# STUDIES ON MECHANISMS INVOLVED IN MACROPHAGE RECOGNITION OF APOPTOTIC NEUTROPHILS

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## ABSTRACT.

Macrophage phagocytosis of apoptotic cells represents a non-inflammatory mechanism for the removal of senescent neutrophils from inflamed sites. The macrophage integrin αvβ3 and CD36 have been proposed to act in concert to bind thrombospondin which forms a molecular bridge between the surface of the macrophage and the apoptotic neutrophil. This thesis has investigated the regulatory mechanisms underlying this macrophage phagocytic system.

Phagocytosis of apoptotic neutrophils by monocyte-derived macrophages was found to be modulated by short term pre-treatment of macrophages with activators of protein kinase C and protein kinase A. In contrast, FcR-mediated phagocytosis was unaffected by protein kinase activation, suggesting specific modulation of this macrophage phagocytic system. Examination of the molecules thought to be involved in this phagocytic pathway revealed that thrombospondin production by macrophages was undetectable after 30 minutes and no change in thrombospondin binding or surface expression of αvβ3 and CD36 occurred following protein kinase activation. However, altered distribution of the β3 integrin subunit did occur with a more localised distribution observed following activation of protein kinase C. Surprisingly, a number of monoclonal antibodies against αvβ3 did not inhibit phagocytosis and αvβ3 was not found to colocalise with the macrophage actin cytoskeleton or with ingested apoptotic neutrophils. These results questions the involvement of αvβ3 in the phagocytosis of apoptotic neutrophils.

Protein kinase A activation was seen to cause disruption of the macrophage actin cytoskeleton. Other implicated inhibitors were subsequently found to disrupt the macrophage actin framework. Since cytoskeletal integrity is required for cell adhesion processes, the adhesion state of the macrophage during phagocytosis of apoptotic neutrophils was investigated. Adherence of macrophages to extracellular matrix proteins was found to increase levels of phagocytosis compared to control conditions. By contrast, very little phagocytosis was observed when the assay was performed in suspension suggesting that the adhesion state of the macrophage is an important factor in regulating phagocytosis of apoptotic neutrophils.

In summary, no evidence was obtained to support the direct involvement of the αvβ3 integrin receptor in this phagocytic system. Macrophage phagocytosis of apoptotic neutrophils can be modulated by activation of protein kinases possibly by affecting the macrophage actin cytoskeleton. Data presented in this thesis suggest that the adhesion state of the macrophage is important in regulating phagocytosis of apoptotic neutrophils. Thus the tissue micro-environment of the macrophage may exert control over macrophage removal of apoptotic cells and hence the process by which inflammation resolves.

## ABREVIATIONS.

| | |
|---|---|
| αvβ3 | Vitronectin integrin receptor |
| α5β1 | Fibronectin integrin receptor |
| AGE | Advanced glycosylation endproducts |
| Arg | Arginine |
| Asp | Aspartamine |
| BSA | Bovine serum albumin |
| C5a | Complement fraction 5a |
| $Ca^{2+}$ | Calcium |
| cAMP | Cyclic AMP |
| CFDA | Carboxyfluorescene diacetate |
| $CO_2$ | Carbon dioxide |
| Col I | Collagen I |
| Col VI | Collagen VI |
| Cys | Cysteine |
| DMSO | Dimethylsulphoxide |
| dbcAMP | Dibutyryladenosine 3'5'-cyclic monophosphate |
| ECM | Extracellular matrix |
| EDTA | Ethylene diamine tetra-acetic acid |
| FITC | Fluorescein isothiocyanate |
| fMLP | N-formyl-L-methionyl-L-leucyl-phenylalanine |
| Fn | Fibronectin |
| Gly | Glycine |
| GM-CSF | Granulocyte macrophage colony stimulating factor |
| H-7 | Isoquinolinylsulfonyl methypiperazine |
| HBSS | Hanks balanced salt solution |
| IBMX | Isobutyl methyl xanthine |
| Ig | Immunoglobulin (-G, -M) |
| IL | Interleukin (-1, -8) |
| Iscove's DMEM | Iscove's Dulbeco's modified Eagles medium |
| LPS | Lipopolysaccharide |
| mø | Monocyte-derived macrophages |
| mAb | Monoclonal antibody |
| $Mg^{2+}$ | Magnesium |
| MLCK | Myosin light chain kinase |
| MNC | Mononuclear cells |

| | |
|---|---|
| MPO | Myleoperoxidase |
| NRS | Normal rabbit serum |
| PdBu | Phorbol 12, 13 dibutyrate |
| PBS | Phosphate buffered saline |
| PBS-T | PBS containing 20% Tween 20 |
| PE-CD14 | Phycoerythrin conjugated CD14 |
| $PGE_2$ | Prostaglandin $E_2$ |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| PMA | Phorbol myristate acetate |
| PMN | Polymorphonuclear granulocytes |
| PPP | Platelet poor plasma |
| RGD(S) | Arginine-Glycine-Aspartamine(-Serine) |
| S.D. | Standard deviation of mean |
| S.E. | Standard error of mean |
| Ser | Serine |
| TBS | Tris buffered saline |
| Thr | Threonine |
| TNF | Tumour necrosis factor |
| Val | Valine |
| Vn | Vitronectin |

