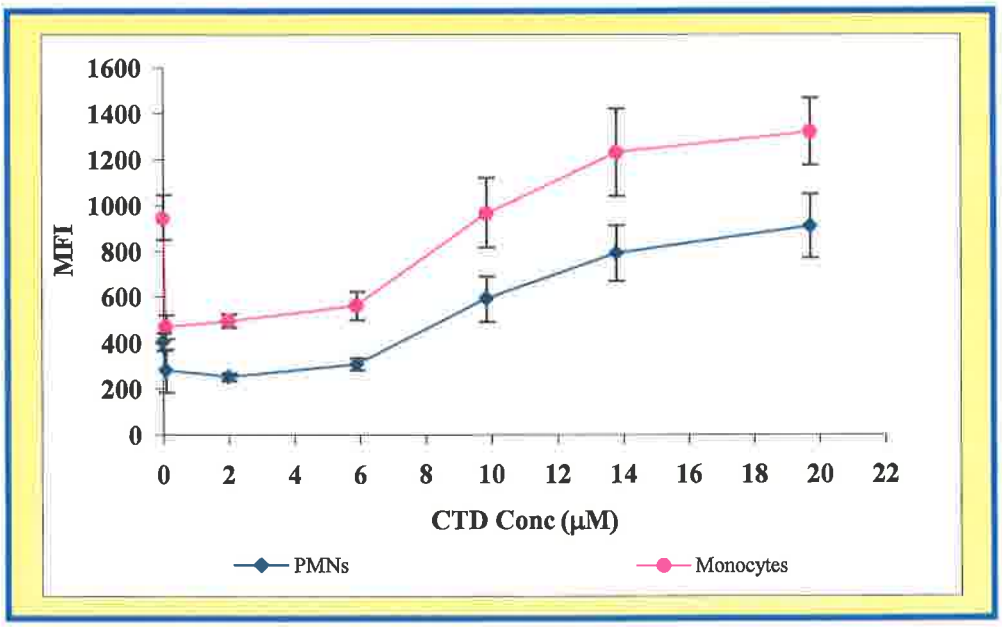


Fig 3.10: Summary of the effect of CTD on F-actin content (MFI) in PMNs ($p<0.001$) and monocytes ($p<0.001$) as measured by flow cytometry. Each point represents the mean ± SEM of n=3. The p-values were determined by ANOVA.

Time course with CTB

Incubation of blood with CTB (10μM) at different time intervals (0-1800 sec) at 37°C showed that no statistically significant changes occurred with incubation time for PMNs (p=0.30) or monocytes (p=0.41) (**Figure 3.11**). The p-values were determined by ANOVA.

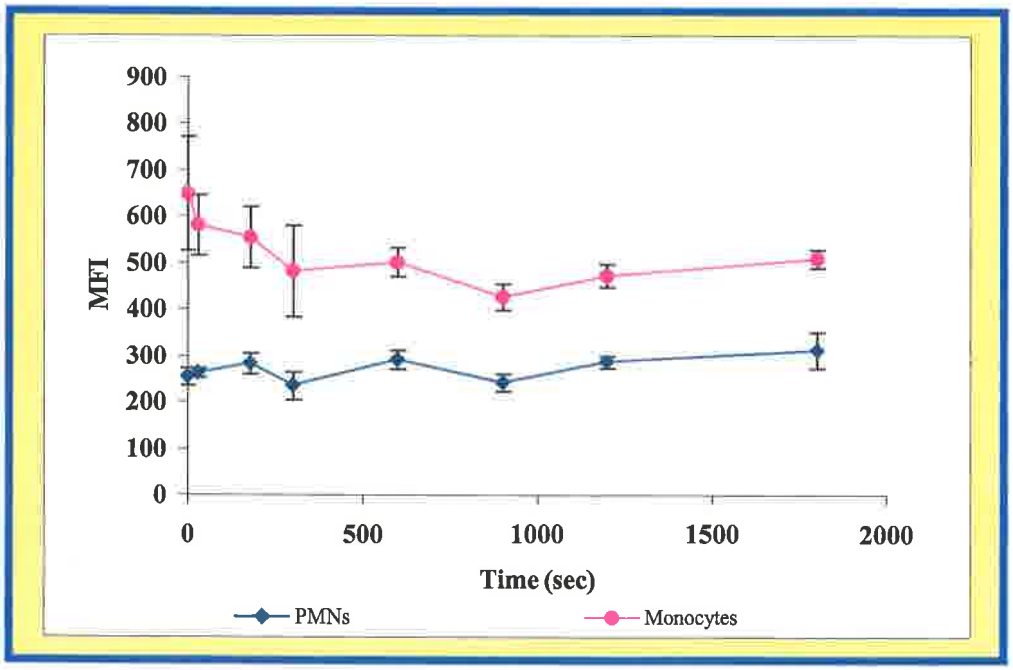
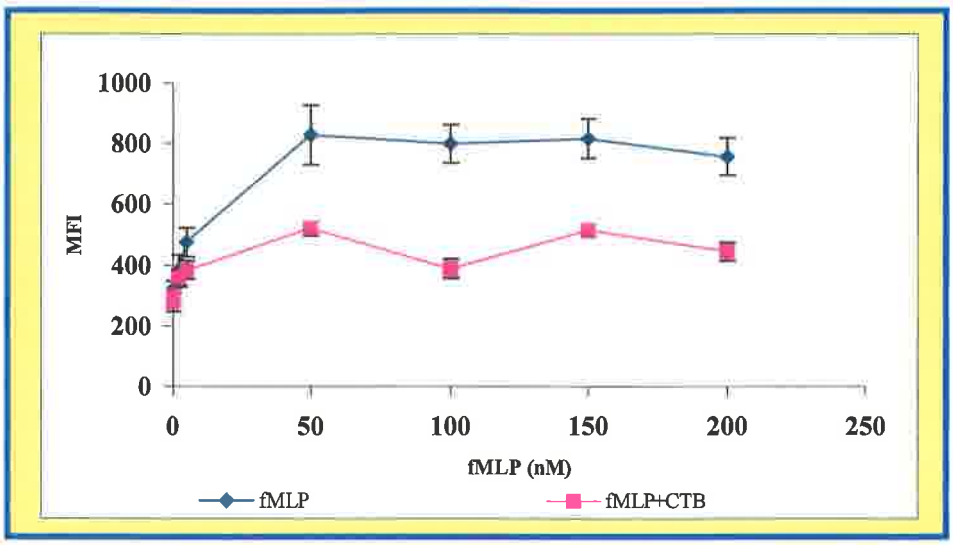


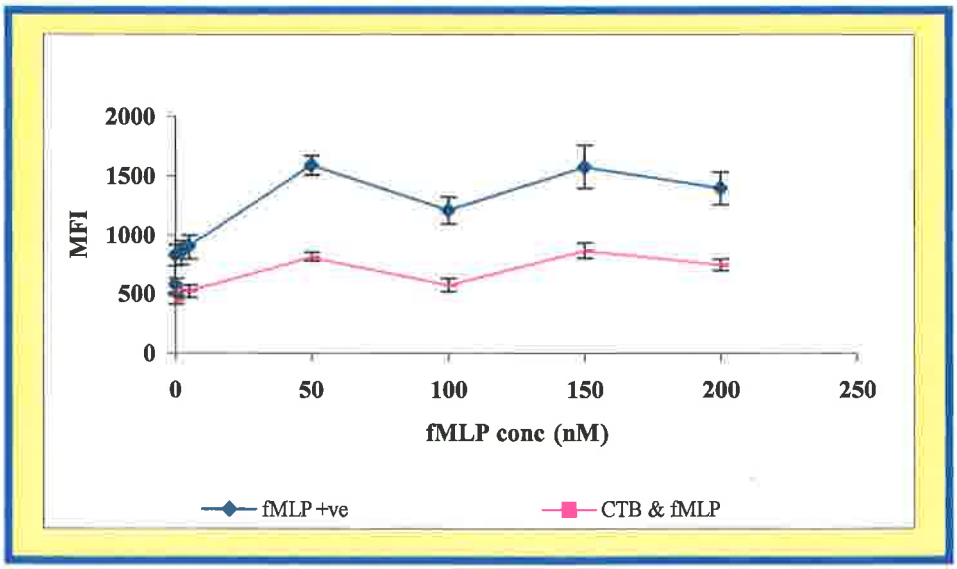
Fig 3.11: Summary of the effect incubation time with CTB (10μM) on F-actin content (MFI) in PMNs (p=0.30) and monocytes (p=0.41) as measured by flow cytometry. Each point represents the mean ± SEM of n=3. The p-values were determined by ANOVA.

3.3.4 Effect of CTB on F-actin content of fMLP stimulated cells

The effect of CTB on F-actin content was studied on fMLP stimulated blood. Blood was stimulated with different concentrations of fMLP (0-200nM) in the presence of CTB (10μM), and incubated for 5min at 37°C. Results (**Figure 3.12**) showed that incubation with fMLP, at various concentrations, increased F-actin content in both PMNs and monocytes, as shown previously. The presence of CTB has a clear effect in lowering F-actin content of these stimulated PMNs ($p<0.001$) monocytes ($p<0.001$) as determined by ANOVA, by inhibiting the activation effect induced by fMLP.



(A)



(B)

Fig 3.12: The effect of CTB on F-actin content of fMLP-stimulated cells (A) PMNs ($p<0.001$) and (B) monocytes ($p<0.001$) was measured by flow cytometry. Points represents mean ± SEM of n=10. The p-values were determined by ANOVA.

3.3.5 Fluorescence Microscopy

Fluorescent microscopic images were captured from different tests to observe the morphological changes occurring as a result of stimulation by PMA or fMLP (**Figure 3. 13**), or of F-actin disruption by CTB and CTD (**Figure 3. 14**). It was clear that both stimulants had a great effect on the distribution and content of F-actin in the cell, as observed by microscopy. Treatment with both fMLP and PMA caused changes in the cell shape from spherical (control) to an irregular, polarised shape. No such changes were observed after treating cells with cytochalasin B; however, small changes were observed after cytochalasin D treatment, with cells exhibiting some blebs.

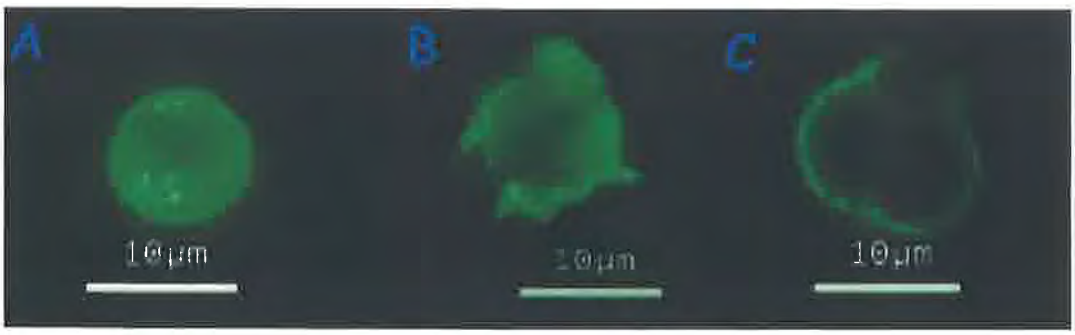


Fig 3.13: Fluorescence microscopy images showing morphological changes of controls (A), cell treated with PMA (B) and cells treated with fMLP (C) for 15 minutes.

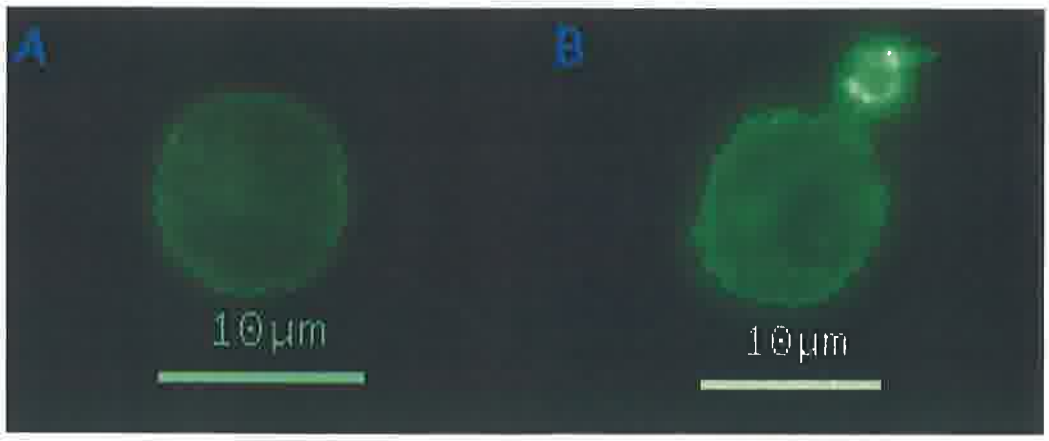


Fig 3.14: Fluorescence microscopy images showing morphological changes of cells treated with cytochalasins B (A) and D (B).

3.4 Discussion

Leukocyte chemoattractants, such as the bacterially derived fMLP or phorbol ester such as PMA have been used to study their effect on actin polymerisation of PMNs and monocytes in whole blood. These molecules induce positive chemotaxis. Chemoattractants play an important role in trans-endothelial migration of leukocytes to sites of inflammation. The aims of the studies in this chapter were two-fold; firstly, to study the effect of PMA and fMLP on F-actin content in PMNs and monocytes. This study enabled us to choose the appropriate stimulant to be used in further investigation. Secondly, to study the effect of cytochalasins (B and D) on F-actin content and to choose the appropriate disruptor for further investigations.

The first studies presented here compare the changes in F-actin content of PMNs and monocytes caused by activation with PMA and fMLP. These studies showed that cells respond differently to each stimulant as measured by flow cytometry and fluorescent microscopy. Specifically, the data demonstrate the following 4 points which are stated briefly here, then discussed in more detail later (a) although both stimulants (PMA & fMLP) cause an increase in F-actin content in PMNs and monocytes, they differ in actin polymerisation rate. This finding agrees with Howard & Wang (1987). The mean rate of fMLP stimulation was greater than the mean rate of stimulation in PMA stimulated cells even though fMLP response was back to baseline within 15 minutes of stimulation. The mean fluorescence intensity was at its maximum and higher in fMLP-stimulated cells than PMA. This could be explained by the fact that although fMLP and PMA have the same signalling pathway involving DAG, fMLP also involves a second part of the pathway

by activating Ca^{2+} (**Figure 1.12**). (b) The effects of PMA and fMLP are dose-independent. The results obtained from dose-response curve showed that actin polymerisation was promoted at a concentration as low as 50nM of both chemoattractants used here (PMA & fMLP). (c) The results obtained from the time-course were similar to those emphasised by Wallace et al (1984) and Howard & Oresajo (1985a, 1985b), and showed that actin polymerisation is increased as early as 60 seconds for fMLP-stimulated cells followed by depolymerisation and after 10 minutes of stimulation with PMA. This delay in activation by PMA could be because PMA is slower than fMLP in entering the cell and PMA may work via a phosphorylated intermediate. (d) Microscopic images were captured after PMA and fMLP stimulation to show the morphological changes which accompanied actin polymerisation.

Initially, as the effect of PMA has been studied, results showed that exposure to PMA (50-250nM) resulted in assembly of actin monomers into F-actin. These results were similar to previous studies (Roos *et al*, 1987, Downey *et al*, 1992, Fernandez et al, 1994) which showed that exposure of neutrophils to protein kinase C (PKC) activators (e.g Diacyglycerol and phorbol esters) induced reorganisation of F-actin and membrane ruffling. However, fMLP, a chemotactic peptide, induced effects such as F-actin reorganisation and pseudopod extension, which cannot be prevented by PKC inhibitors (Fernandez *et al*, 1995).

As a time course was performed for cells being stimulated with PMA or fMLP, results showed that exposure to PMA or fMLP (100nM) resulted in assembly of actin monomers into F-actin. Stimulation by PMA promoted the rate of actin polymerisation within the first minutes but the rate of polymerisation was at its

maximum between 10-15 minutes. Stimulation by fMLP, in contrast, had reached its maximum by 60 seconds (**Fig 3.8**). The delay in response to PMA (**Fig 3.6**) may be attributed to the time necessary for the phorbol ester to cross the plasma membrane and interact with its cytoplasmic receptor. This explanation could be accepted since blood used in these in experiments is activated with PMA before being permeabilised and fMLP binds to its receptor (FPR) on the plasma membrane. Along with flow cytometric results, fluorescent microscope images have shown that fMLP stimulation has a clear effect on cell shape change. After 30 seconds of fMLP activation cells were irregular in shape with large surface projections (**Fig 3.13**).

In the second part of this chapter involving the use of cytochalasins, F-actin disrupting agents, results showed that different cytochalasins can have varying effects on F-actin content and cell morphology. To examine these effects, whole blood samples were used and treated with either cytochalsin B or D. After 5 minutes of exposure to a low concentration of these agents (0.1-6μM), F-actin content in PMNs and monocytes has decreased in cells treated with CTB or CTD. At higher concentrations, CTD treated cells showed an increase in F-actin content (**Fig 3.10**) whereas in CTB treated cells F-actin was more or less stable (**Fig 3.9**). These results indicate that exposing cells to cytochalasin D concentrations greater than 6μM enhance actin polymerisation and cause cell activation. These results agree with those of Mills *et al* (2000), which indicated that exposing cells to cytochalasin concentrations higher than 10μM may not enhance the depolymerisation effect of the cytochalasins, and increasing the concentrations of CTD may reverse the depolymerising effect. Studies by Wilder & Ashman (1991) and Franki *et al* (1992) reported similar findings.

How can the increase in F-actin in the case of CTD treatment be explained? With the splitting of the actin filaments, which are essential to the cytoskeletal network, extra positive barbed filament ends would be available for actin polymerisation. Increasing filament ends further could lead to secondary formation of filamentous aggregates or foci composed mainly of F-actin (Schliwa, 1982; Ting-Beall *et al*, 1995). Thus, this increase in F-actin is likely to be due to an increase in the number of barbed ends. At low concentrations, both CTB and CTD cause slowing of, but do not prevent, filament elongation as measured by the phalloidin-binding flow cytometric assay.

In the last part of this chapter, the effect of CTB on fMLP-stimulated cells was studied. Cells exposed to 10μM CTB and stimulated at the same time with different concentrations of fMLP (0.05-100 nM) showed a significant ($p<0.001$ as determined by ANOVA) reduction in F-actin content (MFI) of PMNs and monocytes. This experiment confirms the effect of CTB as an F-actin disruptor.

Along with a difference in the effect of CTB and CTD on F-actin content is the difference observed in cell morphology obtained from fluorescent microscope images. Cytochalasin D was shown here to have an effect on cell shape with some small blebs observed (**Fig 3.14 B**), whereas in CTB treated cells, cells were spherical and smooth with no bleb formation (**Fig 3.14 A**). This bleb formation is more likely to be related to an alteration in the properties of the F-actin network and the interaction with an actin-binding protein and the cell membrane (Mills *et al*, 2000). In summary, results show that the morphological changes, F-actin disruption, and F-actin depolymerisation is dependent on both the type and the concentration of

cytochalasin used. Based on these results CTB was chosen as the appropriate F-actin disruptor to be used in further investigations.

Chapter Four

Chapter Four: Effect of actin polymerisation on leukocyte deformability in diluted blood

4.1 Introduction

Both leukocyte and erythrocyte deformability are vital for life and flow disturbances have been implicated in various diseases (see Chien *et al*, 1987, for review). For this reason haemorheologists have concentrated on studies of flow properties of both types of cells.

In healthy blood where the concentration of leukocytes is low, leukocyte deformability has little effect on the bulk viscosity of blood and flow in large vessels. However, white blood cells must deform considerably in order to flow through narrow capillaries and their ability to flow is important in determining the overall resistance to flow in the microcirculation (Chien, 1982; Sutton & Schmid-Schonbein, 1992). The rheological properties of PMNs and monocytes, as phagocytic cells, are likely to be extremely sensitive to environmental conditions as they are bound to change on activation.

4.1.1 Association between F-actin and leukocyte deformability

Most circulating leukocytes in the blood stream exhibit low levels of pseudopod projections. Leukocytes with these projections tend to show enhanced resistance to flow in the microvessels (Sutton & Schmid-Schonbein, 1992). The ability of leukocytes, PMNs and monocytes in particular, to deform and to retain their shape is dependent on the mechanical behaviour of the cytoskeleton (Wallace *et al*, 1987; Ting-Beall *et al*, 1995). Activation of white cells is accompanied by cytoskeletal reorganisation, which produce pseudopodia. Actin filaments are the major contributor to this neutrophil cell stiffness, and an increase in the amount of

polymerised F-actin, from conversion of monomeric G-actin, has been correlated with decreased neutrophil deformability (Sheikh *et al*, 1997; Tsai *et al*, 1998; Iano *et al*, 1992; Dubos *et al*, 1992).

4.1.2 Measurement of cell deformability

Variety of techniques and commercially-available instruments have historically been used to study cell deformability, including micropipette techniques.

Studies using micropipettes provide detailed information about the rheology of individual cells. This method is, however, labour intensive so only a limited number of cells maybe studied and the properties of important subpopulation of cells may be missed.

Currently the most widely used techniques for assessing blood cell deformability are filtration techniques. A general description of the principles of filtration methods fellows.

A mathematical model for the steady state flow of a suspension of red blood cells through polycarbonate membranes was provided by Blackshear *et al* (1979). The resistance to flow of pure red blood cell suspensions, relative to that of suspending medium, was measured and used to calculate the ratio of flow rate of suspending medium to the flow rate of a single red cell through a pore. Jones *et al* (1980), Skalak (1981), and Cokelet (1981) used other mathematical models to describe the flow of red blood cell suspensions through polycarbonate membranes. Skalak (1981) described cell resistance to flow in terms of the pressure required to maintain a constant flow rate of cell suspension. The relative resistance of red cells and buffer was described as the β factor. A similar model was described by Cokelet (1981) using different terminology. The proportion of pores, which is evacuated

every second, was described by the proportionality constant (k). The theoretical basis of a commercial Filtrometer for measuring the filterability of red blood cells in pure suspensions was described by Hanss (1983). This author defined an "Index of Filtration" (IF) based on the times taken to filter a fixed volume of buffer and cell suspension respectively. When corrected for haematocrit, the IF gave a measure of the filterability of the red blood cells.

All the theoretical models, described so far, assume a steady flow rate of a homogenous suspension of cells. Buchan (1980) and Alderman *et al* (1981), using the technique of Reid *et al* (1976), highlighted the influence of white cells on the filterability of whole blood through 5μm membranes and concluded that the cause of this problem was pore blocking by white blood cells. A mathematical model, which did not assume a constant flow rate of suspension through polycarbonate membranes, was described by Jones *et al* (1980). Flow profiles of volume filtered versus time, at constant driving pressure, were analysed using a model with rate constants similar to those of Cokelet (1981). Skalak *et al* (1983) extended their model of steady state flow to describe a heterogeneous cell suspension of red and white blood cells and pore blocking by rigid red and white blood cells. Jones *et al* (1984 & 1985) described mathematical models, which extended the steady state treatment described by Blackshear (1979) to analyse of flow profiles at constant pressure with either 3μm or 5μm Nucleopore membranes. These theories are essentially the same as those described by Skalak (1983) but differ in the approach and the terminology. Using a constant pressure system results in a declining flow rate and hence the proportionality constants have a slightly different interpretation to those of Skalak and co-workers. The flow rate of individual cell populations through pores remains constant, as the driving pressure remains constant throughout, although the total flow

rate of suspension decreases. Dormandy *et al* (1985) described a new Filtrometer, the St George Filtrometer, which analysed the initial rate of flow, of a red blood cell suspension, and its immediate decline to give the transit time for red blood cells and the number of pore blocking cells. The pore blocking cells include any slow flowing cells, which have not been given sufficient time to pass through the pores in the membranes (Nash, 1990).