# CHAPTER 1. INTRODUCTION.

## 1.1. CELLULAR EVENTS IN INFLAMMATION.

In the aftermath of an insult to tissue, a stereotypic series of reactions is initiated by the host in an effort to prevent further tissue damage, isolate and destroy the injurious agent and instigate the repair process. This cumulative homeostatic process is known as inflammation. The manifestations of inflammation have been described as far back as Roman times by Cornelius Celsus as rubor, tumor, calor and dolor (redness, swelling, heat and pain) and the term inflammation is derived from the Latin inflamare, meaning to burn.

The immediate and subsequent set of reactions that are induced are known as the acute phase response (Baumann and Gauldie, 1994). A number of factors can cause inflammation including bacteria and other microbes, physical agents (burns, radiation, trauma), chemicals (toxins and caustic substances), necrotic tissue and all types of immunologic reactions. All of these insults are followed by a typical response. The accumulation of leukocytes, primarily neutrophils and monocytes, is the most important feature of the acute phase response. Chemokines are released from the damaged site that control the migration of a variety of cell types into the affected tissue. The recruitment and involvement of leukocytes in the inflammatory response can be summarised as follows:

Margination: as slowing and stagnation of the blood flow occurs leukocytes have increased contact with the endothelium.

Adhesion: involving specific interactions between complementary adhesion molecules on the leukocyte and endothelium.

Emigration: movement of leukocytes between the endothelial cells, through the basement membrane into the extravascular space.

Chemotaxis: migration of cells in response to the chemotactic agents released from the damaged site.

Phagocytosis: engulfment and disposal of injurious particle.

The cell types present in the inflammatory response vary with the age of the lesion and the type of stimuli. However, in most types of acute inflammation, neutrophils predominate in the first 6-24 hours, being replaced by monocytes in 24-48 hours. Monocyte emigration is sustained long after neutrophil emigration has ceased. This is accounted for by chemotactic factors acting differentially on neutrophils and monocytes during different phases of the inflammatory response.

The initial acute reaction to all types of injury lasts from a few minutes to one or two days. The subsequent resolution of this reaction depends on the nature and duration of the inflammatory stimulus, the type of tissue injured and the degree of tissue damage caused by the agent. Acute inflammation may terminate in one of four possible ways:

Resolution: complete restoration of the inflamed site to normal structure and function.

Healing by scar: a result of substantial tissue damage. The lost tissue may be either replaced by the formation of new tissue of the type that was destroyed or the area may be filled by the in growth of newly formed connective tissue (scar tissue).

Suppuration: a result of a persistent stimulus of the type that evokes massive emigration of polymorphonuclear cells. Characterised by

the formation of an inflammatory exudate (pus) containing large numbers of degraded polymorphs and dead tissue cells.

Chronic inflammation: the inflammatory reaction persists for weeks or months after the initial exposure to the damaging agent e.g. pneumonia and arthritis.

Inflammation is considered a pathological response leading to tissue damage and disease and much attention has been paid in the past to the initiation and amplification of this process. However, little work has been directed towards the mechanisms involved in the resolution of inflammation which may provide significant insights as to why some disease states persist rather than resolve.

## 1.2. MACROPHAGES.

### 1.2.1. Origin.

Macrophages are ubiquitously distributed throughout the body and are involved in numerous homeostatic, immunologic and inflammatory processes. Macrophages are derived from common precursors in the adult bone marrow, circulate in the bloodstream as mature monocytes which, under normal steady-state conditions, migrate into tissues and body cavities where they differentiate into macrophages. The majority of resident tissue macrophages are derived from monocytes. However, there is evidence that a small percentage of macrophages derive from local division of non-resident mononuclear phagocytes that have arrived in tissues from the bone marrow before completion of cell division. Under normal steady-state conditions, the macrophage population within

tissues remains constant and is regularly renewed by the influx of monocytes (van Furth, 1992).

### 1.2.2. Macrophage phagocytic receptors.

The wide tissue distribution of macrophages and their phagocytic capacity provide an immediate defence against foreign elements such as bacteria, viruses, fungi and protozoa prior to leukocyte recruitment. Phagocytosis involves the binding of a particle to the surface of the phagocyte and subsequent engulfment of the bound particle. The attachment step is mediated by specific macrophage receptors. Opsonisation of bacteria with complement and/or immunoglobulin-G (IgG) permits macrophage recognition and subsequent phagocytosis of invading micro-organisms.