The above theoretical methods, developed for examining the flow of red blood cells, have been extended to the study of leukocytes (Skalak *et al*, 1987; Lennie *et al*, 1987; Nash *et al*, 1988; Kooshesh *et al*, 1991; Adams *et al*, 1994; Cook *et al*, 1998; Jones *et al*, 1999). Nash *et al* (1988) and Kooshesh *et al* (1991) analysed flow profiles of un-fractionated leukocytes through 5μm membranes. In both studies, the analysis revealed two populations of flowing cells and one minor population of pore blockers. The major population of flowing leukocytes were lymphocytes and granulocytes, with a transit time of about 1 second, while the minor population were monocytes with a transit time about ten times higher. The pore blockers could not be identified as any particular sub-population of cells.

The sensitivity of leukocytes to environmental changes complicates their analysis (Nash *et al*, 1988) and a test with minimum experimental changes to cells before testing is therefore, desirable. A method for analysing the filterability of the majority of leukocytes from the initial flow rate of diluted blood was described by Kooshesh *et al* (1991). This method, using the Cardiff Filtrometer, requires knowledge of the filterability of purified red cells but the transit time for a mixed population of lymphocytes/granulocytes agrees with that derived from analysis of fractionated leukocytes. The method, described by Kooshesh *et al* (1991), was

confined to the first 10 seconds of the flow profile and therefore records the slowest leukocytes (e.g. monocytes) as pore blockers.

The analysis described by Kooshesh *et al* (1991) has been extended to allow calculation of a transit time for monocytes by comparison of the initial and final steady state flow rate of 150 second flow profiles (Adams *et al*, 1994). Cook *et al* (1998) described the use of this model to analyse flow profiles of whole blood. The analysis, using the Cardiff Filtrometer, described by Kooshesh *et al* (1991) is used in these studies. The detail of the model used is described in the method section of this chapter.

4.1.3 Aims

These studies were conducted to investigate the effect of cell activation on the filterability of leukocytes in whole blood suspensions, in order to investigate the relationship between changes in F-actin content and deformability. Leukocyte activation was performed using PMA and fMLP at a variety of concentrations, as these chemoattractants have been widely used for many studies involving leukocyte rheology (Buttrum *et al*, 1994; Frank, 1990; Pecsvarady *et al* 1992). The effects of cytochalasin B, as an F-actin disruptor of fMLP stimulated cells, were also studied, in order to fully investigate the relationship between F-actin and leukocyte filterability.

4.2 Materials and Methods

All materials used in this chapter are from Sigma-Aldrich Company Ltd. unless stated otherwise. The preparation of PBS, PMA, fMLP, cell permeabilisation kit (Harlan) and cytochalasin B have been described earlier in chapters two and three.

4.2.1 Blood samples

Samples used were obtained from healthy volunteers by venepuncture of the ante-cubital vein, and collected into vacutainers containing sufficient tri-pottasium EDTA to give a final concentration of 1.5mg/ml. Filtrations were completed within four hours of blood withdrawal.

4.2.2 Blood counting

Red and partial differential white cells counts were measured using a Coulter® Microdiff automated counter.

4.2.3 Preparations of blood cell suspensions

A. Red blood cell suspension

A sample of blood was centrifuged at 1500 g for 15 minutes at room temperature. The plasma was removed and retained; the "buffy coat" was removed and discarded. Removed plasma was added to the packed red blood cells to return the total volume of to that of the original blood sample. An equal mass (0.5g /2ml blood) of α-cellulose and microcrystalline cellulose (sigmacell type 50) was soaked separately in 10ml of PBS and allowed to stand at room temperature for 15 minutes. The celluloses were then mixed and poured into 5ml syringes plugged with tissue

paper. Each column occupied 2ml and was washed with 10ml PBS prior to use. White cell depleted red cells, 2ml per column, were then allowed to percolate into the column and the red cells were washed off the cellulose with up to 5ml PBS. The number of white cells remaining in the eluate was then counted and was less than 1% of the original volume (Kenny *et al*, 1983). The red blood cell suspensions were diluted with PBS to the required red cell count for each experiment.

B. Whole blood suspension

Blood, from vacutainers was pooled and used immediately. Blood suspensions were diluted with PBS to a concentration of $520x10^6$ml red blood cells. Filtration profiles were recorded over 10 seconds through 5μm Nuclepore polycarbonate filters.

4.2.4 Recording of filtration profiles

The Cardiff filtrometer cell (**Figure 4.1**) which consists of two Perspex parts fixed together securely with wing nuts rendering the unit airtight was used. The upper section consists of an inner chamber, of maximum capacity of 5 ml, surrounded by a closed outer section with two connections to serve as a water jacket if required. The inner chamber has two-inlet points- one for the pressure tube and the other for the introduction of test sample. The lower section holds the wire mesh base plate upon which the filter membranes are places, held still and secured by an o-ring. Between each filtration, the cell is disconnected, dismantled and cleaned thoroughly before replacing the filter for next filtration. The filters used in this filtrometer were from the same batch (Whatman, Kent, UK) with 5μm pore size, 10μm thickness and the total number of pores available are 0.26 million.

A photograph of the whole filtration system is shown in **figure 4.2** and a schematic diagram is also represented in **Figure 4.3**. The filtration cell is interfaced to a BBC Master series Computer. In this experimental system, the flow begins when the pressure is switched on and synchronised with the starting of the clock by the computer. The pressure is generated by an air compressor and controlled by a simple "T" tube immersed into the required depth of water- where a pressure of 98 Pa is generated by immersion in 1 cm of water. The filtration cell is loaded with the blood suspension after calibration with pre-filtered buffer, and the filtration commences when the pressure is switched on. A magnetic valve installed in the pressure line effects the switching on of the pressure. The suspension, flowing through the cell, is collected in a tared container resting on a strain gauge, which is interfaced to the microcomputer via an amplifier and monitors the weight at 20ms intervals. The signal from the strain gauge is amplified and stored in a dedicated memory location in the computer. Thus the volume of filtrate is recorded at time intervals and this constitutes a flow profile. Data was stored in a self-maintained micocomputer's memory from which the data can be printed or transferred to floppy disk.

4.2.5 Cell activation and collection of flow profiles

Blood used in these experiments was from healthy volunteers and was analysed under different conditions: normal, activated with PMA or fMLP, pre-incubated with CTB, and then activated with fMLP. The method used for cell activation was as described in sections 3.2.2 3.2.4 and 3.2.8. Whole blood was incubated with PMA (0.05, 2, 5, 50 and 100 nM) at 37°C for 15 minutes and fMLP (0.05, 2, 5 and 100nM) was incubated at 37°C for 5 minutes, to establish the appropriate concentration, which would cause leukocyte activation. In addition,

whole blood was pre-incubated with CTB (10 μM) for 5 minutes at 37°C before stimulation with fMLP. Just before performing flow profiles under these three conditions, 50μl of blood from each sample was fixed, permeabilised and stained for F-actin content of gated PMNs and monocytes using flow cytometry as described earlier.

Flow profiles were recorded at room temperature and 490 Pa pressure over 10 seconds, for both isolated red cell and diluted blood suspensions. These experiments were performed on blood from 7 healthy subjects, to yield between 16 and 26 separate flow profiles. The collected flow profiles for red blood cells and diluted blood were then analysed to calculate the transit times for flowing leukocytes and the concentration of pore blocking cells in diluted blood.

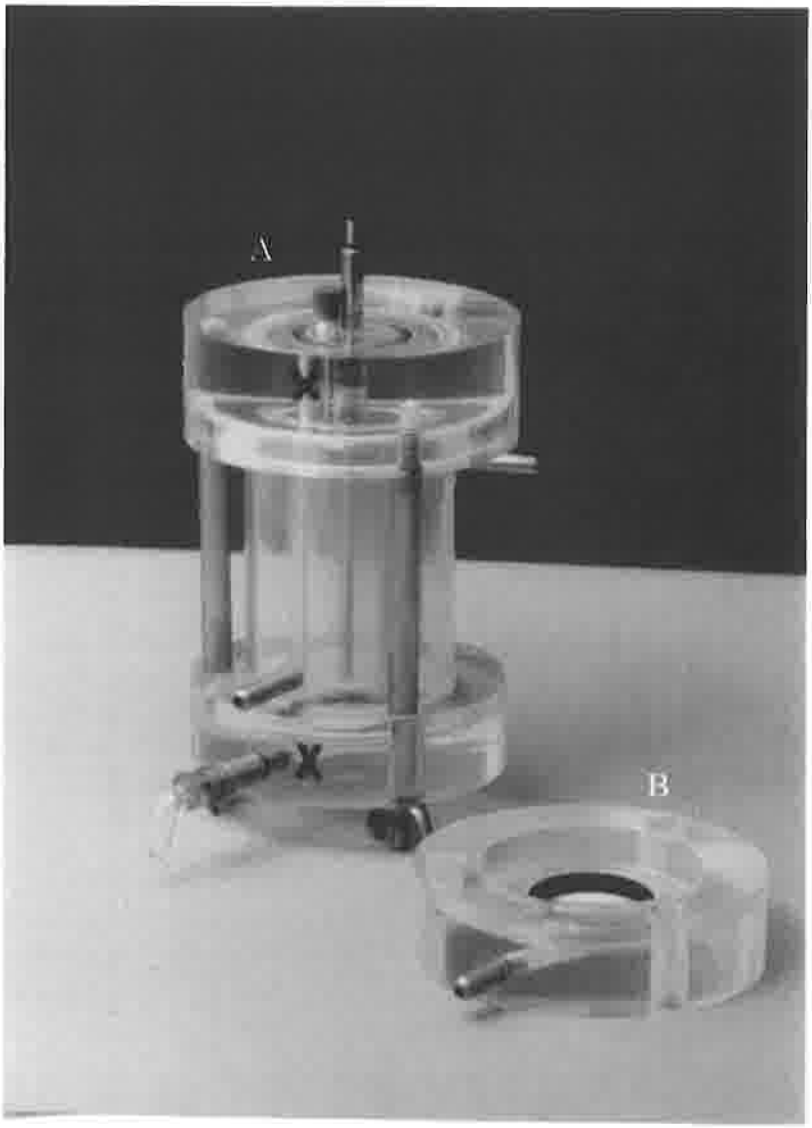


Fig 4.1: Photograph of filtration cell used; (A) represents the complete assembled cell, whilst (B) shows the inner base plate and O ring.

Fig 4.2: The Cardiff Filtrometer

B. Pressure (cm water)

C. Pneumatic valve

D. Strain gauge

E. Amplifier

F. Filtration cell

G. Container which collects eluate

H. Microcomputer

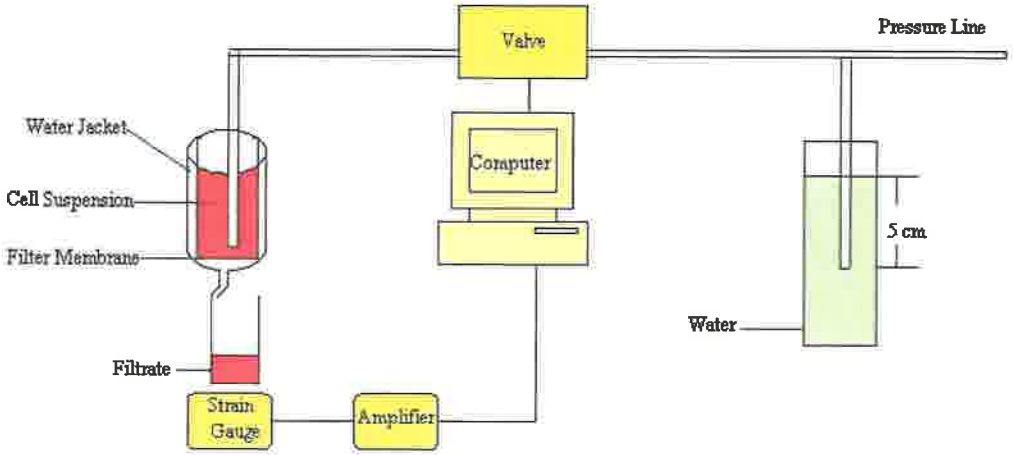


Fig 4.3: A schematic diagram representing the filtration system.

4.2.6 Analysis of flow profiles

Each flow profile was analysed by mathematical models capable of describing the flow profiles of suspensions (purified red blood cells & diluted blood). The analyses of these experimental profiles were achieved by fitting to the most appropriate model (Kooshesh *et al*, 1991; Adams, 1993; Jones *et al*, 1994; Evans 1996). The derivation of the equations is described here:

1. Red blood cell suspensions

The red blood cells are assumed to reach an immediate steady state with membrane pores when the flow rate of suspension should remain constant. The equations describing the rate of occupation of pores by a homogeneous population of cells was given by Jones *et al* (1984, 1985). The predicted steady-state flow rate of suspending medium is given by :-

$$dV/dt = \omega_r Q T \quad \text{.....} \quad (4.1)$$

where:

Q = flow rate of suspending medium through a single pore

T = Total number of pores in the membrane

ω_r = Proportion of pores not occupied by red cells

$$= k_2/(k_1 + k_2) \quad \text{.....} \quad (4.2)$$

where:

k_1 = Proportion of available pores occupied by red cells per second { = [rbc] Q}

k_2 = Proportion of pores, occupied by red cells, evacuated per second { = 1 / Transit Time for a single cell}

[rbc] = concentration of red blood cells

In the presence of pore blocking, the rate of occupation of pores by pore blockers is given by:-

$$dP/dt = k_p [T - P] \quad \text{.....} \quad (4.3)$$

where:

P = the number of pores blocked at time t

k_p = the proportion of available pores being occupied by pore blockers per second

= [pore blockers] Q ω_r

It follows:-

$$P = T (1 - e^{-k_p t}) \quad \text{.....} \quad (4.4)$$

The flow rate of suspending medium is described as:-

$$dV/dt = Q \omega_r [T - P] \quad \text{.....} \quad (4.5)$$

and the flow profile for suspending medium is then described by:-

$$V = \omega_r T Q (1 - e^{-k_p t}) / k_p \quad \text{.....} \quad (4.6)$$

where:

V is volume of suspending medium emerging at time t

The data is fitted to equation 4.6 with ω_r and k_p as unknown parameters and from equation 4.2, the transit time for flowing red blood cells (τ_r) is calculated.

$$\tau_r = (1 - \omega_r) / [rbc]\ Q\ \omega_r \quad \text{.....} \quad (4.7)$$

The concentration of pore blockers in the suspension is calculated from:-

$$\text{Pore Blockers} = k_p / \omega_r Q \quad \text{.....} \quad (4.8)$$

2. Diluted blood

The initial flow rate of diluted blood, relative to that of buffer, was measured exactly as for red blood cell suspensions. The calculated initial relative flow rate ω' is however lower than that for red cells alone. This relative flow rate is compared to the relative flow rate of red blood cell suspensions with red cell counts equal to those of the diluted blood. Flow profiles for diluted blood can be described by equation 4.6 apart from the replacement of ω_r with ω'. The constant ω' is defined by:-

$$\omega' = \omega_r \omega_l \quad \text{.....} \quad (4.9)$$

where ω_l is similar to ω_r but represents the proportion of available pores which are not occupied by "Flowing Leukocytes". It is defined by the appropriate rate constants for entry and exit :-

$$\omega_l = k_4/(k_3+k_4) \quad \text{.....} \quad (4.10)$$

where:

k_3 = Proportion of available pore occupied by Flowing Leukocytes per second

= [Flowing Leukocytes] Q ω_r

k_4 = Proportion of pores occupied by Flowing Leukocytes evacuated per second

= 1/Transit time for Flowing Leukocytes

The data is fitted to the appropriate equation (4.6 with ω' replacing ω_r) as described above. A single transit time for lymphocytes and PMNs is then calculated.

$$\tau_l = (\omega_r - \omega') / (\omega' [\text{Leukocytes}] \omega_r Q) \quad \ldots\ldots\ldots \quad (4.11)$$

where:

[Leukocytes] = the concentration of "Flowing Leukocytes" which equates to PMN + Lymphocytes.

A slower flowing population of blood cells, with a transit time greater than 10 seconds, is recorded as a pore blocker and calculated from equation (4.8) with ω' replacing ω_r.

4.2.7 Statistical methods

Correlation analysis was performed between F-actin content and leukocyte transit time and/ or pore blockers. ANOVA was used to determine the effect of fMLP concentration, with or without CTB, on the pore transit time, the total number of pore blockers, and intracellular F-actin.

4.3 Results

4.3.1 Effect of exposure to PMA

Blood was incubated with various concentrations of PMA (0.05 - 100 nM) at 37°C for 15 minutes. A typical flow profile for blood incubated with 0.05nM PMA and diluted to $520x10^6$rbc/ml is shown in **figure 4.4**. At all concentrations above 0.05 nM, complete pore blocking of the filter occurred almost immediately after the commencement of filtration, and therefore analysis of these flow profiles was not possible. Hence, PMA was not used for any further studies involving leukocyte activation.

4.3.2 Effect of exposure to fMLP

The rheological properties of transit time and number of pore blockers, as well as F-actin content in PMNs and monocytes, before and after fMLP activation, are shown in **Table 4.1**. A typical flow profile for blood incubated with 5nM fMLP and diluted to $520x10^6$rbc/ml is shown in **figure 4.4.** A dose-dependent decrease in diluted blood filterability following fMLP incubation at 37°C for 5 minutes was observed. There was an increase in both leukocyte transit time ($p<0.05$) and the total number of pore blockers ($p<0.001$) in diluted blood as the concentration of fMLP increased (p was determined by ANOVA). There was an increase in F-actin in PMNs ($p<0.001$) and monocytes ($p<0.001$) as the concentration of fMLP increased. The relationship between leukocyte transit time and F-actin content in PMNs is represented graphically in **figure 4.5,** and the r-value for this relationship is 0.217 with $p=0.029$ as determined by correlation analysis. Furthermore, there was a significant relationship between the total number of pore-blockers and F-actin content as the concentration of fMLP increases in both PMNs and monocytes. The r-

value for this relationship is 0.798 with $p<0.001$ for PMNs and 0.763 with $p<0.001$ for monocytes as determined by correlation analysis, and these data are shown in **figure 4.6**.

[fMLP] (nM)	Leukocyte transit time (s)	Pore-Blockers (10^6/ml)	F-actin content (MFI) in PMNs	F-actin content (MFI) in Monocytes
0 (n = 26)	1.33 ± 0.16	0.09 ± 0.01	260 ± 13	445 ± 18
0.05 (n = 20)	1.67 ± 0.22	0.12 ± 0.01	274 ± 13	657 ± 29
2 (n = 16)	1.49 ± 0.21	0.14 ± 0.01	326 ± 18	714 ± 18
5 (n = 16)	2.08 ± 0.3	0.22 ± 0.02	403 ± 31	786 ± 54
100 (n = 21)	2.29 ± 0.27	0.26 ± 0.02	534 ± 39	973 ± 52
p value	< 0.05	< 0.001	< 0.001	< 0.001

Table 4.1: Leukocyte transit time, pore-blockers and F-actin content of gated PMNs and monocytes. All values are means ± SEM. The p values are determined by ANOVA.

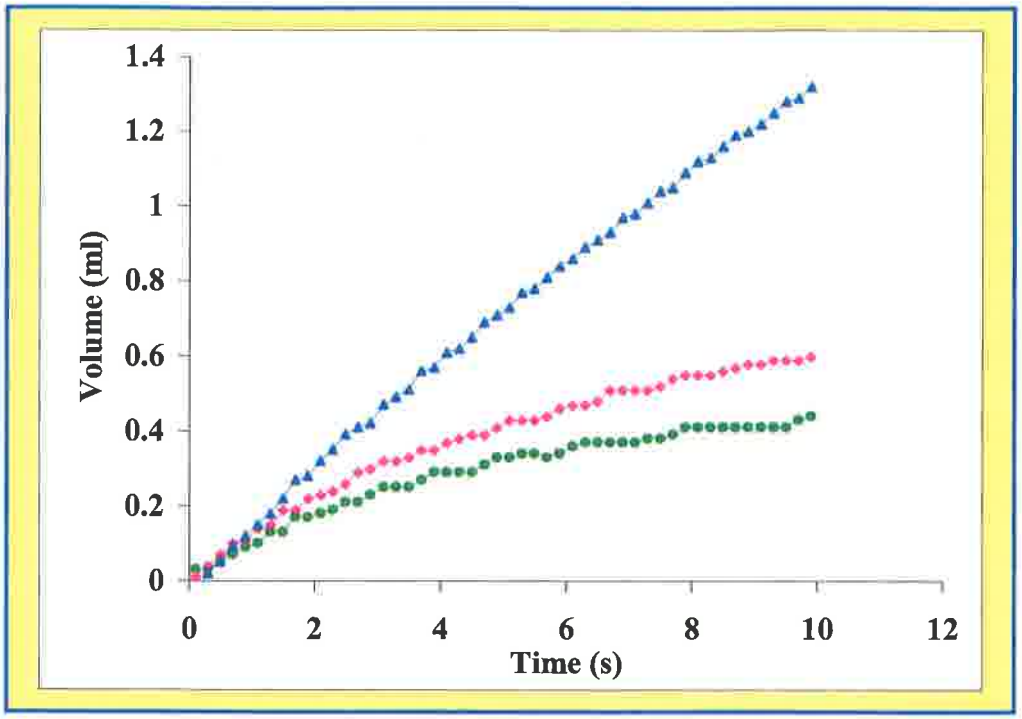


Fig 4.4: Typical flow profiles at $520x10^6$ rbc/ml for un-stimulated blood (blue), blood incubated with 0.05nM PMA (green), 5nM fMLP (pink).

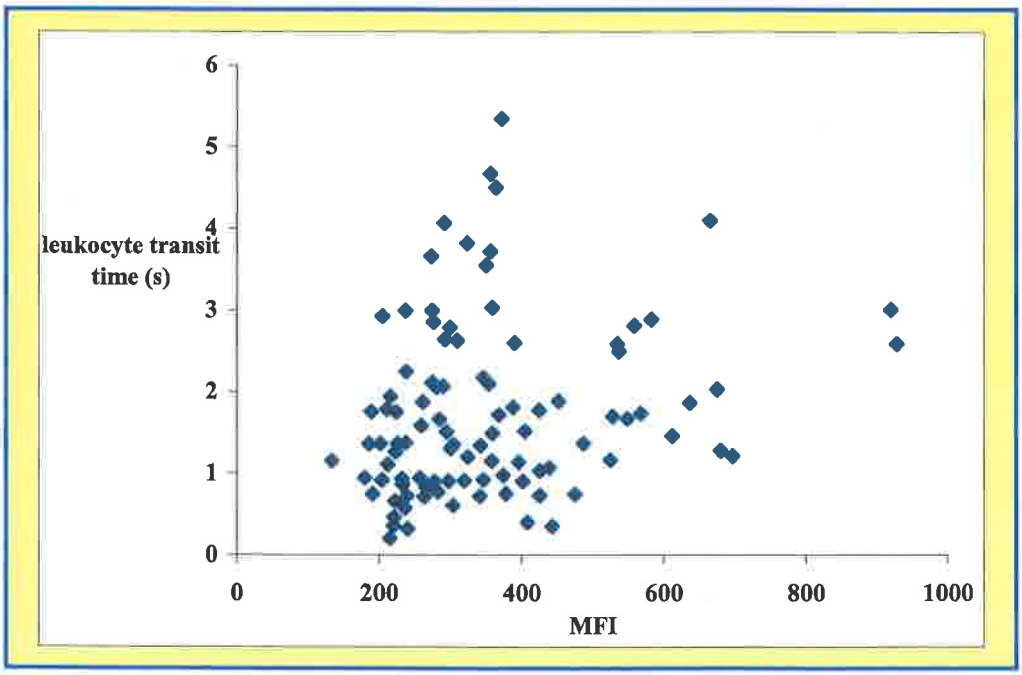
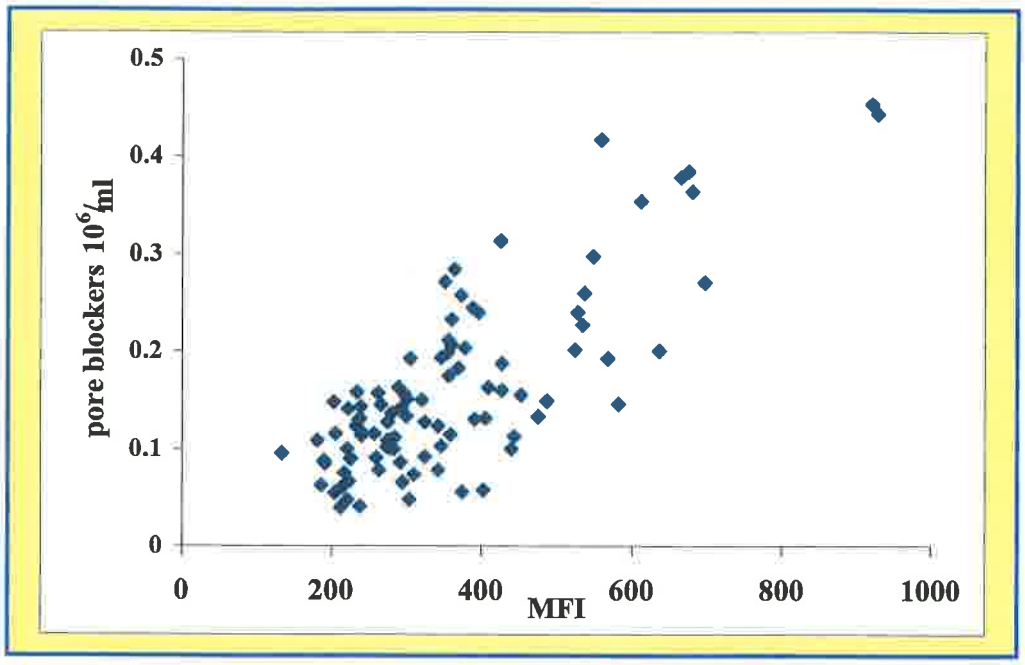
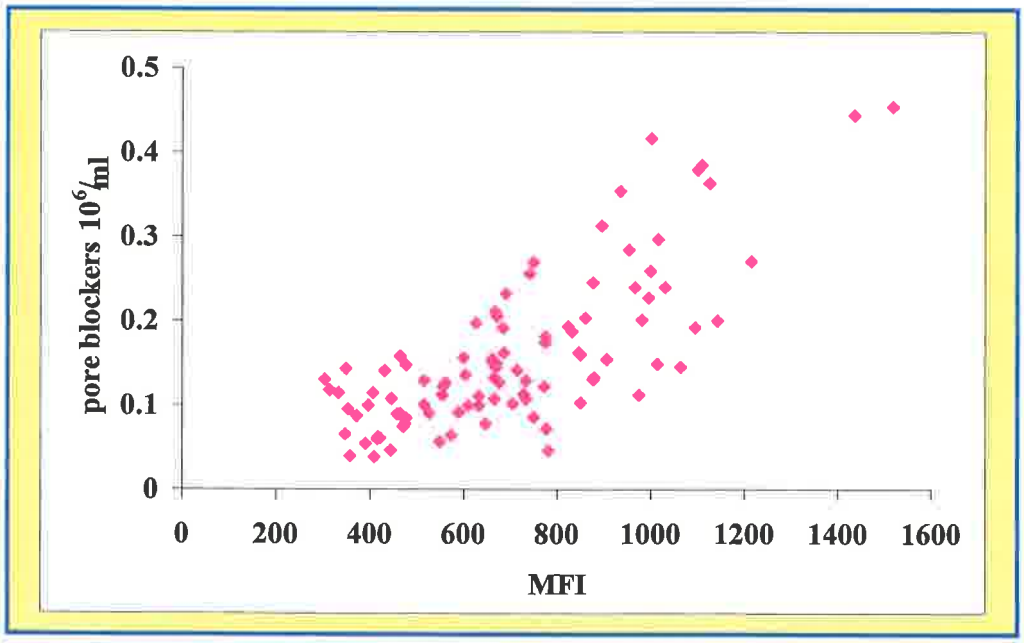


Fig 4.5: The relationship between leukocyte transit time (seconds) and F-actin content of PMNs. Points represent different flow profiles of diluted blood treated with or without various concentrations of fMLP. r=0.217 with p=0.029 as determined by correlation analysis.



(A)



(B)

Fig 4.6: The relationship between pore-blockers and F-actin content of both PMNs (A) and monocytes (B). Each point represent a single flow profile of diluted blood treated with or without various concentrations of fMLP. r =0.798 with p <0.001 for PMNs and r=0.763 with p<0.001 for monocytes as determined by correlation analysis.

4.3.3 Effect of CTB treatment on the flow properties of diluted blood

The initial flow rate of diluted blood relative to buffer was measured in fMLP activated blood and compared with those pre-treated with cytochalasin B (10μM) before fMLP stimulation. The concentrations of fMLP used in these suspensions pre-incubated with CTB were identical to those used for fMLP stimulation alone (0.05, 2, 5, and 100 nM) with incubation time of 5 minutes at 37°C. The leukocyte transit time from filtration of diluted blood was calculated using the appropriate erythrocyte transit time, as described in the methods section to this chapter. The results, presented in **Table 4.2**, confirm an increase in pore transit time with increasing fMLP concentration, and a corresponding increase in the total number of pore blockers detected. These changes were found to be statistically significant with p values of < 0.001 for leukocyte transit time and $p<0.001$ for the total number of pore blockers as determined by ANOVA. The data for cell suspensions pre-incubated with CTB before addition of fMLP are also shown in **table 4.2**. There was no significant change in leukocyte transit time ($p=0.836$) as determined by ANOVA, with the transit time remaining stable at all concentrations of fMLP. There was no significant change in the total number of pore blockers ($p=0.341$) as determined by ANOVA. These data are shown graphically in **figures 4.7 and 4.8**.

FMLP (nM)	0.05	2	5	100	*p value*
A- fMLP only (n=5)					
Leukocyte transit time (s)	2.28 ± 0.18	2.52 ± 0.85	3.34 ± 0.54	3.89 ± 0.56	<0.001
Pore-Blockers (10^6/ml)	0.14 ± 0.01	0.19 ± 0.03	0.21 ± 0.02	0.33 ± 0.03	<0.001
B- fMLP + CTB (n=9)					
Leukocyte transit time (s)	1.72 ± 0.32	1.90 ± 0.35	2.16 ± 0.35	1.88 ± 0.27	P=0.836
Pore-Blockers (10^6/ml)	0.22 ± 0.02	0.22 ± 0.02	0.19 ± 0.02	0.24 ± 0.02	P=0.341

Table 4.2: Leukocyte Transit time and pore-blockers in diluted blood stimulated with varying concentrations of fMLP with or without CTB (10μM). All values represent means ± SEM. The p values were determined by ANOVA.

The effect of CTB on F-actin content was analysed at the same time as filtration, and the data are shown in **table 4.3**. The presence of CTB caused a decrease in the F-actin content, in both PMNs and monocytes. However, although the pre-incubation of blood with CTB before fMLP treatment resulted in lower levels of F-actin than the control suspension, it does not appear to have totally inhibited F-actin formation, as there is a small but progressive increase in MFI seen with respect to fMLP concentration.

[fMLP] nM	PMNs		Monocytes	
	fMLP	CTB + fMLP	fMLP	CTB + fMLP
0.05	388 ± 78	317 ± 56	860 ± 183	456±73
2	457 ± 105	386 ± 63	902 ± 188	522 ± 82
5	552 ± 104	409 ± 72	940 ± 230	538 ± 97
100	811 ± 133	469 ± 65	1288 ± 244	677 ± 117
p value	<0.001	0.004	0.015	0.008

Table 4.3: The effect of CTB (10μM) on the F-actin content (MFI) of un-stimulated and stimulated PMNs and monocytes. All values represent means ±SEM. The p values were determined by ANOVA.

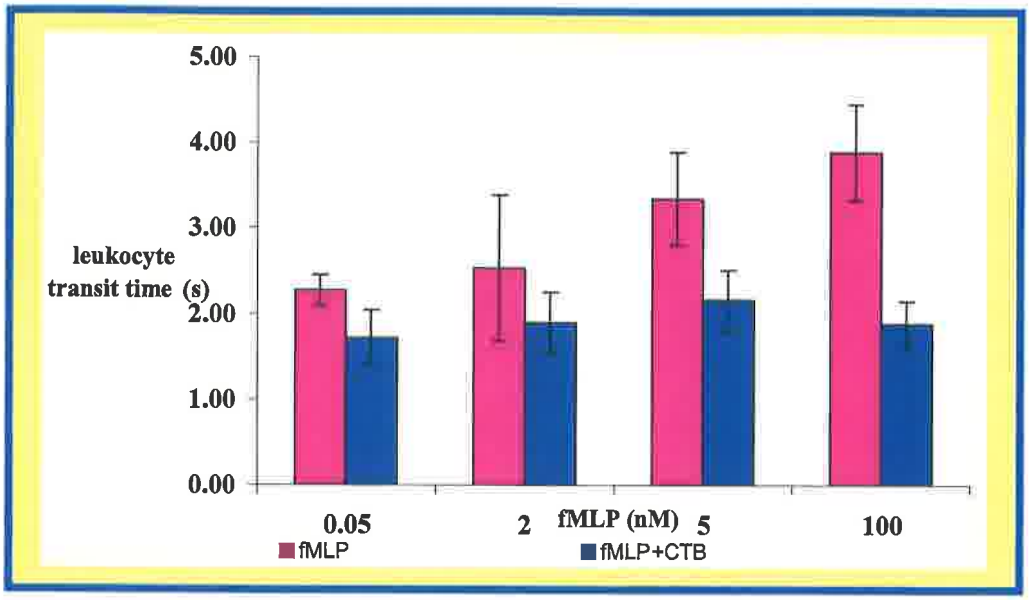


Fig 4.7: The effect of increasing fMLP concentration (nM) on the leukocyte transit time (s) of diluted blood treated with or without CTB (10μM). Points represent Means ± SEM of n = 8. The p value <0.001 (fMLP) and p=0.836 (fMLP+CTB) as determined by ANOVA.

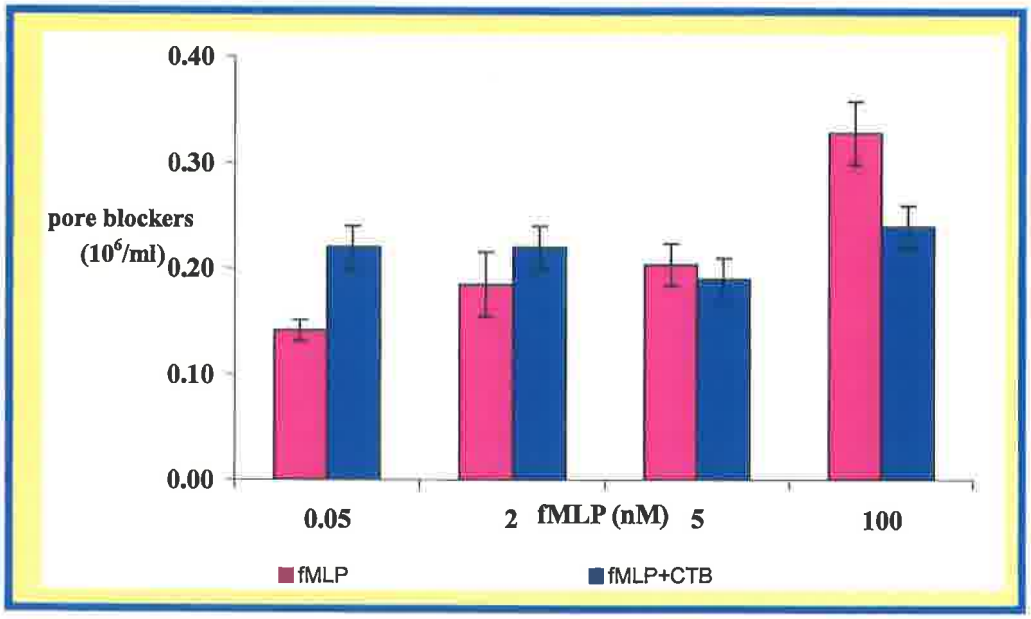


Fig 4.8: The effect of increasing fMLP concentration (nM) on the concentration of pore blockers (10^6/ml) in diluted blood treated with or without CTB (10μM). Points represent Means ± SEM of n = 8. The p value <0.001 (fMLP) and p=0.341 (fMLP+CTB) as determined by ANOVA.

4.4 Discussion

The purpose of this study was to correlate F-actin formation in leukocytes with their filtration properties, as measured in whole blood. Deformability of white cells was assessed by measuring the time required for the cells to completely pass through a single-capillary sized pore (5μm) in a thin polycarbonate membrane. Cells were driven through the pore by 490 Pa (5 cm H_2O). Leukocyte pore transit times were measured in diluted blood, from the initial flow rate and the transit time of erythrocytes filtered at the same concentration. Cells not completing their passage through the pore were recorded as pore-blockers

During the initial experiments, to assess the effect of leukocyte activation on cell deformability, PMA was used. At concentrations of PMA between 2-100nM and incubation time of 15 minutes at 37°C, complete pore blocking of the filter occurred, hence, because of the inability of the cells to transit the pore, it was not possible to quantify this response. To facilitate data collection, a lower concentration was used and at 0.05 nM PMA, cells were able to pass through the filter pores. However this concentration was too low to promote adequate actin polymerisation, which can be detected by flow cytometry. To overcome this problem, increasing the pressure or increasing the filtration time may be beneficial, but using PMA is more time consuming than fMLP due to the necessary longer incubation time, which makes it difficult to perform all filtrations within four hours of venepuncture. Hence, fMLP was used here, to measure the mechanical behaviour of fMLP-activated leukocytes, as it has been used successfully in previous studies using a variety of techniques.

Initial experiments evaluated changes in leukocyte deformability in diluted blood in the normal state and in response to stimulation with the chemotactic compound fMLP. Whole blood was stimulated with fMLP for an optimum time of 5 minutes at 37°C; during this time cells are activated, but it avoids the problems of maximal activation which occurs within 2 minutes, and of not exhibiting a detectable response to fMLP as studied by Frank (1990) and described in chapter three. The leukocyte pore transit time increased with increasing concentrations of fMLP, from 1.33±0.16 seconds in the controls to 2.29±0.27 seconds at 100 nM fMLP with $p<0.05$ as determined by ANOVA, and there was a corresponding increase in the total number of pore blockers from 0.09±0.01 to 0.26±0.02 with $p<0.001$ as determined by ANOVA. At high concentrations of fMLP, the number of pore blocking particles is close to the total number of PMNs and monocytes in the suspension. The calculated transit time is likely to be a global transit time for lymphocytes and some faster flowing PMNs, which are able to flow through the filter in less than 10 seconds and are, therefore, not recorded as pore blockers. If lymphocyte rheology remains normal, it is possible that the recorded changes in transit time may be an underestimate of the actual changes in transit time of the PMN sub-populations.

These studies have confirmed a dose-dependent increase in the rigidity of fMLP-activated cells, as found by others. Worthen *et al* (1989) measured cell stiffness in response to fMLP and noted a seven-fold increase in the average stiffness of cells. Kawaoka *et al* (1981) measured the minimum pressure necessary for aspiration of the neutrophil into the micropipette after stimulation with fMLP. Buttrum *et al* (1994) studied the rheological responses of the neutrophil to different types of stimulation and showed that incubation of human neutrophils with fMLP

resulted not only in decreased neutrophil deformability, but also in increased neutrophil size, and progressive bipolar-shaped forms.

The role of F-actin in the mechanical changes, in response to fMLP stimulation, was evaluated by simultaneously measuring deformability changes and F-actin content in leukocytes. F-actin content increased in leukocytes stimulated with fMLP at 37°C for 5 minutes, and this was true for both PMNs ($p<0.001$) and monocytes ($p<0.001$) as determined by ANOVA. Stimulation with fMLP, at 37°C, produces a rapid rigidification of cells within 5 minutes of exposure. At low concentrations of fMLP (0.05 nM), increases in F-actin content of 5% and 47% for PMNs (from 260±13 in controls to 274±13) and monocytes (from 445±18 in controls to 657±29), were measured. At higher concentrations of fMLP, the F-actin content of both PMNs and monocytes had increased to over 100% of the control value (from 260±13 to 534±39 (PMNs) and from 445±18 to 973±52 (monocytes)). Increases in F-actin content in neutrophils of 33% (Wallace *et al*, 1984) and 50% (Frank, 1990) were measured by others, at a concentration of 0.1 nM and within 2 minutes, although these measurements were made sooner after activation, and cells activated with fMLP peak in actin polymerisation within 1 minute as observed previously (**Fig 3.8**).

Studying the rheological properties of leukocyte incubated with various concentrations of fMLP at 37°C for 5 minutes, revealed a relationship ($r=0.217$ with $p=0.029$) between leukocyte transit time and F-actin content as determined by correlation analysis. Previous results (Kooshesh *et al*, 1991) have indicated that monocytes are less filterable than other leukocytes and were recorded as pore-blockers. Results obtained from experiments performed in this chapter agree with

these findings, and a good correlation was observed between the F-actin content of monocytes and the total number of pore blockers ($r=0.763$ with $p<0.001$) as determined by correlation analysis. This increase in the total number of pore blockers in monocyte, suggests the presence of a fast flowing monocytes that become more rigid and unable to flow within 10 seconds, and therefore were determined as pore blockers. Another significant correlation was also observed between the F-actin content of PMNs and the total number of pore blockers ($r=0.798$ with $p<0.001$) as determined by correlation analysis. These correlations would indicate that many PMNs have become too rigid to flow through the pores after fMLP treatment, and are therefore detected as pore blockers. Various studies by others using different filtration techniques have revealed that changes in cell rigidification are caused by the formation of a gel-like actin network (Schmid-Schonbein *et al*, 1981; Pollard, 1986; Worthen *et al*, 1989; Frank, 1990). This gelation formation can occur with just small changes in F-actin content, thus allowing large changes in cellular deformability in response to changes in F-actin in some of the cells. Previous work has shown that the retention of cells in filters is primarily due to changes in the state of actin assembly and organisation (Smith *et al*, 1984; Worthen *et al*, 1989); Worthen *et al* (1989) studied the effect of fMLP activation on neutrophil transit time and reported that the decreased ability of these activated cells to transit pores in polycarbonate membranes is not as a result of increased adhesion of the stimulated cells to the membranes.

Hence, to further investigate the involvement of F-actin polymerisation on leukocyte filterability in this system, cells were treated with CTB (10μM), which, as described previously, disrupts the formation of cellular F-actin. Experiments performed to study the effect of CTB on deformability and F-actin content included

three groups, i.e., normal cells, cells treated with fMLP (0.05, 2, 5, 100 nM) and cells pre-incubated with CTB (10 μM) and then stimulated with fMLP at 37°C for 5 minutes. Pre-incubation of cells with CTB before fMLP stimulation substantially eliminates the deformability changes associated with the fMLP chemotactic response. This was true for the leukocyte transit time from diluted blood at all fMLP concentrations, which decreased when cells were pre-incubated with CTB. This observation clearly confirms that F-actin content is the major determinant of changes in leukocyte transit time. Although the concentration of pore blockers is slightly increased in the presence of CTB, this concentration remained stable at all fMLP concentrations. Pecsvarady *et al* (1992) and Buttrum *et al* (1994) studied the effect of cytochalasin B on the rigidity of activated neutrophils by filtration and micropipette studies and found that CTB inhibits the marked rigidification that follows activation. Tsai *et al* (1994) reported that changes in F-actin structure most probably account for the dramatic reduction in cytoplasmic viscosity and cortical tension. However, it is still unknown whether the decrease of F-actin content is the primary mechanism for these changes in neutrophil cytoplasmic viscosity. The interpretation of their results was partly based on the finding of Wallace *et al* (1987) that CTB reduces F-actin content. Such a reduction in cytoplasmic viscosity may also have an effect on the leukocyte pore transit time, and on the concentration of pore-blockers.

In conclusion, these studies confirm previous observations by other workers that leukocyte deformability is dependent, in part, on the structure and integrity of the actin cytoskeleton. However, these studies, in contrast to other workers who always isolate leukocytes, were performed in diluted whole blood, and this is true for both filtration and flow cytometric measurements. This has the advantage that prior isolation of leukocytes, which may be detrimental to their structure and function, is

not necessary, and this is obviously an advantage when studying aspects of leukocyte activation. In addition, measuring leukocyte rheology in blood or diluted blood is also more physiologically appropriate, in being more like the *in vivo* circulation.

Chapter Five

Chapter Five: Haemorheological effects of particulate air pollution

5.1 Introduction

In epidemiological studies in humans, increased exposure to airborne particulate matter with an aerodynamic diameter of less than 10 microns (PM10) has been implicated in increasing asthma symptoms, cardiovascular malfunction, hospital admissions and morbidity and mortality rates (Anderson *et al*, 1991; Schwartz, 1994; Schwartz, 1999; Peters *et al*, 1997; Seaton *et al*, 1999). Although different reasons have been postulated to link inflammatory changes induced by particle inhalation to such cardiovascular alterations, the exact biological causes of the increased morbidity and mortality are unclear. It is thought that the correlation between air pollutants and mortality is likely to be due to individuals who have pre-existing heart or lung conditions, but the causative mechanisms of the final cardiovascular insult are, as yet, uncertain.

Although the inflammatory and permeability changes in the lung after dust exposure have been studied previously, the mechanisms of how these are manifested systemically in the blood are not yet fully understood, despite investigations in this area (Peters 1997, Seaton 1999). Cigarette smoke is known to be detrimental to leukocyte rheology, with neutrophil exposure to cigarette smoke causing a marked distortion of cell shape with bleb formation, and decreased deformability (Buttrum *et al* 1994). Also, chronic smokers have decreased blood filterability and increased levels of plasma elastase (Drost *et al* 1993).

Research has shown that equivalent masses of small particles are more inflammatory to the lung than larger sized particles of similar composition (Osier *et al*, 1997) and studies in combustion emission particulates have provided direct

evidence that the soluble transition metal component promotes lung injury (Dreher *et al*, 1997; Kodavanti *et al*, 1998; Lambert *et al*, 2000). Particulate air pollution that has 50% of organic and inorganic particles with an aerodynamic diameter of $\leq 10\mu m$ is known as PM10 (Pope & Dockery, 1999). PM10 consists of a heterogeneous mixture of particles that include minerals, metal oxides, sea salt, biological components and soot. In urban locations the soot element, especially ultra fine diesel exhaust particles (DEP), accounts for 20-80 % of airborne PM10 arising from vehicular activities (DoE, 1995). Cabosil, an amorphous silican dioxide that forms individual ultra fine particles, has also been used for studies of lung inflammation.

The inflammatory and permeability changes following instillation of small masses of DEP (used as a surrogate for PM10) into rat lung have been well documented (Murphy *et al*, 1998; Reynolds *et al*, 2001), and the overall impression is that DEP has a low bio-reactivity in the lung of a healthy rat. Therefore, in order to further investigate the epidemiological evidence linking air pollution and cardiovascular malfunction, perhaps the model for delivery of particulates should not be a healthy lung, but instead a 'compromised' system. Thus, through compromisation, the lung represents a sensitive individual who may prove to be more susceptible to the PM10 pollution episodes, and therefore to DEP intratracheal instillation.

The work described in this chapter was part of a collaborative approach to investigate toxicological, biochemical and haematological changes after exposure to particulate air pollution. This work was carried out in conjunction with the Lung and Particle Research group at Cardiff University, on a well-defined animal model.

5.1.1 Aims

The goal of the studies described here is to test the hypothesis that exposure to airborne particulate pollution causes cardiovascular malfunction by alteration of rheological parameters.

To this end, initial investigations will involve F-actin measurement of the phagocytic cells (PMNs and monocytes) in rats, to determine if dust inhalation causes cellular activation and hence alterations in F-actin content. Further studies will then be performed, with the following aims. Firstly, to compromise the lung in order to generate a model system which broadly approximates to sensitive individuals, with some impairment of lung function. This will be done using bleomycin, an antineoplastic antibiotic, which has previously been shown to produce changes in lung inflammation and permeability followed by epithelial repair. Two different model systems will then be generated, in order to investigate the changes in compromised lungs at different stages of damage and repair. In **model 1** (dust instilled 3 days post-bleomycin) the lung is at the peak of oedematous response, whilst in **model 2** (dust instilled 7 days post-bleomycin), the lung is at the peak of epithelial repair. The second aim of this study is to use these compromised lung models to study the bioreactivity and circulatory haematological changes induced by instillate deliveries of DEP and Cabosil particles on the lung. This will also enable the investigation of any association between changes occurring at the lung surface with effects on the circulatory system. To this end, various haematological and haemorheological measurements were made. Full blood counts were performed to investigate any quantitative changes in erythrocyte and leukocyte populations. Plasma viscosity was also measured; this is known to be a sensitive and useful

measurement of acute-phase protein changes, which can occur as a result of infection or inflammation.

5.2 Materials and Methods

5.2.1 Collection and preparation of PM10

Airborne dust was collected within and proximal to Park Slip West opencast coal mine using mobile Negretti PM10 heads and polycarbonate Millipore collection filters (Jones *et al*, 2002) in a co-operation with the operators, Celtic Energy. Milligram quantities were collected for a thorough and detailed characterisation of the particle present.

5.2.2 Collection and preparation of diesel exhaust particles (DEP) and Cabosil

DEP was collected under dry, outdoor conditions from a 1985 Japanese ISE41, 1500cc tractor burning Esso City Low Sulphur diesel fuel and 20/30 light engine oil mixture. It was operated at 10mph and 2000 rpms and collections were made with a Negretti L30, high volume air sampler operating at a flow rate of 30L/min attached to a Total Suspended Particulate selective-inlet head. The sampler head was placed directly behind the engine exhaust pipe and collection was made on preweighed Millipore DA (55mm) filters with a pore size of 0.67μm. A known weight of DEP was resuspended in 0.15M saline and 'wetted' (suspended in solution) by 15 min sonication (BeruBe *et al* 1999). Preweighed samples of Cabosil (Aerosil 380) (British Drug Houses, UK) were resuspended in 0.15 M saline and 'wetted' (suspended in solution) by 15 min sonication.

5.2.3 Animals and experimental protocol

Male Sprague Dawley rats (200 g) were purchased from Charles River (UK) and were acclimatised within the animal holding facility for one week prior to

instillation. The animals were kept on wire-bottom cages with pelleted food and tap water *ad libitum.*

5.2.4 Blood samples and cell counting

Blood was collected via cardiac puncture into tri-potassium EDTA (15%). Cell counts were perfomed on a Serono-Baker System 9000 automated cell counter (Bio-Stat Diagnostic Systems, Stockport, Cheshire, UK), which is calibrated to measure veterinary samples such as rat. A full blood count was performed, which included red cell indices, leukocyte numbers, including a three-part leukocyte differential count (granulocytes, lymphocytes and monocytes) and platelets (PLT).

5.2.5 F-actin measurement

Materials and methods used for F-actin measurement by flow cytometric analysis are fully described in chapter 2. In addition to this previously used method, which uses FITC-phalloidin to label F-actin (FL1), two other labelling systems were used to ensure that the 'gated' cells were PMNs and monocytes. Firstly, R-PE (R-phycoerythrin) anti-rat Granulocyte monoclonal antibody was used in fluorescence channel 2, FL2, an antibody that reacts with rat peripheral blood granulocytes (0.4μg/L), with R-PE monoclonal immunoglobulin isotype standard (0.4μg/Ll) as a control (Becton Dickinson, San Jose, CA, USA). Secondly, biotin anti-rat Mononuclear phagocyte was used (1.4μg/L) with biotin mouse IgG_1 monoclonal immunoglobulin isotype standard as a control (Becton Dickinson, San Jose, CA, USA). Cocktails containing three labels at the appropriate concentration were added to the whole blood after fixation and along with the permeabilsation step (CPK). The biotin labelling method also required PerCP (streptavidin-peridinin chlorophyll-a protein (0.1μg/L)) (FL3), a second-step reagent for the indirect staining of cells in

combination with the biotinylated primary antibodies described above (Becton Dickinson, San Jose, CA, USA). The PerCP fluorochrome was added after performing the last wash and was incubated for a further 30 minutes in the dark. After incubation another wash step was performed and cells were re-suspended with 0.5ml PBS before flow cytometric analysis. Two sets of Isotype controls were prepared to contain FITC and PE and FITC and Biotin. In addition, a suspension of unstained cells was run through the flow cytometer.

Since more than one label was used for Flow Cytometry, compensation was performed between FL1 & FL2 and between FL2 & FL3 to avoid fluorochrome overlap by which some of the fluorescence from one fluorochrome passes to the other detector intended to measure the fluorescence from the other and vice versa. The more common fluorescent dyes used in flow cytometry used in flow cytometry have overlapping emission spectra. Emission filters in the flow cytometry minimise the overlap of these signals but do not completely eliminate them. FACSComp determines the amount of compensation needed for this remaining spectral overlap so that the unwanted signal can be electronically subtracted from the signal of interest. For example, the maximum emission of FITC is 515nm and PE is 580nm, but because the two fluorochromes emit over a range of wavelengths, their emission spectra overlap (**Figure 5.1**). FACSComp adjusts the electronic circuitry of the cytometer to remove the overlapping PE signal from the FL1 detector (FL1-%FL2) and the overlapping FITC signal from the FL2 detector (FL2-%FL1). FACSComp removes the overlapping PE signal from the FL3 detector (FL3-%FL2), for three-colour set up. Because PerCP does not overlap into the FL2 detector, FL2-%FL3 compensation is not necessary.

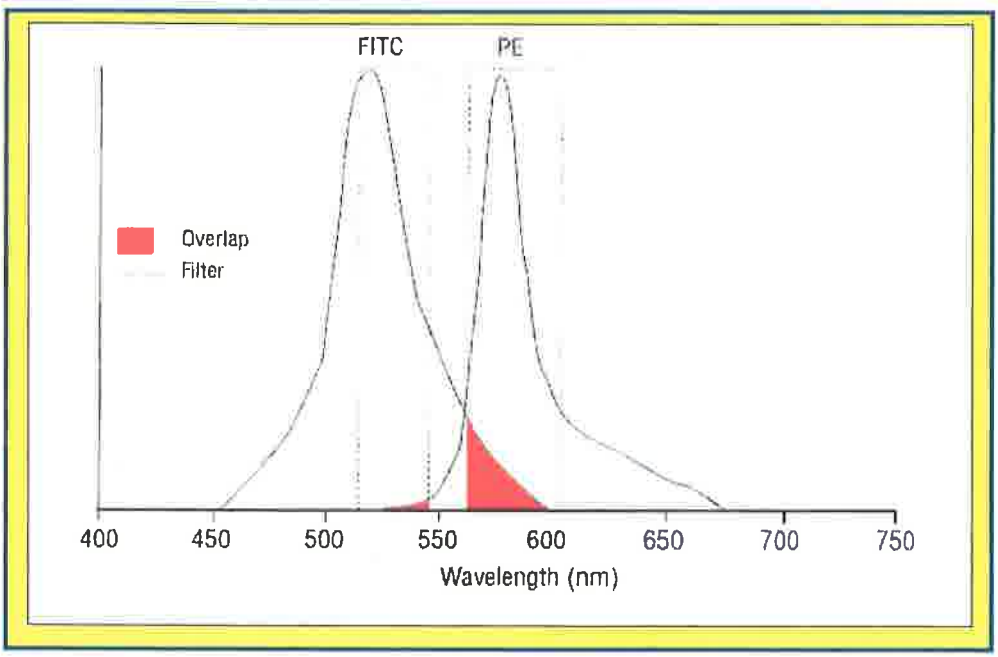


Fig 5.1: Emission spectra of FITC and PE.

5.2.5.1 Animals used for F- actin measurement

Two groups of animals were used. The animals were lightly anaesthetised with Halothane before receiving intratrachael instillations. Controls were instilled with saline only (n=5), whilst the others were instilled with PM10 (2.5 - 10mg, n=10). Animals were sacrificed 3 days and 6 weeks after dust instillation. Blood samples were collected as described above and fixed immediately for actin content measurements

5.2.6.Compromisation of lung function & dust instillation

Compromisation of lung function was produced by the intratracheal instillation of 0.5 unit bleomycin (Kyowa Hakko Ltd., Slough) in 0.5 ml of 0.15 M

saline at time zero. A set of control animals accompanied each group that were administered bleomycin and these animals received saline only. To check for adequate compromisation, two groups of animals (bleomycin and sham treated controls) were sacrificed at 3 days post-bleomycin instillation (day 3).

These two groups of animals (compromised and controls) were then treated with DEP or Cabosil dusts, 3 days post-bleomycin instillation (Model 1) or 7 days post-bleomycin instillation (Model 2). The animals were lightly anaesthetised with Halothane before receiving a single intratracheal instillation of 1 mg DEP or 1 mg Cabosil, suspended in 0.5 ml of 0.15 M saline. Controls received saline only. There were five animals in each treatment group, and they were sacrificed at one-week post-dust instillation (referred to from here on as day 10 and day 14). Blood samples were collected as described above.

5.2.7 Plasma viscosity

Blood samples were centrifuged at 1400g for 10 minutes, and the plasma was aspirated. Samples were stored immediately at -80^{0}c, and were assayed on a Coulter viscometer II (Coulter Electronics Ltd, Luton, Beds, UK) in one batch in order to avoid any assay drift.

5.2.8 Conventional toxicological analyses

Conventional toxicological analyses were performed by the Lung and Particle Research group as described previously (Murphy *et al*, 1998). These measurements included lung surface protein content of lung lavage (Bradford, 1976; Reynolds & Richards, 2001), lung:body weight ratio, free cells in the lungs and surfactant levels.

5.2.9 Statistical analysis

All data manipulation and statistical analysis was performed with Minitab 12 software. Correlations were performed to establish if there are any relationships between lung surface and systemic markers, and these correlations used 'pooled' samples of all the data of each animal used in the compromisation & dust instillation study (n=80).

5.3 Results

5.3.1 F-actin content in PMNs and monocytes after PM10 treatment

In this experiment, three different fluorescent labels were used in order to enable the correct positioning of the gates for rat PMNs and monocyte analysis by flow cytometry. Unfortunately, it was not possible to achieve this, as there was too much fluorescence overlap between the labels, even despite the compensation procedure employed. Hence, adequate flow cytometric dot plots could not be obtained. Due to time constraints, estimated gates were adjusted so that the percentage of cells were identical to that identified using a Serono-Baker System 9000 automated cell counter as shown in **figure 5.2**.

The F-actin content of PMNs and monocytes 3 days after PM10 exposure compared with control is shown **in figure 5.3**. Flow cytometric results showed MFI values of 366±74 in control PMNs compared with 399±13 in those exposed to PM10 *in vivo,* with no statistical difference detected between them (p=0.69) as determined by two-sample t-test. For monocytes, the MFI increased from 453±76 in controls to 606±36 in the PM10 group, although this increase was not statistically significant (p=0.14) as determined by two-sample t-test. The same measurements were also made 6 weeks after PM10 exposure (**Figure 5.4**). Again, the MFI, hence F-actin content, was unchanged in the PMN population, being 324±48 in controls and 343±34 in the PM10 group (p=0.76) as determined by two-sample t-test. However, the MFI of the monocyte population increased from 362±41 in controls to 484±36 in the PM10 treated animals, and this increase is significant (p=0.05) as determined by two-sample t-test.

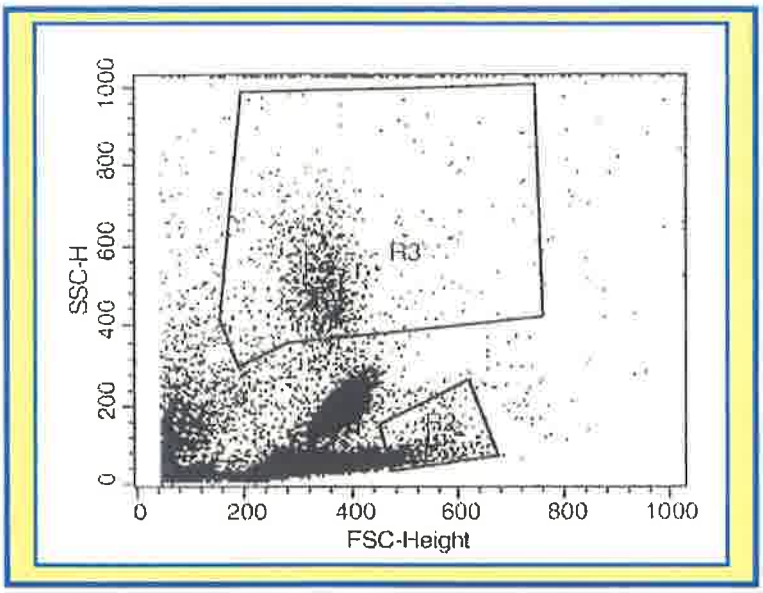


Fig 5.2: Estimated gates of PMNs (R3) and monocytes (R2).

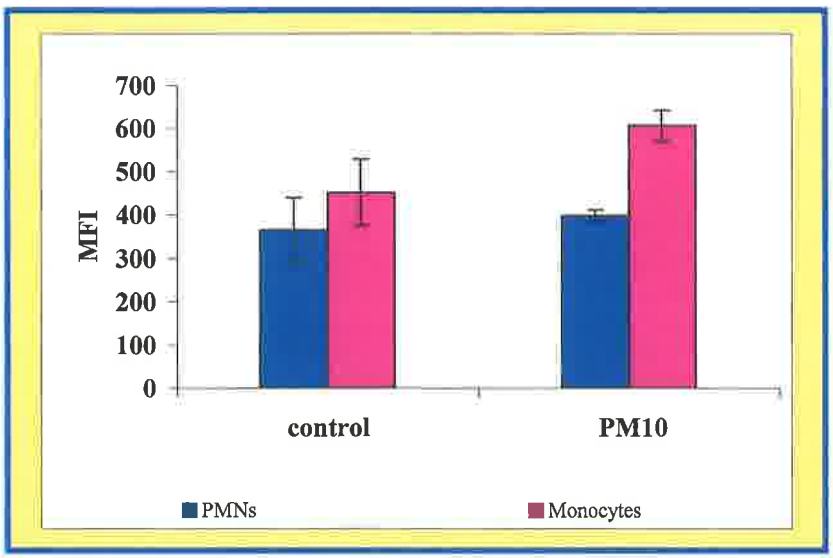


Fig 5.3: MFI (F-actin content) of PMNs and monocytes 3 days after PM10 instillation. Data represent mean ± SEM (n=4 for controls, n=9 for PM10). p = 0.69 in PMNs and p=0.14 in monocytes as determind by two-sample t-test.

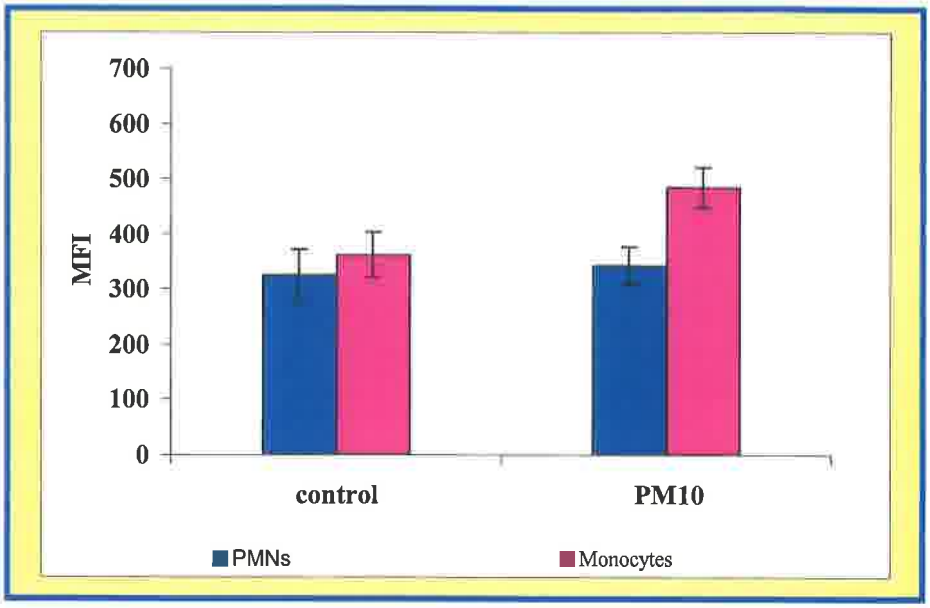


Fig 5.4: MFI (F-actin content) of PMNs and monocytes six weeks after PM10 instillation. Data represent mean ± SEM (n=5 for controls and n=10 for PM10). p=0.76 in PMNs and p=0.05 in monocytes as determined by two-sample t-test.

The above data are shown graphically together in **figure 5.5**, with the control groups for 3 days and 6 weeks after PM10 treatment having been pooled together. It can be seen that whilst there were no measureable changes in the F-actin content of the PMNs (p=0.369), the monocyte F-actin content, which was higher 3 days after PM10 treatment, had returned to close to control values after 6 weeks (p=0.004) as determined by ANOVA.

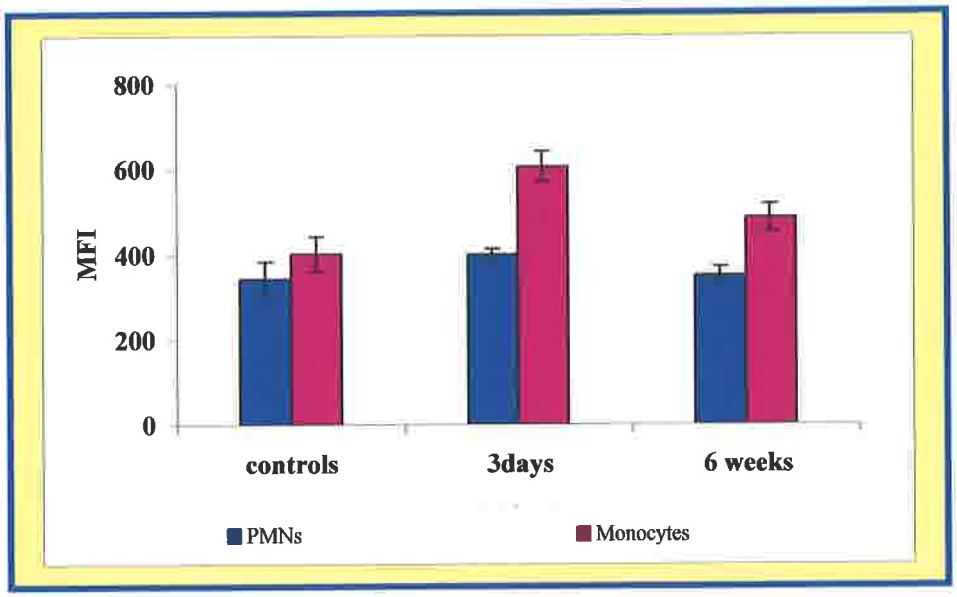


Fig 5.5: Summary of MFI (F-actin content) in PMNs (p= 0.369) and monocytes (p=0.004) 3 days and 6 weeks post PM10 treatment. Data represent mean ± SEM (n=9 for controls and 3 days, n=10 for 6 weeks). The p-values were determined by ANOVA.

5.3.2 Compromisation of lung function & dust instillation

5.3.2.1 Compromisation- 3 days post-bleomycin instillation (day 3)

The data for this initial group compromised with bleomycin compared with controls is shown in **tables 5.1 and 5.2**. In the bleomycin-treated group, there is an obvious inflammatory status and oedema within the lung, as shown by the significant increase ($p<0.001$) in acellular protein and free cells (mainly neutrophils, $p=0.02$), as determined by two-sample t-test. Acellular protein and free cells are good indicators of lung permeability. The red cell indices increased significantly ($p=0.023$ for erythrocytes and $p=0.03$ for Hct as determined by two-sample t-test) in the compromised animals (erythrocyte count from 5.72 x 10^{12}/L to 6.80 x 10^{12}/L, haematocrit from 34.8% to 41.3%). The apparent decrease in the blood leukocyte

count does not reach statistical significance ($p=0.28$) as determined by two-sample-test), although it is worth noting that a corresponding increase in free cells (mainly neutrophils) in the lungs was measured. There was an increase in the plasma viscosity from 1.39 ± 0.02 to 1.41 ± 0.02, which did not reach statistical significance and there was no changes were seen in the platelet count.

Type	Leukocytes (10^9/L)	Erythrocytes (10^{12}/L)	Hct (%)	Platelets (10^3/L)	Plasma Viscosity (mPas)	Lymphocytes (10^9/L)	Midrange (10^9/L)	Granulocytes (10^9/L)
Control	6.29 ± 0.56	5.72 ± 0.18	34.8 ± 1.00	1056 ± 64	1.39 ± 0.02	6.10 ± 0.55	0.12 ± 0.02	0.07 ± 0.03
Compromised	**4.98 ± 0.96**	**6.80 ± 0.31**	**41.3 ± 2.00**	**1145 ± 77**	**1.41 ± 0.02**	**4.85 ± 0.94**	**0.09 ± 0.02**	**0.04 ± 0.00**
P value	0.28	0.023	0.03	0.40	Ns	Ns	Ns	Ns

Table 5.1: Haematological changes seen 3 days after compromisation with bleomycin (day 3). All data shown as mean ± SEM (n=5) for each group). The p values were determined using two Sample test.

Note

Midrange: Cells comprises mainly monocytes.

Type	Protein (mg/lung)	Lung/Body weight ratio	Free cells (10^6/lung)	Surf. (mg)
control	2.83 ± 0.318	0.0043 ± 0.0002	4.40 ± 0.41	1.01
Compromised	5.02 ± 0.253	0.0055 ± 0.0004	7.84 ± 1.07	1.53
p-value	<0.001	<0.05	0.02	

Table 5.2: Permeability and inflammatory changes seen 3 days after compromisation with bleomycin (day 3). All data shown as mean ± SEM (n=5) for each group). The p values were determined using two Sample t-test.

Abbreviation: Surf = surfactant.

5.3.2.2 Model 1 (day 10) – dusts instilled at peak of lung oedema

Data from control and bleomycin-compromised lungs are shown in **tables 5.3 and 5.4**, with the main aim being to look at the effects of dust instillation on control (unshaded) compared with compromised (shaded) lungs. It can be seen that although in these measurements made on day 10, compromisation resulted in significant permeability and inflammatory changes within the lung, there were no significant changes seen in any of the haematological measurements. Thus, the haematological changes seen above, at 3 days post-bleomycin, were no longer apparent.

The toxicological data of the control group alone agree with previous studies, which show silicon dioxides, such as cabosil, to be more reactive biologically than DEP (Murphy *et al*, 1998). Compromisation with bleomycin resulted in permeability changes within the lung, as shown by a mean increase in protein (1.10 ± 0.16mg in controls, 3.69 ± 0.50mg in compromised ($p=0.008$)) and a corresponding increase in lung: body weight ratio ($p=0.011$) as determined by two-sample t-test. Dust instillation, of both DEP and cabosil, caused some significant changes in both the control and compromised animals. For DEP, the protein was higher in the compromised group ($p=0.011$), with a corresponding increase in the lung: body weight ratio ($p=0.003$) as determined by two-sample t-test. Surfactant levels were again higher in the compromised animals. The effect of cabosil was also detrimental, with increases seen in lung surface protein ($p=0.041$) and lung: body weight ratio ($p=0.018$) in the compromised group as determined by two-sample t-test. Hence, there is little doubt that the detrimental lung changes seen here due to dust instillation are markedly worse in the compromised animals, and this is true for both DEP and cabosil.

Instillate 2	Leuko-cytes (10^9/L)	Erythro-cytes (10^{12}/L)	Hb (g/dL)	HCT (g/dL)	PLT (10^3/L)	Plasma Viscosity (mPa.s)	Lymphocytes (10^9/L)	Midrange (10^9/L)	Granulocytes (10^9/L)
Saline	6.41 ± 0.38	6.53 ± 0.12	14.0 ± 0.20	39.3 ± 0.5	985 ± 100	1.41 ± 0.01	6.07 ± 0.35	0.22 ± 0.04	0.12 ± 0.05
DEP	6.24 ± 0.91	6.61 ± 0.21	13.8 ± 0.31	39.6 ± 1.0	1013 ± 42	1.40 ± 0.02	6.0 ± 0.87	0.19 ± 0.03	0.05 ± 0.01
Cabosil	7.70 ± 1.00	6.76 ± 0.12	13.6 ± 0.57	39.9 ± 1.2	976 ± 120	1.43 ± 0.01	7.44 ± 0.99	0.20 ± 0.05	0.06 ± 0.02
Saline	6.90 ± 0.55	6.81 ± 0.17	14.0 ± 0.17	39.8 ± 0.7	992 ± 80	1.42 ± 0.02	6.61 ± 0.53	0.23 ± 0.04	0.06 ± 0.02
DEP	8.04 ± 1.10	6.88 ± 0.12	14.2 ± 0.20	40.9 ± 1.0	1030 ± 37	1.43 ± 0.03	7.76 ± 1.03	0.23 ± 0.04	0.05 ± 0.01
Cabosil	7.22 ± 0.66	6.94 ± 0.14	14.3 ± 0.19	41.6 ± 0.9	1048 ± 66	1.43 ± 0.02	6.95 ± 0.64	0.22 ± 0.03	0.05 ± 0.01

Table 5.3: Conventional haematological measurements in control and bleomycin-compromised animals (shaded) in MODEL 1 (day 10, peak of oedematous response). All data shown as mean ± SEM (n=5 for each group).

Instillate 2	Free cells (10^6/lung)	Lung/Body weight ratio	Protein (mg/lung)	Surf. (mg)
Saline	5.69 ± 0.41	0.0037 ± 0.0001	1.10 ± 0.16	1.05
DEP	6.16 ± 0.85	0.0036 ± 0.0001	2.57 ± 0.13	1.76
Cabosil	7.67 ± 0.87	0.0044 ± 0.0008	2.15 ± 0.50	2.20
Saline	7.58 ± 0.80	0.0063 ± 0.0006	3.69 ± 0.50	1.92
DEP	10.67 ± 2.29	0.0053 ± 0.0003	4.55 ± 0.42	2.51
Cabosil	10.09 ± 0.49	0.0066 ± 0.0006	3.65 ± 0.22	3.28

Table 5.4: Toxicological measurements in control and bleomycin-compromised animals (shaded) in MODEL 1 (day 10, peak of oedematous response). All data shown as mean ± SEM (n=5 for each group).

Instillate 2	Leuko-cytes (10^9/L)	Erythro-cytes (10^{12}/L)	Hb (g/dL)	HCT (g/dL)	PLT (10^3/L)	Plasma Viscosity (mPa.s)	Lymphocytes (10^9/L)	Midrange (10^9/L)	Granulocytes (10^9/L)
Saline	8.08 ± 0.47	6.87 ± 0.23	14.2 ± 0.31	39.9 ± 0.8	1016 ± 20	1.41 ± 0.01	7.87 ± 0.47	0.16 ± 0.01	0.05 ± 0.01
DEP	6.60 ± 0.21	7.07 ± 0.13	14.6 ± 0.04	40.8 ± 0.6	1100 ± 31	1.43 ± 0.01	6.38 ± 0.20	0.18 ± 0.01	0.04 ± 0.01
Cabosil	*	7.02 ± 0.20	14.5 ± 0.13	41.0 ± 0.6	965 ± 21	1.46 ± 0.03	*	*	*
Saline	7.58 ± 1.3	6.93 ± 0.19	14.1 ± 0.25	40.2 ± 0.9	1048 ± 47	1.42 ± 0.02	7.34 ± 1.28	0.20 ± 0.03	0.04 ± 0.01
DEP	8.12 ± 1.3	6.97 ± 0.17	14.2 ± 0.37	41.8 ± 1.4	1027 ± 26	1.44 ± 0.03	7.87 ± 1.25	0.20 ± 0.05	0.05 ± 0.01
Cabosil	5.77 ± 0.38	7.02 ± 0.07	14.3 ± 0.09	40.5 ± 0.2	1029 ± 61	1.43 ± 0.01	5.58 ± 0.38	0.15 ± 0.01	0.04 ± 0.00

Table 5.5: Haematological measurements in control and bleomycin-compromised animals (shaded) in MODEL 2 (day 14, peak of epithelial repair). All data shown as mean ± SEM (n=5 of each group).
* = Not determined.

Instillate 2	Free cells (10^6/lung)	Lung/Body weight ratio	Protein (mg/lung)	Surf. (mg)
Saline	5.96 ± 0.72	0.0036 ± 0.0001	4.05 ± 0.48	3.05
DEP	7.12 ± 0.48	0.0038 ± 0.0003	1.64 ± 0.40	2.23
Cabosil	9.64 ± 2.03	0.0046 ± 0.0003	5.28 ± 0.64	3.62
Saline	11.26 ± 1.52	0.0060 ± 0.0004	5.69 ± 0.36	3.53
DEP	10.97 ± 2.35	0.0052 ± 0.0004	5.78 ± 0.48	2.92
Cabosil	11.63 ± 0.87	0.0051 ± 0.0003	5.33 ± 0.67	4.53

Table 5.6: Toxicological measurements in control and bleomycin-compromised animals (shaded) in MODEL 2 (day 14, peak of epithelial repair). All data shown as mean ± SEM (n=5 of each group).

5.3.2.3 Model 2 (day 14) – dusts instilled at peak of epithelial repair

In this model (day 14), the epithelial repair process is at its maximum level. All data are shown in **tables 5.5 and 5.6**. Again, there were no measurable changes seen in any of the haematological measurements. Although, there were fluctuations in the plasma viscosity, these changes did not reach statistical significance ($p>0.1$). However, the compromisation with bleomycin is still having an obvious effect on the lungs, as shown by the protein (4.05 ± 0.48mg in controls, 5.69 ± 0.36mg in compromised, $p=0.028$), the lung:body weight ratio (0.0036 ± 0.0001 in controls, 0.0060 ± 0.004 in compromised, $p=0.0037$), and the free cell recruitment (5.96 ± 0.72 in controls, 11.26 ± 1.52 in compromised, $p=0.025$). However, the effects of instillation of DEP and cabosil are less dramatic here, and cabosil instillation did not result in any statistically significant increases of parameters in the compromised group.

5.3.3 Statistical correlations

Correlations were made between the measured parameters of lung permeability and damage, and the systemic haematology of every animal in this study, and some interesting observations were made. Plasma viscosity was shown to correlate significantly with free cell numbers ($r=0.280$ with $p=0.014$, **Fig 5.6**), lung/body weight ratio ($r=0.282$ with $p=0.013$, **Fig 5.7**) and lung acellular protein ($r=0.279$ with $p=0.014$, **Fig 5.8**) as determined by correlation analysis.

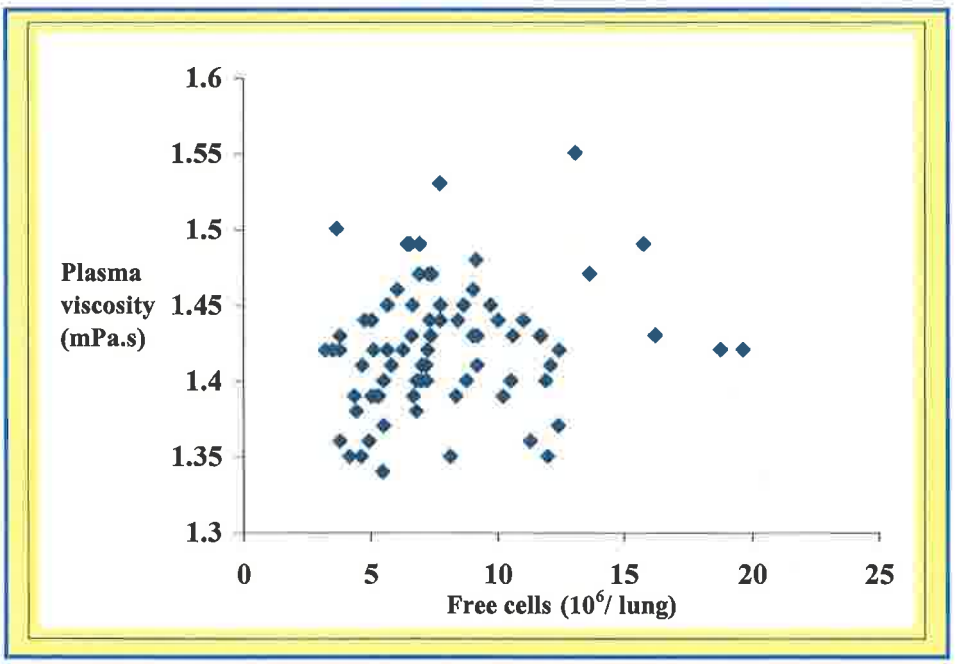


Fig 5.6: The relationship between free cells in the lung and plasma viscosity (r=0.28 with p=0.014 as determined by correlation analysis).

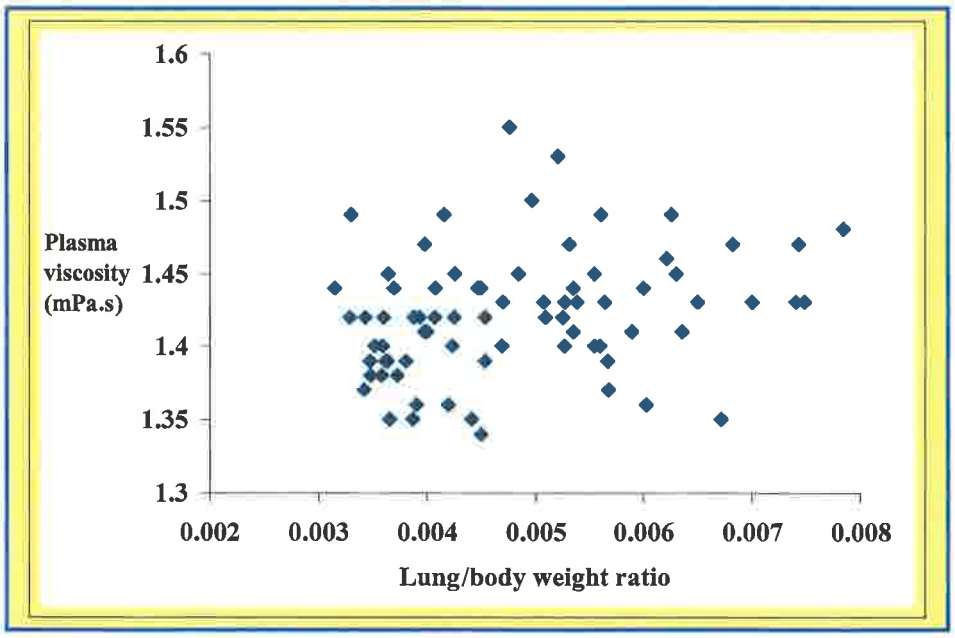


Fig 5.7: The relationship between lung/ body weight ratio and plasma viscosity (r=0.282 with p=0.013 as determined by correlation analysis).

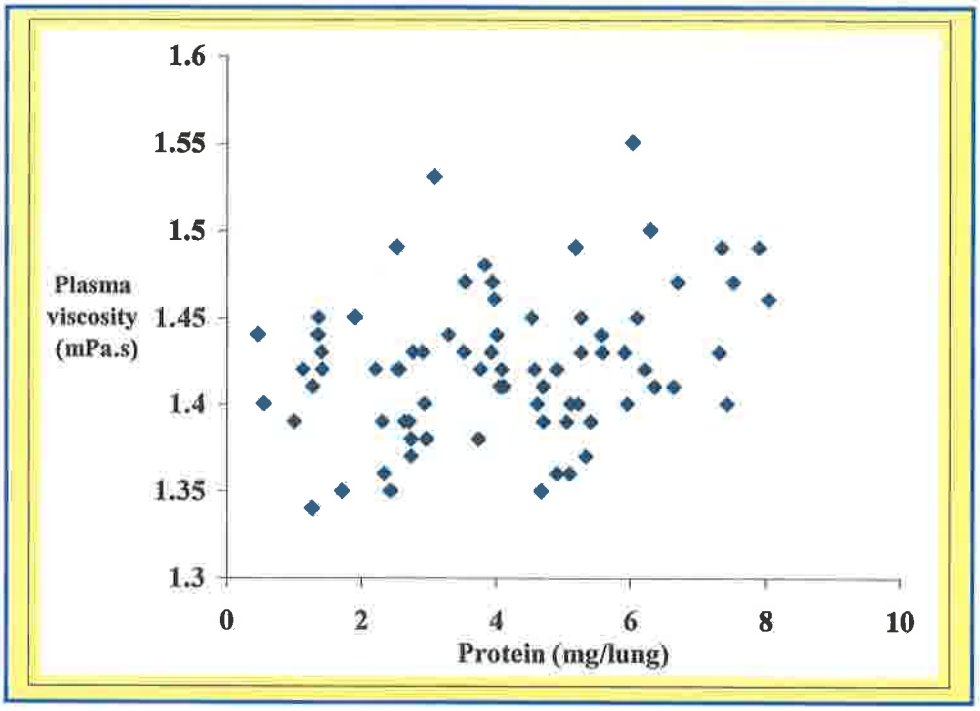


Fig 5.8: The relationship between acellular protein in the lung and plasma viscosity (r=0.279 with p=0.014 as determined by correlation analysis).

5.4 Discussion

Air pollution can be considered as a rheologically detrimental environmental stimulus, akin to cigarette smoking, which is currently known to cause leukocyte activation. Time series analysis of PM10 pollution and mortality suggests that the effect of increasing PM10 concentration by $10\mu g/m^3$ above the minimally accepted values, increases total mortality in the respiratory and cardiovascular daily mortality by 1% and 3.4% respectively (Mukae *et al*, 2001). The aims of this investigation can be divided into three main approaches. Firstly, lungs were instilled with PM10 to study the effect of this instillation on the F-actin content of PMNs and monocytes. Secondly, the lungs were compromised with bleomycin, in order to represent a model of sensitised individuals, such as asthmatics, who may respond differently to the pulmonary challenge of dust inhalation. Thirdly, these models were used to examine the effects of DEP and Cabosil instillation on both the lung surface and the circulatory system in a compromised lung system as compared to a control model, and to establish if there were any links apparent between the lung surface and the systemic circulation. The findings are now discussed in this order below.

In the experiments performed here, to measure the effect of inflammatory stimuli such as PM10, F-actin content was measured in rats using estimated gates of PMNs and monocytes using flow cytometric analysis. Measurements were made at different time points: 3 days and six weeks after PM10 instillation (when the inflammatory response elicited has returned to normal). Difficulties were encountered whilst trying to measure F-actin content in rat cells by flow cytometry. Two labels were used, specific to rat phagocytes, in an attempt to clarify the leukocyte sub-populations. Despite this, it was not possible to adequately identify and therefore gate the PMNs and monocytes separately. Fluorescence overlap

occurred, despite attemps to compensate for this, although it is possible that over-labelling was a causative factor. Further investigations using each probe separately in controls, or use of other rat phagocyte-specific markers would undoubtedly help identify the flow cytometric sub-populations. However, it is worth noting that rat leukocytes are predominantly lymphocytes (approximately 90%), with a much smaller proportion of neutrophils and monocytes than in humans. Hence, the success of the above experiments may be improved by isolation of phagocytic cells, but this is problematical in rats where a maximum of 5 ml blood is obtained. Therefore, FITC-phalloidin labelled cells were used, with estimated gates.

After three days, PM10 instillation resulted in no difference in F-actin content of PMNs, whilst a possible increase in F-actin content of monocytes was seen, although this was not statistically significant. After six weeks, again no changes were seen in the F-actin content of PMNs, although in this case, the increase in F-actin content of monocytes almost reached statistical signifcance ($p=0.05$). This suggests that in the monocytes at least, an activation process has been elicted, which is still in evidence six weeks after PM10 treatment. It is possible that PM10 particles could activate cells indirectly via the activity of alveolar macrophages in the lung, which phagocytose the dust particles and accordingly produce mediators such as cytokines. These mediators could activate peripheral blood cells, with activation resulting in reassembly of the leukocyte cytoskeleton, leading to changes in the F-actin content. Mukae *et al* (2000) used rabbits to study whether alveolar macrophages (AM) play an important role as a source of inflammatory mediators responsible for the systemic response such as bone marrow stimulation after PM10 exposure. This study showed that AMs are capable of producing numerous mediators such as growth factors and cytokines. Thus particulate air pollution seems to activate F-actin formation in

monocytes, although this response is less after six weeks, when the F-actin content has decreased towards the initial control level.

Compromisation with bleomycin induced an obvious inflammatory, oedematous response within the lung after 3 days, and there are corresponding haematological changes. Large increases in the red cell indices were seen in the compromised animals. It is possible that, as the lungs have become oedematous, as shown by increased lung surface protein, 'circulatory dehydration' due to fluid accumulation in the lungs has caused a haemoconcentration effect. However, this is unlikely as no measurable changes were seen in the plasma viscosity. It is more likely that the increase in the red cell indices in the compromised animals arises due to defective pulmonary function leading to less efficient gaseous exchange in the lungs; this in turn leads to production of erythropoietin, hence increased haematocrit, although no measure of reticulocyte count was made to confirm this. It is also plausible that the increased red cell indices is simply because of more erythrocytes entering the peripheral circulation, as rats are known to have a large splenic reserve, although it is not clear why bleomycin treatment would cause this. Nevertheless, increased haematocrit in humans is strongly predictive of cardiovascular ischaemic events (Toghi *et al* 1978, Lowe *et al* 1992), and thus such changes induced by bleomycin instillation alone could predispose the animals to cardiovascular complications. Speculatively, this may mean that deaths recorded on days of high air pollution are a result of the increased sensitivity in individuals whose lung epithelium is already suffering permeability changes (as a result of disease), than those in which it is healthy. In addition to these changes seen in the red cell indices, the systemic leukocyte count decreases in the compromised animals, being 79% of the non-compromised figure. Although this decrease is not statistically significant, it

is worth noting that in these compromised lungs more free cells (neutrophils) are present on the lung surface, and it is possible that the systemic leukocyte count is depressed due to this large influx of phagocytic cells to the lungs. It is well known that the pool of sequestered neutrophils normally present in the lungs is markedly enhanced in acute lung inflammation, due in part to decreased deformability, because of the induction of phagocytic cells to the site of inflammation to phagocytose the dust particles.

In addition to the above time point of 3 days post-bleomycin instillation, two models of lung surface compromisation were generated; in model 1 (day 10), dusts were instilled at the peak of lung oedema. In model 2 (day 14), dusts were instilled when the lung epithelium is maximally repairing. The generation of compromised lungs in these groups of animals was successful in both models 1 and 2, as shown by significant increases in two markers of epithelial permeability. The lung surface protein, a sensitive indicator of lung permeability changes, was increased in compromised lungs compared to controls in both models. The elevation of the lung:body weight ratio to above 0.006 in both models confirms this increased oedema after compromisation; values below about 0.004 are usually a good indicator of a heathy lung which is free of significant infection. In model 2, compromisation also resulted in increased numbers of free cells, indicating a progressive increase in inflammation. However, for both these models, in which all measurements were made much later than for the initial compromisation discussed above, no haematological changes were measured at all.

With regards to the instillation of DEP and cabosil, the results clearly show that the lung damage suffered was markedly worse in the bleomycin-compromised

animals. In other words, the damage suffered by the lung because of bleomycin instillation alone was exacerbated by the additional instillation of the dust samples. Interestingly, the effects of DEP and cabosil instillation were mostly confined to Model 1, where the lung epithelium is at the peak of oedematous damage. The repairing epithelium (Model 2) saw less dramatic differences between the saline treated controls and the dust treated animals. Logically, this suggests that an epithelium in repair plays a more effective role in lung protection than one suffering permeability changes. Again, this may indicate that mortality recorded on high air pollution days results from increased susceptibility in individuals whose lung epithelium is already suffering permeability changes, rather than those in which it is healthy or at a state of repair.

In model 1 and 2, in which all measurements were made at between 10 and 14 days after the initial bleomycin treatment, neither the compromisation nor the subsequent dust instillations had any measurable effects on the cell counts. However, on pooling all data from the whole study, strong relationships were found between the plasma viscosity and the lung surface markers of free cell numbers, lung: body weight ratio and lung acellular protein. These relationships may be partly due to the induction of an acute phase response by the instilled dusts leading to small increases in fibrinogen, although plasma fibrinogen wasn't measured as part of this study, and no statistically significant changes were seen in the plasma viscosity. Nevertheless, plasma viscosity is a well-known marker of cardiovascular risk (Yarnell *et al,* 1991), and is likely to lead to increased coagulability of the blood. The correlation seen here between plasma viscosity and lung damage undoubtedly account, in part, for the pathological mechanisms linking air pollution to cardiovascular mortality. Although there were changes in the plasma viscosity, these changes did not reach statistical

significance. Plasma viscosity was found to be increased in individuals during an air pollution episode in Central Europe, as part of the MONICA-Augsburg survey (Peters *et al*, 1997), even after adjustment for meteorological features associated with raised plasma viscosity. Also, Seaton *et al* (1999) found that estimated personal exposure in humans to PM10 showed a negative correlation with haemoglobin concentration, packed cell volume (PCV) and red cell count. They suggested that increased sequestration of red cells was occurring in the circulation, resulting in ischaemic damage. The authors claim that this was possibly due to alterations in red cell adhesiveness, although it is not obvious why this would occur, and no such haematological changes were observed here. It is worth remembering that whilst the lung is the primary site encountered by the dusts, the circulatory system is secondary and hence any changes seen haematologically may be decreased in magnitude and hence more difficult to detect unless sample sizes are increased significantly. Since the greatest haematological changes were seen in the earliest measurments made after compromisation, it is possible that measurement of haematological indices would be more appropriately made sooner after compromisation and dust instillation. This is particularly pertinent for phagocytic leukocytes, whose systemic counts can alter within hours of infection and inflammation. Measurements of more appropriate indicators of inflammation may yield more information in this type of study; plasma levels of C-reactive protein or cytokines which induce neutrophil accumulation into inflammatory sites, would be plausible candidates for studies such as this. Bleomycin-induced lung injury has previously been associated with increased neutrophil elastase activity (Azuma *et al*, 1998), and hence plasma elastase measurements may yield additional information about neutrophil behaviour.

In summary, fluctuations seen in the plasma viscosity were small, and hence, did not reach statistical significance. However, it is possible that any increase in plasma viscosity induced by air pollutants may precipitate congestive heart failure more readily in the presence of pre-existing cardiovascular or respiratory disease, and further investigation of such correlations will undoubtedly help elucidate the intermediate steps between primary damage in the lung and secondary changes in the blood. Further investigation is needed in this field to establish a relationship between the effects of particulate air pollution and F-actin content of phagocytic cells.

Chapter Six

Chapter Six: Effect of PPAR γ agonists on actin polymerisation in PMNs and monocytes

6.1 Introduction

Chronic diseases such as diabetes, obesity, cancer and cardiovascular disease are responsible for the majority of deaths in developed societies. These diseases have complex causes involving genetic, environmental and nutritional factors. There is considerable evidence that a group of nuclear hormone receptors, called peroxisome proliferator-activated receptors (PPARs) are important in these diseases (Spiegelman, 1998; Demetri *et al*, 1999; Walker *et al*, 1999; Minamikawa *et al*, 1998). The fact that PPAR ligands can be pharmacologically synthesised in the form of drugs such as thiazolidinediones (TZDs) and fibrates has instigated a huge research effort into PPARs and their role in inflammatory disease (Kersten *et al*, 2000).

6.1.1 Peroxisome proliferator-activated receptors

PPARs are a group of the nuclear hormone receptor super family of ligand-dependent transcription factors that are predominantly expressed in adipose tissue, the adrenal gland and the spleen. Activation of target gene transcription depends on the binding of the ligand to the receptor (**Figure 6.1**), and these receptors regulate glucose and lipid homeostasis. The pharmacology of synthetic PPAR ligands demonstrated the role of these receptors in regulating glucose and lipid homeostasis and established their utility as molecular targets for the development of drugs for the treatment of diabetes and cardiovascular disease (Oberfield *et al* 1999). Three types of Peroxisome proliferator-activated receptors have been identified; α, ß (also called δ, NUC-1 or FAAR) and γ, which are all activated by naturally occurring fatty acids

or fatty acid derivatives (Auwerx, 1999) and pharmacological agents such as TZDs, of which are rosiglitazone and glitazones are 2 examples (Delerive *et al*, 2001). Since PPARs are activated by various fatty acid metabolites as well as several drugs used in the treatment of metabolic disorders, they translate nutritional, pharmacological and metabolic stimuli into changes in the expression of genes. The PPAR α protein is expressed most in brown adipose tissue, liver, kidney, heart, skeletal muscles and vascular wall, whereas, PPAR ß is expressed in almost all tissues except the adipose tissue. The PPAR γ protein is expressed in white and brown adipose tissues and to a lesser extent in colon, the retina and some parts of the immune system such as neutrophils and monocytes (Vaidya *et al*, 1999; Kersten *et al*, 2000).

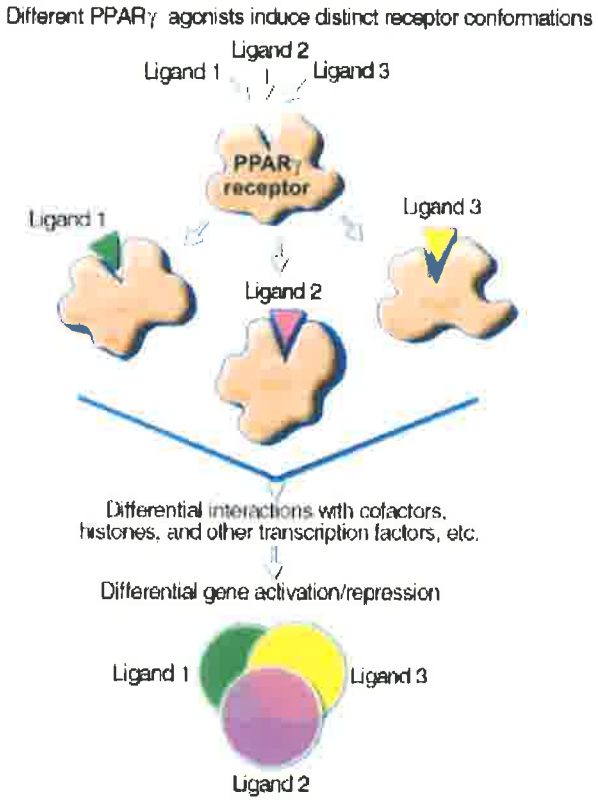


Fig 6.1: Different PPAR ligands bind to the ligand- binding site leading to a differential interaction with cofactors, histones and other transcription factors, etc.As a result of these interactions, each PPAR ligand-receptor complex lead to differential but overlapping pattern of gene expression (Reproduced from Glass & Rosenfeld, 2000).

6.1.2 Peroxisome proliferator-activated receptor Gamma (PPAR γ)

One of the best-characterised receptors which has important roles in adipogenesis and insulin sensitisation is PPAR γ. It has been implicated in the pathogenesis of type II (non-insulin dependent) diabetes mellitus. The agonists to PPAR γ are used in the treatment of this disease where patients suffering this disease showed an increased risk of atherosclerosis and its clinical complications (Glass, 2001). Peroxisome proliferator-activated receptor γ plays an important role in adipogenesis and energy balance (**Figure 6.2**). Furthermore it has been described as a powerful player in general transcriptional control of numerous cellular processes with implications in cell cycle control, carcinogenesis, inflammation, atherosclerosis and immuno-modulation (Auwex, 1999).

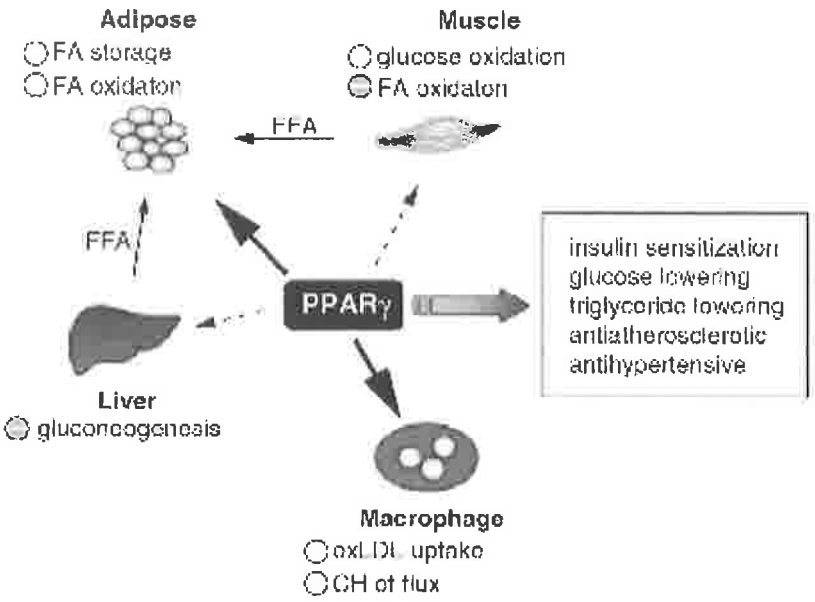


Fig 6.2: Activation of PPAR γ either stimulates or represses key metabolic pathways in adipose, muscle, and liver tissue and in macrophages, and promotes the flux of free fatty acids (FFAs) from muscle and liver to adipose tissue. In addition, PPAR γ activation also increases macrophage-mediated cholesterol (CH) metabolism (Modified from Willson *et al*, 2001).

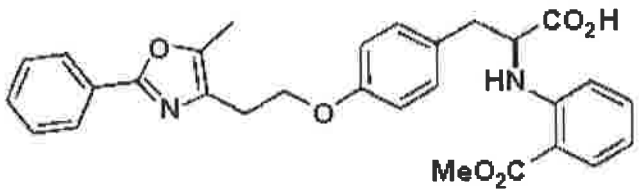
6.1.3 Peroxisome-proliferator activated receptor gamma agonists

Several agents have been shown to be PPAR γ agonists, which are agents that promote differentiation of fibroblasts into adipocytes. Examples of these agents are prostanoids, such as 15-deoxy-Delta prostaglandin J_2, as well as members of a new class of oral antidiabetic agents, the thiaziodinediones and a variety of non-steriodal anti-inflammatory drugs (Jiang *et al*, 1998). Thiazolidinediones (TZDs: troglitazone, pioglitazone, ciglitazone and rosiglitazone) are pharmacological compounds that bind to PPAR γ with a high affinity and stimulate transcription of target genes. One closely related agonist to rosiglitazone is a Glaxo-Wellcome drug, GW 7845 (**Figure 6.3**), which belongs to the TZD class of drugs that acts on PPAR γ. Pioglitazone was also shown to increase gene expression through a region of its promoter that had been identified as a PPAR γ binding site (Tontonoz *et al*, 1994). The TZDs can function either as a full or partial agonist or antagonist depending on cell type and sequence of recognition (Auwex, 1999). Although activation of PPAR γ appears to have protective effects on atherosclerosis, it is still uncertain whether PPAR γ agonists prevent the development of CVD. Further understanding of PPAR γ function could lead to the development of new therapies for various diseases such as diabetes, atherosclerosis, obesity and cancer. Since it has been suggested that TZDs may have functions other than antidiabetic agents and may reduce inflammation and various aspects of cardiovascular disease in diabetes (Buchan & Hassel, 2000), they may have an effect on intracellular F-actin content, and therefore are worthy of investigation here.

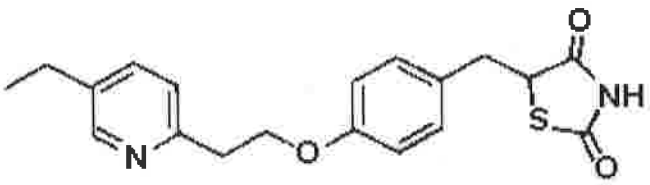
6.1.4 PPAR γ and Leukocytes

Both monocytes and PMNs are known to express large quantities of PPAR γ (Jiang *et al*, 1998; Ricote *et al*, 1998). The PPAR γ agonists inhibit production of monocyte inflammatory cytokines such as TNF-alpha (Jiang *et al*, 1998). Experiments investigating the effect of prostaglandin (15 deoxy-Delta prostaglandin J_2 ($dPGJ_2$)), a naturally occurring PPAR γ agonists on cytokine production, have shown that adding prostaglandin and an inducer (e.g PMA or LPS) at the same time inhibited cytokine production (Jiang *et al*, 1998, Vaidya *et al*, 1999). These PPAR γ agonists also have an effect on expression of gelatinase B, a matrix metallo-proteinase that is up regulated during macrophage activation and contributes to tissue damage in acute and chronic inflammation (Ricote *et al*, 1998).

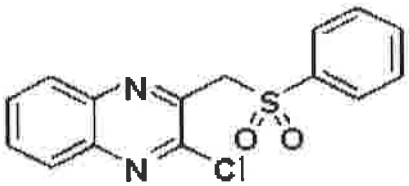
Peroxisome-proliferator activated receptor gamma, which may bind and mediate responses to $dPGJ_2$, is expressed on neutrophils in addition to G-protein coupled receptors that initiate responses to prostaglandins (Greene *et al*, 1995; Vaidya *et al*, 1999). The prostaglandin $dPGJ_2$ inhibits the adhesion-dependent oxidative burst by blocking the early step in the signalling pathway and this inhibition was not associated with PPAR γ (Vaidya *et al*, 1999). However, little is known about PPAR γ with respect to neutrophil function.



GW 7845



Pioglitazone



L-764406

Fig 6.3: Different structures of PPAR γ agonists (GW 7845 & pioglitazone) and PPARγ antagonist (L-764406).

6.1.5 Aim

PPAR γ agonists are increasingly being used to treat insulin sensitivity in Type II diabetes. Recent evidence suggests that these drugs have anti-inflammatory effects over and above their insulin regulatory function. One of the most important and life-threatening complications of Type II diabetes involves increased inflammation that includes a decrease in leukocyte deformability. Therefore, this section will investigate whether PPAR γ agonists are able affect leukocyte actin polymerisation as measured by flow cytometry.

6.2 Materials and Methods

All chemicals were purchased from Sigma-Aldrich Company Ltd unless stated otherwise.

Preparations of PBS, fMLP are the same to those used in earlier chapters. The cell permeabilisation kit from Harlan was also used as described earlier (section 2.2.1).

6.2.1 Drugs used and preparations

Preparation of Glaxo-Wellcome 7845 Agonist

Glaxo-Wellcome 7845 (GW 7845), a thiazolidinedione (TZD) PPAR γ agonist was received from supplier in aliquots (0.01M) and kept frozen at –80°C until used. Appropriate concentrations were prepared using DMSO.

Preparation of Glaxo-Wellcome L-764406 Antagonist

The antagonist L-764406 was received from the supplier in aliquots (0.01M) and was kept frozen at –80°C until used. Similar concentrations to the agonist were used in a single test as recommended by the supplier.

Preparation of Pioglitasone

Pioglitasone, a PPAR γ agonist, was received in aliquots from Glaxo-Wellcome with a stock concentration of 0.01M. A concentration of $1x10^{-5}$ M was prepared using DMSO in a single test as recommended by the supplier.

6.2.2 Blood samples

Blood containing sufficient EDTA i.e disodium Ethylenediaminetetraacetic acid was collected from healthy volunteers by the same method used earlier (section 2.2.2). All blood samples were stored at room temperature during laboratory procedures and used within six hours of venepuncture.

6.2.3 Effect of GW 7845 and L-764406 on un-stimulated cells

For each sample to be analysed, 0.5ml of un-stimulated whole blood was incubated with $6x10^{-5}$ M of drug for 1hour at 37°C as recommended by the supplier. After incubation, fixing the cells with the cell permeabilisation kit stopped the effects of the drug. A control sample was prepared by adding 0.5ml of blood with DMSO in an equivalent volume to that of drug and incubated for one hour at 37°C.

6.2.4 Fixing and Permeabilising cells

Controls and cells treated with drug were fixed using the cell permeabilisation Kit (section 2.2.3.1). Phalloidin (FITC conjugated) was used in same concentration as described earlier ($1.6x10^{-6}$ M). Cell suspensions were prepared by adding 0.5ml of PBS to the fixed and permeabilised cells.

6.2.5 Flow cytometric analysis

Cell suspensions of whole blood samples were prepared and analysed using flow cytometry as described earlier (section 2.2.3.3). Cells were analysed immediately.

6.2.6 Effect of GW 7845 and L-764406 on stimulated cells

Whole blood (0.5ml) was incubated with $6x10^{-5}$M GW 7845 or L 764406 for one hour at 37°C. After incubation, cells were stimulated by adding 10μl of fMLP at a concentration of 5nM for 5minutes at 37°C. After 5 minutes cells were fixed, permeabilised and stained as described earlier. A control sample was prepared by incubating whole blood (0.5ml) without drug for one hour at 37°C and adding the same volume of fMLP (10μl) for 5 minutes at 37°C.

6.2.7 Dose response of drug (GW 7845)

Blood (0.5ml) was incubated for 1 hour with different concentrations of GW 7845 (0.25, 0.5, 1, 2 and $6x10^{-5}$M).

6.2.8 Cell viability

A viability test was performed to ensure that the concentration ($6x10^{5-}$M) of drug (GW 7845) used in these experiments does not have any detrimental effect on leukocytes. Trypan Blue solution was used as one of several stains recommended in dye exclusion procedures for viable cell counting. This method is based on the principle that live cells do not take up Trypan Blue, whereas dead cells do. There are a number of methods, available for determining cell viability. The Trypan Blue method confirms that cells have intact membranes and the method has been criticized for not excluding same toxic effects. However, it has been used to determine cell viability in several studies (Altman *et al*, 1993; Tonks *et al*, 2003). A cell suspension was prepared by adding 0.5ml of 0.4% Trypan Blue solution to a test tube. PBS (0.3ml) and 0.2ml of blood suspension (cells treated with drug) were added together and mixed thoroughly. Red blood cells were lysed with 5% actetic acid. The mixture

was allowed to stand for 5-15 minutes. A hemacytometer was used to count cells by placing a cover slip in place and using a Pasteur pipette to transfer a small amount of cell suspension mixture to both chambers. Cells were counted in both chambers. Starting with chamber 1, cells in the 1mm Centre Square and four 1mm corner squares were counted. The same procedure was repeated for chamber 2.

Cell viability (%) = total viable cells ÷ total cells x 100

Cell morphology was also studied by light microscopy to assess any changes in cell shape.

6.2.9 Time course with GW 7845 of un-stimulated cells

Whole blood was incubated with the drug for 2 hours, during which samples were removed at different time points for measurement of F-actin content in PMNs and monocytes. These time points were 5, 10, 15, 30, 60, 90 and 120 minutes. Whole blood was incubated with $6x10^{-5}$M of drug at 37°C. At each time point, a volume of DMSO equivalent to the drug was added and incubated for the 5 minutes at 37°C. Cell suspensions were prepared for analysis immediately after incubation.

6.2.10 Time course with GW 7845 of stimulated cells

The same procedure and same time points as for un-stimulated blood were used here, except blood was stimulated with fMLP (5 nM) for 5 minutes. Whole blood was incubated with $6x10^{-5}$M of drug at 37°C. At each time point, fMLP (5 nM) was incubated with cell suspensions for 5 minutes at 37°C before fixation takes place to stimulate the cells.

6.2.11 Effect of pioglitazone on F-actin content

The same procedure used in treating blood with GW 7845 was repeated here using another type of TZD, pioglitazone. The recommended concentration ($1x10^{-5}M$) was used on un-stimulated and stimulated cells (fMLP).

6.2.12 Dose response of pioglitazone

To investigate the dose-response relationship of pioglitazone, blood (0.5 ml) was incubated for 1 hour with different concentrations of pioglitazone (0.25, 0.5, 1 and 2 $x10^{-5}M$) before flow cytometric analysis.

6.2.13 Statistical Analysis

All experiments were performed at a minimum of triplicates, but experiments involving the antagonist (L764406) were performed in duplicate only. Results are reported as means ± standard error (SEM). Differences were analysed using one-way analysis of variance (ANOVA) and two-sample t-test.

6.3 Results

6.3.1 Effect of GW 7845 on MFI

To test whether the drug has an effect on F-actin content, flow cytometric analysis was used where F-actin content of gated PMNs and monocytes (stimulated and un-stimulated) in whole blood was expressed as the mean fluorescent intensity (MFI) of cells as described in previous chapters.

Blood treated with PPAR γ agonist (GW 7845) at a concentration of $6x10^{-5}M$ showed a decrease in F-actin content (MFI), which was not statistically significant in PMNs ($p=0.21$) and significant in monocytes ($p\leq0.001$) after applying a two-sample t-test (**Figures 6.4 and 6.5**).

Stimulated cells

Blood treated with PPAR γ agonist ($6x10^{-5}M$) and then stimulated with fMLP (5nM) showed a significant decrease in MFI of both PMNs ($p\leq0.001$) and monocytes ($p\leq0.001$) compared to cells being stimulated with fMLP without any drug treatment (**Figure 6.4 and 6.5**).

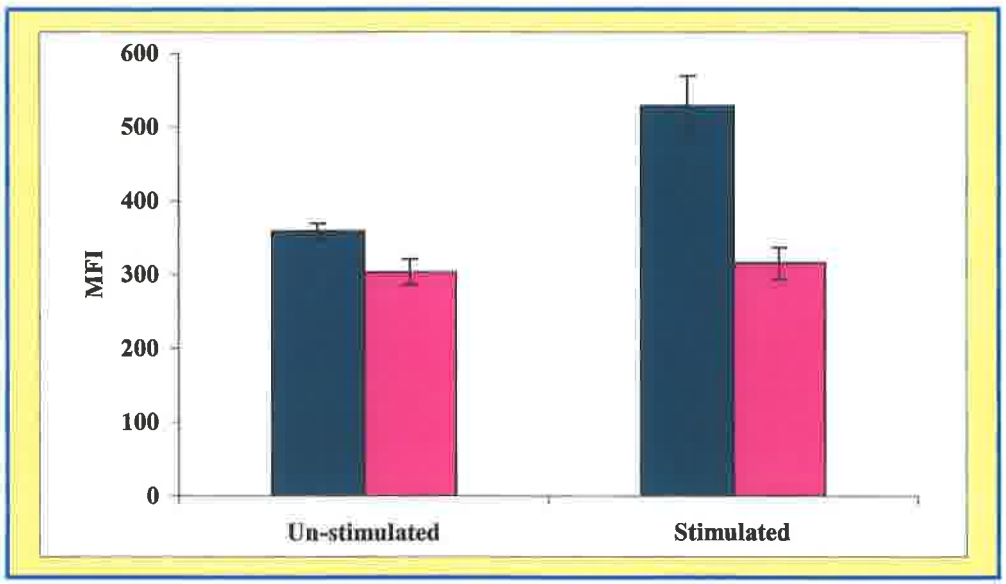


Figure 6.4: Effect of the PPAR γ agonist (GW 7845) on F-actin content of un-stimulated and stimulated (fMLP 5nM) PMNs. Cells incubated with (pink) and without (blue) GW 7845 ($6x10^{-5}$M) for one hour at 37°C. Points represent mean ± Standard error (n=10). Two sample t-test between drug –ve and drug +ve. $p=0.21$ for un-stimulated cells and $p<0.001$ for stimulated cells.

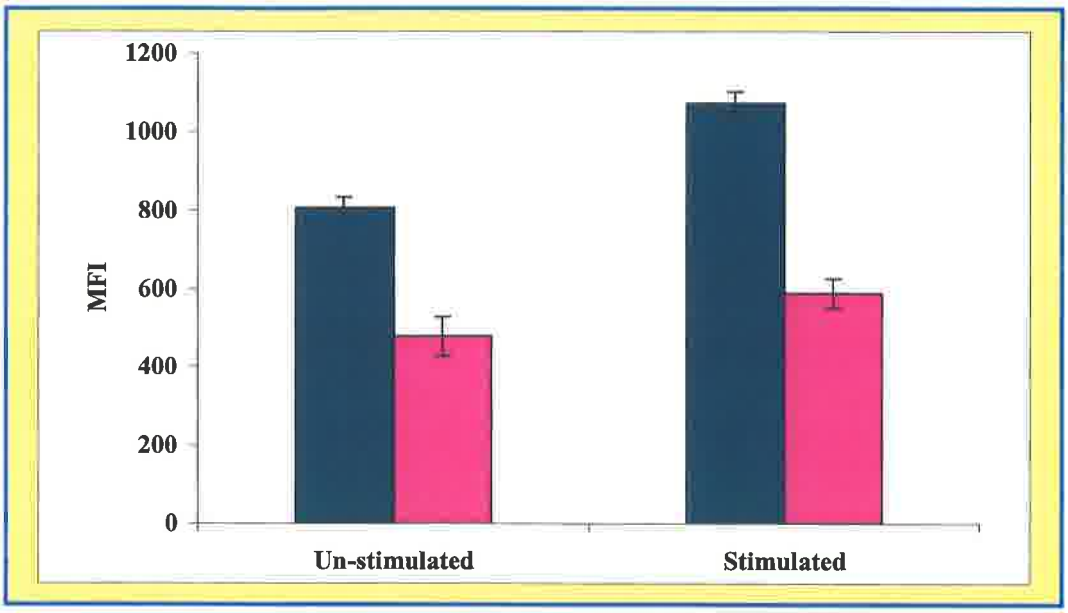


Figure 6.5: Effect of the PPAR γ agonist (GW 7845) on F-actin content of un-stimulated and stimulated (fMLP 5nM) monocytes. Cells incubated with (pink) and without (blue) GW 7845 ($6x10^{-5}$M) for one hour at 37°C. Points represent mean ± Standard error (n=10). Two sample t-test between drug –ve and drug +ve. $p<0.001$ for un-stimulated cells and $p<0.001$ for stimulated cells.

Dose response of GW 7845

Results showed that increasing the concentration of GW 7845 has an effect on F-actin content, namely that as the concentration of drug increases, actin depolymerisation increases. However, this apparent decrease in F-actin content was not statistically significant for either the PMNs (p=0.795) or the monocytes (p=0.639), although monocytes showed more sensitivity to drug concentration than PMNs (**Figure 6.6**).

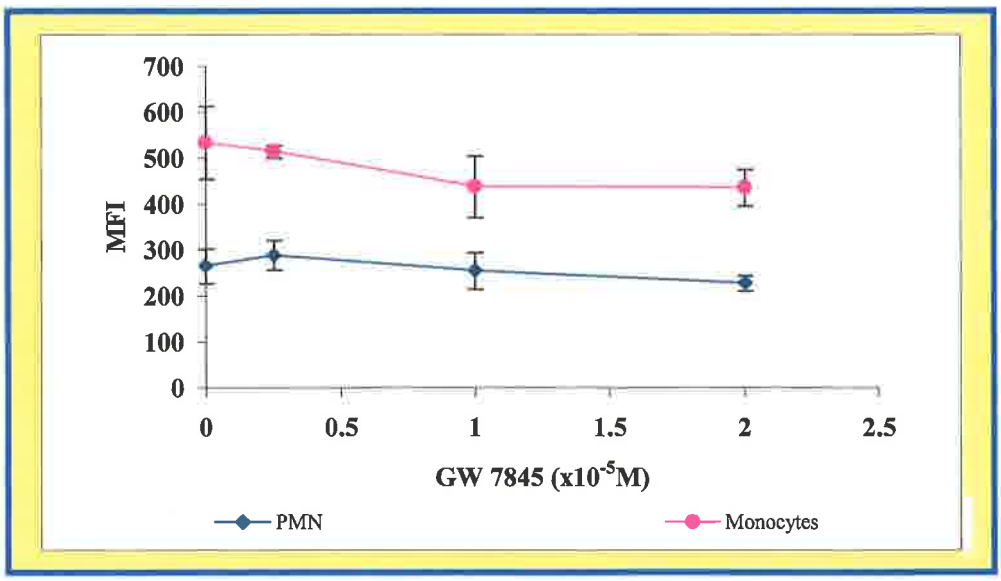


Fig 6.6: The effect of GW 7845 dose on MFI of un-stimulated PMNs (p=0.795) and monocytes (p=0.639). Points represent means ± SEM of n = 3 or 5. The p-values were determined by ANOVA.

Time course with GW 7845

Flow cytometric analysis of cells incubated with the agonist for up to two hours showed a change in F-actin content compared to the control at each time point (5, 10, 15, 30, 60, 90 and 120). Results showed that incubation with the drug reduces F-actin (MFI) dramatically within 5 minutes of incubation and this effect has continued for 15 minutes. Between 30 minutes and 2 hours the level of F-actin remains reasonably stable, although it is still much lower than that at time zero. This effect was evident in both PMNs ($p=0.018$) and monocytes ($p=0.012$) as measured by ANOVA (**Figure 6.7**).

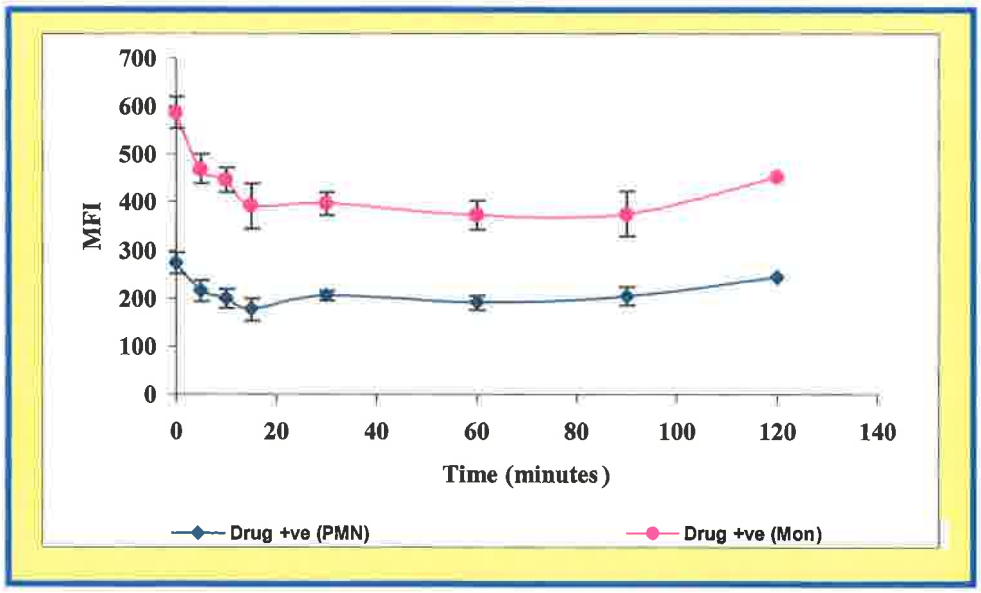


Fig 6.7: Effect of incubation time with GW 7845 ($6x10^{-5}M$) on the F-actin content of PMNs ($p=0.018$) and monocytes ($p=0.012$). Points represent mean ± SEM ($n=5$). The p-values were determined by ANOVA.

Stimulated cells

Flow cytometric analysis of cells incubated with the agonist for up to two hours and then stimulated with fMLP at different time points showed a decrease in the F-actin content in PMNs and monocytes. The MFI values were higher in this case, due to the use of fMLP-stimulated cells. The pattern of results was similar to that of unstimulated cells, in that there was a dramatic decrease in F-actin content of drug-treated cells after 5 minutes, followed by a stabilisation of F-actin levels over the 2-hour period. Applying ANOVA showed no statistical difference in PMNs ($p=0.194$) but a significant difference in monocytes ($p=0.006$). Overall, these results showed that stimulation of cells after drug treatment inhibited the fMLP-induced activation, shown by the decreased F-actin content (**Figure 6.8**).

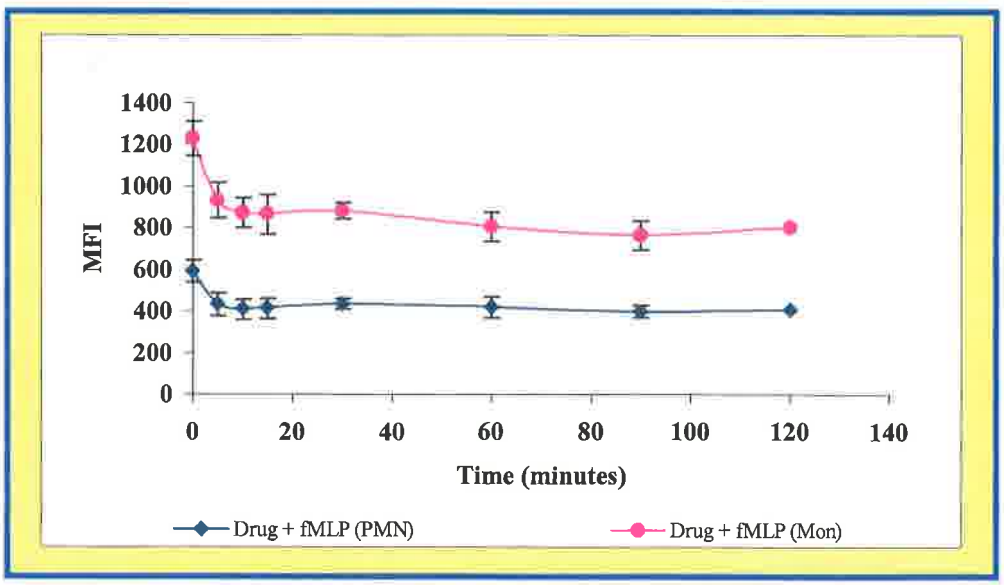


Fig 6.8: Effect of incubation time with the GW 7845 ($6x10^{-5}M$) on F-content of stimulated PMNs ($p=0.194$) and monocytes ($p=0.006$). Points represent mean ± SEM ($n=5$). The p-values were determined by ANOVA.

6.3.2 Effect of antagonist L 764406

Cells treated with the antagonist ($6x10^{-5}M$) for 1 hour without stimulation with fMLP showed a decrease in the F-actin content in both cell types. For PMNs (**Figure 6.9**), the MFI decreased from 421±6 in controls to 251±6 in drug treated, and the corresponding MFI values were 945±7 and 488±8 in monocytes (**Figure 6.10**). Cells treated with antagonist and then stimulated with fMLP also showed a decrease in the F-actin content of both PMNs and monocytes (**Figure 6.9 and 6.10**). In this case the mean MFI decreased from 542±37 to 312±1 in fMLP-stimulated PMNs, and from 1263±36 to 657±21 in monocytes. No statistical significance testing was performed on these data as each test was performed in duplicate for each subject. Further tests could not be performed due to lack of drug availability.

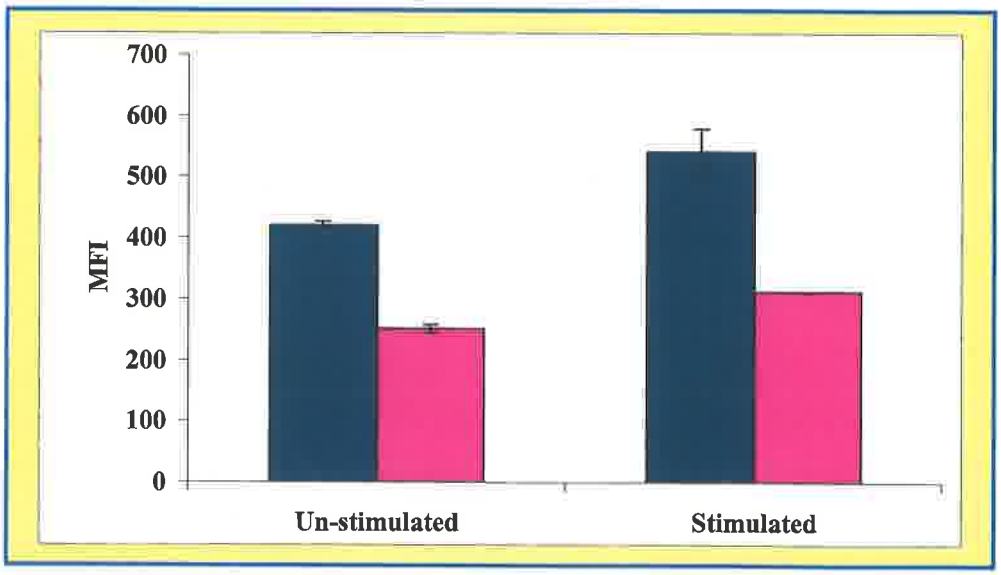


Fig 6.9: Effect of the PPAR γ antagonist (L764406) on F-actin content of un-stimulated and stimulated (fMLP 5nM) PMNs. Cells incubated with (pink) and without (blue) L764406 ($6x10^{-5}M$) for one hour at 37°C. Points represent mean ± Standard error (n=2).

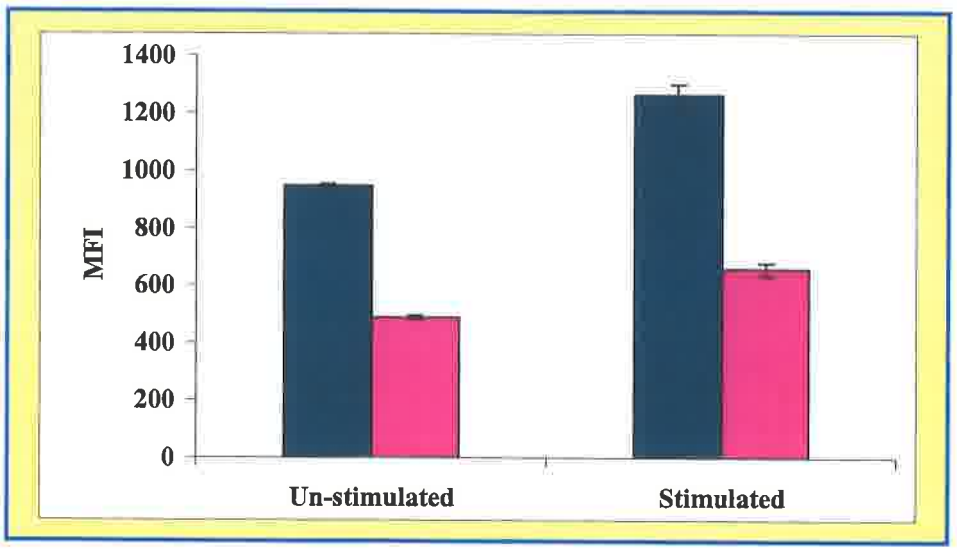


Fig 6.10: Effect of the PPAR γ antagonist (L764406) on F-actin content of un-stimulated and stimulated (fMLP 5nM) monocytes. Cells incubated with (pink) and without (blue) L764406 ($6x10^{-5}$M) for one hour at 37°C. Points represent mean ± Standard error (n=2).

6.3.3 Effect of agonist (pioglitazone) on MFI

The incubation of un-stimulated cells with pioglitazone resulted in no alteration in the F-actin content of gated PMNs in whole blood (p=0.74, **Figure 6.11**). However, in monocytes, it is possible that pioglitazone promotes actin depolymerisation with the MFI decreasing from 528±64 to 444±15, although this change does not reach statistical significance (p=0.26, **Figure 6.12**) as determined by two sample t-test. For cells treated with pioglitazone and then stimulated with fMLP a similar picture emerges. In PMNs, there was no change seen in the F-actin content (p=0.65, **Figure 6.11**), but there was a significant decrease in the F-actin content of the monocytes (p=0.016, **Figure 6.12**) as determined by two sample t-test, with the MFI decreasing from 853±129 to 580±71 in fMLP stimulated, drug treated cells.

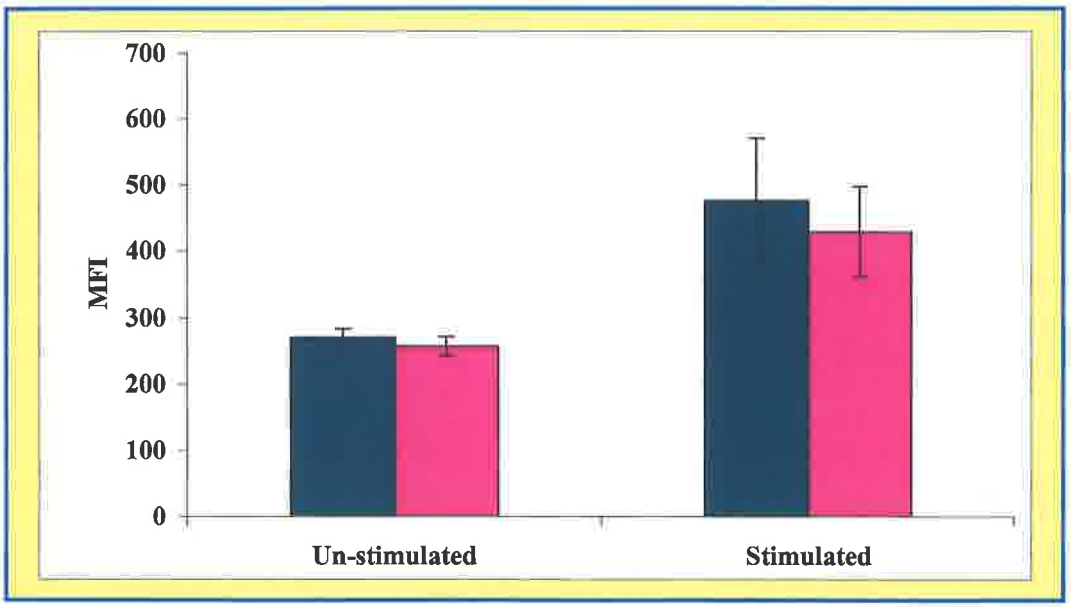


Fig 6.11: Effect of the PPAR γ agonist (pioglitazone) on F-actin content of un-stimulated and stimulated (fMLP 5nM) PMNs. Cells incubated with (pink) and without (blue) pioglitazone ($1x10^{-5}$M) for one hour at 37°C. Points represent mean ± Standard error (n=5). Two sample t-test between drug –ve and drug +ve. P=0.74 for un-stimulated cells and p=0.65 for stimulated cells.

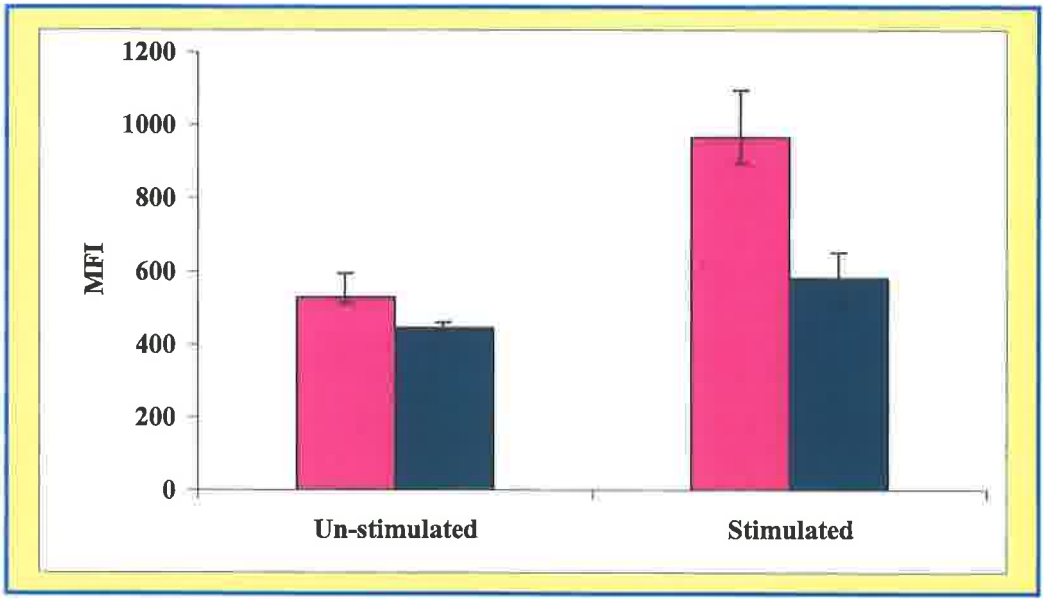


Fig 6.12: Effect of the PPAR γ agonist (pioglitazone) on F-actin content of un-stimulated and stimulated (fMLP 5nM) monocytes. Cells incubated with (pink) and without (blue) pioglitazone ($1x10^{-5}$M) for one hour at 37°C. Points represent mean ± Standard error (n=5). Two sample t-test between drug –ve and drug +ve. p=0.26 for un-stimulated cells and p=0.016 for stimulated cells.

Dose response of Pioglitazone

The results for Pioglitazone were similar to those of GW 7845 in that increasing drug concentration seems to increase the rate of actin depolymerisation, although this is more obvious in the monocytes than the PMNs ($p=0.488$) as determined by ANOVA. In the PMN population, this possible decrease in F-actin content is only seen at concentrations higher than $1x10^{-5}M$, but in the monocytes the effect is more pronounced, and is obvious from the lowest concentration of pioglitazone ($0.5x10^{-5}M$), although this is not obvious statistically ($p=0.213$) as determined by ANOVA. These results indicate that again, the monocytes showed more sensitivity to drug concentration than PMNs (**Figure 6.13**).

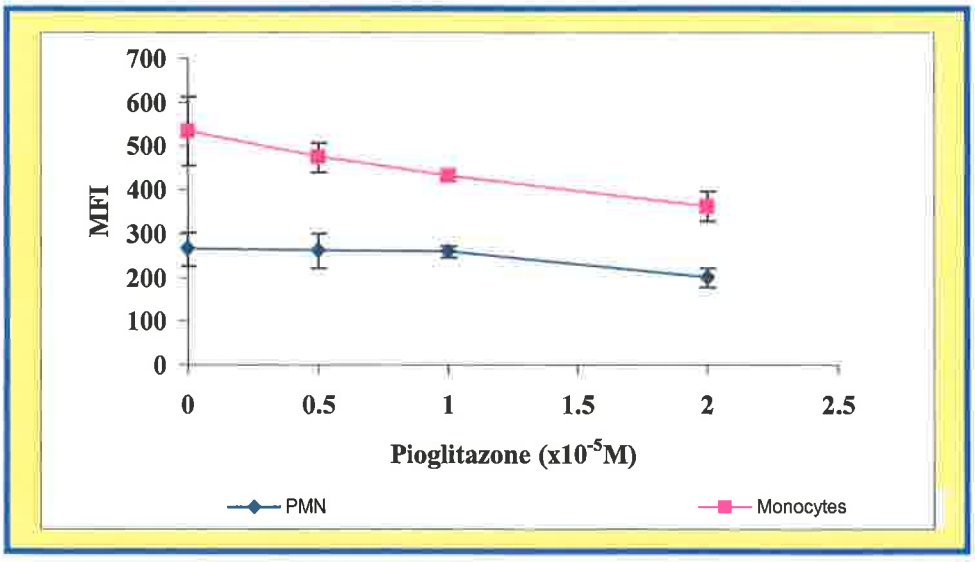


Fig 6.13: The effect of pioglitazone ($x10^{-5}M$) dose on MFI of un-stimulated PMNs ($p=0.488$) and monocytes ($p=0.213$). Points represent means ± SEM of n = 3 or 5. The p-values were determined by ANOVA.

6.3.4 Cell viability

Cell viability tests using Trypan Blue solution showed that after counting on a haemocytometer, more than 98% of cells treated (either with GW 7845 only or with fMLP stimulation and GW 7845 treatment) did not take the stain. Cells also maintained their normal spherical shape. These observations suggest that treatment with the drug and fMLP did not affect cell viability, even at the highest concentration of drug used.

6.4 Discussion

Recently, various studies have suggested the potential role of PPAR γ in regulation of inflammatory processes. Since the polymerisation of F-actin in diabetes is well recognised (Clodi *et al*, 1998) and increased neutrophil F-actin has been reported (Pecsvarady *et al*, 1994; Sulochana *et al*, 2001; Advani *et al*, 2002) this study was performed. It has been shown that insulin induces actin polymerisation in several cell types and that actin remodelling after insulin stimulation expedites the translocation of insulin-responsive glucose transporter 4 (GLUT-4), from intracellular membranes to the cell surface to enhance glucose uptake into fat and muscle cells (Bose *et al*, 2001; Emoto *et al*, 2001; Patki *et al*, 2001). Numerous studies have pointed to abnormalities in the regulation of GLUT-4 as the primary cause of insulin resistance in humans (Garvey *et al*, 1993, 1998). Other studies on muscle cells have indicated that interfering with actin remodelling by pharmacological agents or by high glucose and insulin used to induce insulin resistance stops actin polymerisation and largely prevents GLUT-4 insertion into the plasma membrane in response to insulin (Tong *et al*, 2001)

In the study presented here, PMNs and monocytes, cells that are involved in inflammatory responses, were treated with anti-diabetic drugs of the TZDs group (GW 7845 and Pioglitazone) used in type II diabetes (insulin resistant) as a specific ligand for PPAR γ. Some evidence was found here that treatment with these drugs has an effect on the F-actin content of normal and stimulated PMNs and monocytes, although most of the changes seen were more prominent in the monocyte sub-populations. This effect observed on the F-actin content raises a question; Do PPARγ agonists have an effect on cell viability thus causing F-actin to decrease? This is

unlikely to happen, since the concentrations of drugs used were very low, and the maintenance of cell viability during these experiments was confirmed using the Trypan Blue viability test.

Several lines of evidence indicate that GW 7845 reduces F-actin content in PMNs and monocytes by mechanisms independent of PPAR γ. First, experiments were performed using PPAR γ antagonist to compare with the effects obtained by PPAR γ agonist, as it would be likely that they would have opposing effects. However, the antagonist caused a reduction in F-actin content similar to that caused by the agonist, and this was true for both PMNs and monocytes, although statistical significance testing was not applied due to the small number of experiments done. This observation suggests that GW 7845 and L764406 are working via other mechanisms; possibly binding to some receptors other than the nuclear receptor (PPAR γ), namely cytoplasmic protein receptors or cell surface such as tyrosine kinase and GTP-binding protein-linked receptors. The possible mechanism by which agonist and antagonist are acting to reduce F-actin content of cells is that these ligands have multiple binding sites other than PPAR γ to bind other receptors. Secondly, the drug GW 7845 reduces F-actin dramatically within 5-15 minutes although these changes then stabilise between 30 mins and 2 hours of incubation. These immediate changes in F-actin content suggest that they were not as a result of changes in gene transcription via PPAR γ because changes mediated by alterations in gene transcription would be expected to take a considerably longer time, of at least 30 minutes.

The possibility of the involvement of cytokines in promoting actin polymerisation is unlikely, as *de novo* expression of cytokines takes longer than 5

minutes. To this end, the possibility of the involvement of actin depolymerisation factor/cofilamentous protein (ADF/cofilin), which binds to both F-actin and actin monomers, is raised. Cofilin promotes the depolymerization of actin filaments, which is required for a variety of cellular responses such as the formation of lamellipodia and chemotaxis. Cofilin's effect occurs at the minus end with an increase in the off rate of filaments (Maciver *et al*, 1998). The F-actin binding and depolymerising activities of cofilin are regulated by phosphorylation where phosphorylation of cofilin is known to block these activities (Morgan *et al*, 1993). Phosphatidylinositol 4-phosphate (PIP) and 4,5-bis-phosphate (PIP2), membrane lipids that bind to the actin binding domains of ADF/cofilin and are capable of inhibiting ADF/cofilin binding to actin in vitro (Yonezawa *et al*, 1991; Kusano *et al*, 1999). The un-phosphorylated cofilin is responsible for actin depolymerisation. No experimental evidence, however, currently exists to support the hypothesis that phosphorylation reactions are responsible for the changes in F-actin content, after drug treatment, and this is an important avenue for future work.

In conclusion GW 7845 and poiglitazone, known PPAR γ agonists, reduce F-actin content. They may affect actin by binding to receptors other than PPAR γ and hence could alter other signalling pathways.

Chapter Seven

Chapter Seven: General Discussion

It is well documented that changes in the rheologic behaviour of leukocytes contribute to the pathology of cardiovascular diseases and other various diseases (see general introduction). Hence, studying the causative factors behind leukocyte activation, such as F-actin polymerisation and leukocyte deformability, will be of great benefit both in understanding leukocyte mechanics and in treating cardiovascular diseases.

Phagocytosis, a process that is required as a response to external stimuli, is of fundamental importance for a wide diversity of organisms, and is known to be an actin-dependent process (Kaplan, 1977). In general, it is known that activation of phagocytic cells triggers an increase in F-actin content, in both neutrophils (Downey *et al*, 1992, Anderson *et al*, 2000) and monocytes (Greenberg *et al*, 1991, Nagaish *et al*, 1999). This thesis investigated some aspects of the mechanical properties of leukocytes, by assessing cell activation and cytoskeletal assembly on leukocyte shape and deformability. This investigation was based, initially, on quantification as well as visualisation methods for accurately measuring F-actin involvement in cytoskeletal organisation. Then, the effects of this cytoskeletal organisation on leukocyte deformability were investigated. This thesis also describes the use of a well-characterised animal model for studying the circulatory effects of particulate air pollution, including measurement of F-actin formation. Finally, these studies also investigated the effects of some specific pharmacological agents on cytoskeletal organisation.

Activation of phagocytic cells, which leads to changes in the F-actin content, maybe obtained using fMLP and PMA. An optimisation of the conditions was performed using a simple method based on fixing, permeabilising and labelling F-actin with either FITC-phalloidin or Oregon green® 514 phalloidin. Whole blood samples were used since isolation procedures such as density gradient centrifugation, are time consuming and could cause cell activation. The permeabilisation procedure is necessary since phalloidin is a large molecule, thus not able to enter the cell. Based on flow cytometric analysis and fluorescence microscopy, FITC-phalloidin was chosen as the appropriate probe over Oregon green® 514 phalloidin to label F-actin with minimal activation or morphological changes. The optimum concentration of FITC-phalloidin is 1.6μM, which is low enough to decrease the non-specific binding to obtain a high staining intensity, to reduce cost and decrease the degree of toxicity. This concentration was used throughout the studies.

To induce cell activation, whole blood was treated with either fMLP or PMA. Chemoattractant treatment, with both PMA and fMLP, stimulated cellular response by dramatically increasing the F-actin content, accompanied by morphological changes and these observations have been made by others (Howard & Oresajo, 1985a; Coates *et al*, 1992). During activation, cells showed a different response to each chemoattractant with respect to: the amount of polymerised actin, the cell morphology and the mechanical behaviour of cells. The rate of activation and the time required to achieve maximum activation varied between fMLP and PMA. A rapid F-actin formation was observed between 30-60 seconds for fMLP-stimulated cells whereas activation in PMA-stimulated cells was between 10-15 minutes. This difference could be attributed to the fact that fMLP binds to its receptor on the plasma membrane, whereas, PMA crosses the bi-lipid plasma membrane to interact

with its cytoplasmic receptor. Findings agree with Haward & Wang (1987), which showed that both actin polymerisation rate and the mean fluorescent intensity are higher in fMLP-stimulated cells than in PMA. Although, PMA induces a slower increase in F-actin content through the activation of protein kinase C (PKC), the increase was constant. Djafarzadeh & Niggli (1997) showed that activation with PMA resulted in dephosphorylation of a phospho-protein, which has been shown to be identical to cofilin, a protein implicated in actin filament remodelling. Dephosphorylated cofilin, enriched at the plasma membrane could locally sever actin filaments, leading to an increase in free filament ends for actin assembly. After that cofilin is dephosphorylated, possibly as a consequence of PKC activation, and recruited to sites of actin assembly (Djafarzadeh & Niggli, 1997). Binding of fMLP to its cell surface receptor triggers a series of cytoplasmic events that result in the activation of the cytoskeleton. This binding stimulates local F-actin formation by a mechanism not involving cofilin. The fMLP receptor undergoes affinity state changes modulated by G-proteins which consist of α, β, and γ subunits (Malech *et al*, 1985, Jin *et al*, 2000). Ligand binding causes a conformational change in the receptor that promotes the exchange of GTP for the GDP on the alpha subunit inducing the release of the α from the β γ complex. The α subunit interacts with phospholipase C (PLC, Park *et al*, 1993) whereas, the β γ subunit interacts with phosphoinositide 3 kinase enzymes (PI-3K, Clapham & Neer, 1993). PLC cleaves phosphotidyinositol-4,5-biphosphate (PIP_2) to generate the second messengers inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). The PI-3 kinases phosphorylate inositol phospholipids to produce PIP, PIP_2 and PIP_3. PIP_2 and PIP_3 are implicated in a wide variety of cellular processes, one of which is the reorganisation of the actin cytoskeleton (Toker & Cantley, 1997; Ramech & Cantley, 1999). Various studies

have used PI-3 kinase inhibitors to study PI-3 kinase activity in cellular processes, and it has been shown that fMLP-stimulated actin polymerisation in neutrophils seems to be only partially affected by PI-3 kinase inhibitors (Niggli & Keller, 1997). These findings suggest that there is also a PI-3 kinase independent pathway leading to actin polymerisation and the main function of PI-3 kinase is in detection and signalling of a chemotactic gradient (Cicchetti *et al*, 2002). The activity of PI-3 kinase has been linked to the activation of a family of small GTP-binding proteins related to Ras GTPases, which includes Rho, Rac and Cdc-42. The small GTPases are active regulators of cell shape change and motility in neutrophils (Katanaev, 2001; van Nieuw Amerongen & van Hinsbergh, 2001). The small GTPases, for example, Rac activates several pathways to stimulate actin polymerisation in three ways: a) nucleation of new filaments, b) extension from the barbed ends of the existing filaments or c) severing of filaments (Carpenter, 2000).

Morphoplogically, upon activation, cells change shape from round to an irregular shape with bleb formation and finally into a polar shape, and apparently shift F-actin content from diffuse to polar (Howard & Oresajo, 1985a), and might accumulate polymerised actin in the region first engaged by stimuli yielding saturated fluorescence intensity. Cells with polarised shape are characterised by a leading actin rich pseudopodia and a tail like rear end. This localization of F-actin at the leading end is a hallmark of cell polarisation that requires PIP_3 synthesis. Also, Sadhu *et al* (2003) showed that treatment of neutrophils with fMLP resulted in a significance increase in the mean surface area. The active forms of the small GTPases lead to different morphological changes, for example, cdc-42 leads to the formation of filopodia, Rac induce lamellipodia and membrane ruffles and Rho cause stress fiber and focal actin assembly (Nobes & Hall, 1995). Chodniewicz & Zhelev

(2003) used inhibitors of PI-3 kinase and G-protein coupled receptors in a micropipette assay to measure the rate of extension of single pseudopods from non-adherent human neutrophils during fMLP stimulation. Their results demonstrated that the polymerisation of 80% of the F-actin in the pseudopods region is PI-3 kinase dependent, while the remaining 20% is not. This is in agreement with the observations of Niggli & Keller (1997) stated above, that fMLP-stimulated actin polymerisation in neutrophils is only partially inhibited by PI-3 kinase inhibitors. However, fMLP stimulated pseudopod extensions were completely abolished in cells treated with G-protein coupled receptor inhibitors. Furthermore, the possibility for PLC dependence on the rate of pseudopod extensions was tested using PLC inhibitor and showed that this inhibitor had no effect on the rate of pseudopod extensions, however it completely inhibited the release of intracellular calcium. They also indicated that the PI-3 kinase-independent pseudopod extension in the neutrophil is dependent on NADPH oxidase using studies with NADPH oxidase inhibitors.

Cytochalasins were used to confirm that changes observed here in the F-actin content, are directly as a result of actin polymerisation. Initial studies used both CTB and CTD, but it was apparent that CTB was a more appropriate choice of F-actin disruptor due to the following results. Firstly, flow cytometric results showed that at higher concentrations of CTD, F-actin content was higher than control indicating enhancement of actin polymerisation, which agrees with Mills *et al* (2000). Furthermore, morphological observations using fluorescence microscopy revealed morphological changes in cell shape from spherical to irregular with some bleb formation in cells pre-treated with CTD, but CTB pre-treated cells were more or less similar to control. Lastly, the cellular response to CTD was more variable than with CTB, which showed good stability throughout a range of concentrations. Hence,

CTB was used for all studies henceforth. The effect of CTB was studied in fMLP-stimulated cells by exposure to different concentrations of fMLP. Flow cytometric analysis showed a significant ($p<0.001$ as determined by ANOVA) reduction in F-actin content as a result of CTB treatment by inhibiting the stimulatory effect of fMLP. It is well known that cytochalasin binds to the high-affinity end of the actin filament and blocks addition of monomer thus inhibiting actin polymerisation, and the results here confirm this mechanism.

To correlate F-actin formation in leukocytes with their filtration properties, a study was performed using whole blood in optimised conditions. The role of F-actin in the mechanical changes, in response to fMLP stimulation, was evaluated by simultaneous measurement of deformability changes and F-actin content in leukocytes. Leukocyte deformability was assessed using the Cardiff filtrometer to measure the time required for leukocytes to completely travel through a single, capillary sized pore (5μm) in a thin polycarbonate membrane, and also the number of pore-blockers. A pressure of 490 Pa (5cm H_2O), to drive cells through the pore, was used which was ideal to initiate the energy required for different blood suspensions to flow through the pore. Leukocyte transit times were measured in diluted blood using the initial flow rate and the transit time of erythrocytes filtered at the same concentration. Evaluation of changes in leukocyte deformability in diluted blood in the normal state and in response to PMA and fMLP was performed. Stimulation of cells with PMA at all concentrations used caused complete pore blocking; hence it was not possible to quantify this response. As a result of failing to obtain any filtration data from PMA stimulated cells, fMLP was used as a second stimulant. There was a marked and a significant increase in the transit time of leukocytes in diluted blood as fMLP concentration increased, with a corresponding increase in F-

actin content. This dose dependent increase in the cell rigidity, as measured by filtration, has been observed by others (Kawaoka *et al*, 19981; Worthen *et al*, 1989; Buttrum *et al*, 1994). At higher concentrations of fMLP, cells showed increased rigidity with a trebling of their pore transit times, and at the highest concentrations of fMLP were sometimes unable to flow at all. Additionally, there was a marked and a significant ($p<0.001$ as determined by ANOVA) increase in the total number of pore-blockers as the concentration of fMLP increased. Monocytes are less filterable than other leukocytes and are recorded as pore-blockers over 10 seconds in this filtration system (Kooshesh *et al,* 1991). These results agree with the findings of Kooshesh *et al* (1991) although recording flow profiles over a longer period of time (eg. 300 seconds) would enable measurement of the longer pore transit times of monocytes (Evans *et al*, 2001; Cook *et al*, 1998). In fMLP activated cells, there was a good correlation observed between the F-actin content of monocytes and the number of pore-blockers ($r=0.763$ with $p<0.001$ as determined by correlation analysis), and therefore it is likely that these cells were 'true' pore blockers, which would not flow through the filter in any length of time. Furthermore, another significant correlation ($r=0.789$ with $p<0.001$ as determined by correlation analysis) was seen between F-actin content of PMNs and the concentration of pore-blockers, which may mean that many PMNs have become too rigid to flow after fMLP treatment, and are therefore detected as pore-blockers.

In the presence of CTB, the increases in pore transit time seen in the presence of fMLP were abolished, and there was no increase in the number of pore blockers. This finding, that CTB inhibits the marked rigidification that follows activation has previously been reported in other studies using filtration techniques (Pecsvarady *et al*, 1992; Buttrum *et al*, 1994). Hence, these results confirm the remarkable ability of

PMNs and monocytes to undergo rapid mechanical changes resulting in decreased deformability and eliminate cell swelling and changes in the lipid membrane as the causes of reduced deformability results, therefore, implicate F-actin as the primary structure responsible for these mechanical changes.

Several studies suggested that increasing PM10 above minimal accepted values increases total mortality in respiratory and cardiovascular diseases (Mukae *et al*, 2001). Particulate pollution in the form of cigarette smoking is known to cause leukocyte activation, hence particulate air pollution, a real 21st century problem, could also be rheologically detrimental. Therefore, a collaborative, *in vivo* study using an animal model was performed, to investigate toxicological and circulatory effects. Exposure to PM10 resulted in no changes in the F-actin content of PMNs ($p=0.69$ as determined by two-sample t-test), with a possible, but not statistically significant increase ($p=0.14$ as determined by two-sample t-test), of monocyte F-actin. This suggests that PM10 instillation elicited some activation of monocytes, which is still in evidence 6 weeks after PM10 instillation ($p=0.05$ as determined by two-sample t-test). It is possible that phagocytosis of dust particles in the lungs, by alveolar macrophages, induces further circulatory leukocyte activation *in vivo* due to secretion of cytokines and other inflammatory mediators (Mukae *et al*, 2001).

Further studies in this area focussed on the effects of two environmentally common dusts, although despite much evidence of pulmonary inflammation, evidence of haematological alteration was very sparse. Despite this, there was a strong correlation between the plasma viscosity and other toxicological measurements such as lung free cell number ($r= 0.280$ with $p=0.014$ as determined by correlation analysis), lung:body weight ratio ($r=0.282$ with $p=0.013$ as determined

by correlation analysis), and lung acellular protein ($r=0.279$ with $p=0.014$ as determined by correlation analysis). These findings are possibly part of the story linking air pollution to cardiovascular risk, as plasma viscosity is a known marker of this (Yarnell *et al*, 1991). Theoretically, using an *in vivo* model of inflammation such as this is ideal for studying leukocyte activation and inflammation, although there are certain practical problems. For instance, using a rat model means a maximum of 5 ml of blood can usually be obtained, which can limit the rheological analyses one can perform. Studies are also limited by the number of animals available (due to costs and licence conditions), and if relatively small changes in measured variables are occurring, large numbers of animals need to be studied in order to reach a statistical significance.

Consideration of the relationship between F-actin polymerisation and diabetes, using PPAR γ agonists that are used in type II diabetes, should provide more beneficial information in pharmacological intervention. Pre-treatment of cells with different PPAR γ agonists resulted in decreases in the F-actin content of PMNs and monocytes and also inhibited the stimulatory response caused by fMLP. These responses instantly decreased F-actin content within 5 minutes and are unlikely to be caused by PPAR γ activation and changes in gene transcription as such changes would result in a far longer lag period before drug effects were observed. The drugs used may mediate their effect on F-actin by some mechanism independent of PPAR γ activation. Identifying the mechanism, by which drugs cause actin depolymerisation, could be used to prevent inappropriate leukocyte activation. The suggested mechanism by which the PPAR γ agonist is causing a decrease in the F-actin content of PMNs and monocytes may be explained in two ways: firstly, the presence of other biding sites on the agonist may allow the binding to other receptors such as plasma

membrane or cytoplasmic receptors before binding to the nuclear receptor. Secondly, PPAR γ agonists may inhibit phosphorylation of ADF/cofilin causing a depolymerisation process since the un-phosphorylated cofilin is responsible for causing actin depolymerisation to occur. These suggestions require further investigation and the effect of PPAR γ agonists on signalling pathways should be investigated.

Evaluation of the circulatory consequences of activation of PMNs and monocytes requires a detailed knowledge of their rheological response. Thus, the work presented here carried out comparative studies of the properties of PMNs and monocytes in whole blood, exposed to different types of stimuli (PMA, fMLP and particulate air pollution) and different pharmacological agents. These studies support the importance of F-actin in determining cell rigidity, with all measurements made on whole blood with no prior isolation of cell sub-populations. To this end, it is hoped that a greater understanding of this relationship between actin polymerisation and blood cell deformability could facilitate pharmacological intervention. For instance, drugs that could inhibit inappropriate activation and hence actin polymerisation would improve haemorheological disturbances, particularly in the microcirculation. However, the association between abnormal rheology and physiological or clinical relevance is not always clear, hence, studies on pharmacological agents which improve some aspects of leukocyte function should be shown to have a clinically beneficial endpoint. PPAR γ could be such a drug, although the mechanism by which F-actin content is decreased is less well understood. Identifying other receptors that PPARγ agonists could bind are potential targets for therapeutic intervention, raising hope for victims of cardiovascular diseases.

7.1 Future directions

1. In order to achieve a full understanding of the process by which filamentous actin (F-actin) is activated and its involvement in pathogenesis such as cardiovascular diseases, the exact association between this protein and CVD need to be elicited. The mechanisms by which mechanical signals are transduced to F-actin are still not completely understood; therefore, further studies involving these signalling pathways, and those of inflammatory cytokines, could be of great benefit. The exact role of actin binding proteins in activating F-actin need further study as these proteins are responsible for actin polymerisation and depolymerisation processes. The *in vitro* interaction of PIP and PIP_2 with different actin binding proteins, such as profilin and gelsolin (Hartwig & Yin, 1988), suggests that these phosphoinositides might be involved in actin polymerisation, but the precise role of these actin-binding proteins remains to be elucidated. Furthermore, based on the suggestion that MAP kinases may mediate responses in the membrane, cytoplasm, nucleus, or cytoskeleton (van Biesen *et al*, 1996), studying the relationship between MAP kinases, actin polymerisation and cell deformability in activated cells using techniques such as western blotting is required. Upon activation by G-protein coupled receptors, MAP kinases translocate to the nucleus, where they phosphorylate and activate nuclear transcription factors involved in DNA synthesis and cell division (Julius *et al*, 1989).

2. Currently, it is common practice in the field of haemorheology to separate out cell sub-populations and study them individually, by filtration or micropipette methods. Development of the Cardiff Filtrometer to separate the pore transit times of lymphocytes and PMNs during filtration of blood would be of great benefit. Detailed dissection of deformability by micromanipulation can yield information on the visco-elasticity of phagocytic leukocytes, and study of the membrane elasticity, in

conjunction with F-actin formation, would be advantageous. In addition to deformability changes, leukocyte adhesion is also part of the rheological phenomenon so studies, which include measurements of the adhesive state, such as plasma levels of circulating leukocyte adhesion molecules, will undoubtedly be useful. Correlating F-actin formation with both cell deformability (by filtration or otherwise) and cell adhesion would yield a more complete rheological picture of blood flow *in vivo.*

3. In the animal model used here, there is good potential to get information on the leukocyte properties and activation *ex vivo.* The inflammatory insults are well defined and well timed, and the harvested blood cells have been circulating *in vivo* after the insult. Measurement of plasma fibrinogen would be possible and useful here, particularly in view of the correlations seen between the plasma viscosity and lung markers. Other appropriate indicators of inflammation and leukocyte adhesion would yield more information, although filtration studies, on the Cardiff Filtrometer at least, are not possible due to inadequate blood volumes obtained.

4. Further studies on PPAR γ agonists may help to establish the mechanism by which a decrease in the F-actin content was observed in normal and stimulated blood pre-treated with PPAR γ agonist. The signalling pathways of calcium and phosphate could be involved to some extent to cause actin depolymerisation. Since the un-phosphorylated cofilin is responsible for actin depolymerisation processes (Arber *et al*, 1998; Matsui *et al*, 2002), the role of ADF/cofilin could be studied also by investigations with dominant LIM1 kinase. At present, cofilin is the only known substrate of LIM1 kinase that could affect the reorganisation of actin cytoskeleton, which is known to inhibit the depolymerisation activity of cofilin through

phosphorylation of cofilin, therefore causing actin polymerisation. In addition, the role of Ca^{2+} maybe studied as various studies had indicated that Ca^{2+} plays a pivotal role for many responses, such as actin polymerisation, in PMNs stimulated by fMLP (Lew, 1989). Gremm & Wegner (2000) studied the effect of increasing [Ca^{2+}] on gelsolin, the actin capping protein, and showed that the activity of gelsolin has increased by binding to the barbed end of actin filaments and inhibiting polymerisation, therefore, causing depolymerisation of actin to occur.

5. Potential for further studies of the effect of PPAR γ agonists on F-actin polymerisation *in vivo* can be possible at this stage as a current study is possibly approaching this effect. Using diabetic patients before and after pharmacological therapy with TZDs could approach this study. Prior to the commencement of the drug traement, samples can be taken from each subject. On treatment with PPARγ, samples can be taken on a regular interval to observe any changes in F-actin polymerisation *in vivo*.

7.2 Conclusion

Intracellular F-actin content can be assessed in whole blood by staining F-actin with FITC-phalloidin. The concentration of intracellular F-actin can be semi-quantitatively assessed by flow cytometry. Morphological changes associated with actin polymerisation can be visualised by fluorescence microscopy and the spatial arrangement of F-actin within the cell can be assessed. These whole blood methods allow determination of F-actin within leukocytes with minimal manipulation of cells prior to assay and experimental artefacts are, therefore, minimised.

Increased actin polymerisation is associated with changes in cell rheology. Increased intracellular F-actin is significantly correlated with increased leukocyte

transit time (r= 0.217 with p=0.029) and pore blocking (r=0.798 with p<0.001 for PMNs and r=0.763 with p<0.001 for monocytes). Incubation of cells with cytochalasin B reduces actin polymerisation and normalises leukocyte rheology. Actin polymerisation is, therefore, the major cause of changes in leukocyte filterability, and is a simple and reproducible measure of changes in leukocyte rheology.

Exposure to particulate air pollution is an inflammatory stimulus that results in changes in blood rheology. In a rat model, exposure to particulate air pollution results in increased leukocyte actin polymerisation and increased plasma viscosity. These results support the hypothesis that changes in blood rheology is the mechanism by which increased exposure to particulate air pollution causes increased myocardial infarction.

Incubation of blood with pharmacological agents, which modify PPAR γ receptor function, reduces leukocyte actin polymerisation. This effect is independent of the PPAR γ receptor. These agents may be used to reduce actin polymerisation and improve leukocyte rheology in a range of disease states.

In conclusion, actin polymerisation is a major determinant of changes in leukocyte rheology. Actin polymerisation is increase by exposure to a range of exogenous stimuli and can be reduced by a number of pharmacological agents.

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