The macrophage has three surface receptors that recognise the Fc domain of IgG (Schriber *et al.*, 1992). FcRI (CD64: 72kD) is a high affinity receptor for IgG1 and IgG3, expressed mainly on macrophages and monocytes. FcRII (CDw32: 40kD) has a lower affinity for IgG than FcRI and is expressed on a variety of cell types including macrophages, platelets, eosinophils and B cells. The third receptor, FcRIII (CD16: 51-73kD), is found on neutrophils, natural killer cells and macrophages, but not monocytes and has intermediate affinity for IgG1 and IgG3. Ligation of Fc receptors induces the release of reactive oxygen intermediates, arachidonic acid metabolites and other mediators of inflammation. As well as mediating avid binding and phagocytosis of particles coated with immune complexes, Fc receptors are implicated in the removal of senescent erythrocytes. Kay (1975) describes the *in situ* attachment of IgG to senescent human erythrocytes thus making them susceptible to macrophage phagocytosis.

Many micro-organisms are capable of activating the complement cascade, and C3b and C3bi, fragments of the third component, are major opsonins. CR1 (CD35) is a glycoprotein, molecular weight 160-250kD, found on all phagocytic leukocytes and recognises C3b. CR3 and CR4 bind C3bi and are found on all phagocytic leukocytes, with regulated expression observed upon monocyte-macrophage maturation. CR3 and CR4 are composed of two non-covalently linked subunits (CD11b/CD18 and CD11c/CD18 respectively) and are members of the β2 family of integrins (Hynes, 1992). Phagocytosis via CR1 and CR3 differs from that of FcR-mediated phagocytosis in that receptor binding alone is not sufficient to induce phagocytosis. Activation of the receptors, e.g. by substrate bound fibronectin (Wright *et al.*, 1983) or PMA (Wright *et al.*, 1982), is necessary to promote subsequent phagocytosis. Furthermore complement receptor-mediated phagocytosis does not result in the release of inflammatory mediators and may provide an alternative non-antagonistic pathway for the clearance of opsonised particles during inflammation.

Glucose reacts non-enzymatically with amino acid groups of a wide range of proteins throughout the body, including cell membrane proteins on erythrocytes (Millar *et al.*, 1980), to form advanced glycosylation endproducts (AGE). Macrophages also express receptors for AGE and these receptors have been shown mediate phagocytosis of AGE-modified erythrocytes (Vlassara *et al.*, 1987).

### 1.2.3. Macrophage functions.

Macrophages have diverse biological roles (Auger and Ross, 1992). The wide tissue distribution of macrophages provides an immediate defence against foreign elements such as bacteria, viruses, fungi and protozoa

prior to leukocyte recruitment. Fc receptors and complement receptors enable macrophages to recognise and phagocytose opsonised micro-organisms (section 1.2.2). Phagocytosis can also occur without opsonisation if the micro-organism expresses surface molecules that are directly recognised by the macrophage e.g. carbohydrate residues. Furthermore, macrophages assist in cell-mediated immune responses against pathogens, producing immunomodulatory cytokines and regulating immunological responses. Phagocytosed antigen is processed and presented to lymphocytes in conjunction with Class I or II molecules of the major histocompatability complex (Unanue and Allen, 1987) thereby stimulating antigen-specific T cells. Macrophages are a source of many important secretory substances including cytokines and enzymes, cytokine and enzyme inhibitors, growth factors, hormones, complement components, coagulation factors and extracellular matrix proteins (catalogued in Auger and Ross, 1992). Many of these secretory products are induced by inflammatory or infectious stimuli and have roles in host defence and the inflammatory response. Macrophages are also believed to be involved in defence against tumours, secretory products inhibiting tumour cell growth and leading to lysis of neoplastic cells (Adams and Hamilton, 1992 and references therein).

### 1.2.4. Macrophages in inflammation.

Tissue macrophages are central to initiating the acute phase response. Macrophages that have immigrated into sites of inflammation have a large and diverse potential for further development. Upon activation (Adams and Hamilton, 1984; Paulnock, 1992), resident macrophages acquire increased capabilities for killing of micro-organisms and tumour cells. Activated macrophages also display maximal secretion of

inflammatory mediators such as tumour necrosis factor (TNF)-α, prostaglandin $E_2$ ($PGE_2$), interlukin (IL) -1 and IL-6 (Keshav *et al.*, 1990). These cytokines act on stromal cells e.g. fibroblasts and endothelial cells, causing a secondary release of cytokines including chemotactic factors for neutrophils (e.g. IL-8) and monocytes (e.g. monocyte chemoattractant protein). During an inflammatory reaction, there is increased recruitment of peripheral blood monocytes to the perturbed site where they differentiate into macrophages. The transition from monocyte to macrophage occurs over approximately 48 hours and involves considerable increase in size, the number of cytoplasmic organelles and surface molecules involved in effector function thereby enhancing the potential capabilities of the differentiated cell.

### 1.3. FATE OF THE NEUTROPHIL AT INFLAMED SITES.

Inflammation evolved as a regulated process which results in the removal of invading pathogens and/or damaged cells and repair and remodelling of host tissue. The neutrophil is the first cell recruited to damaged areas and has evolved a variety of biological agents (listed in Bainton, 1992) to aid bacterial killing. The ultimate fate of the large numbers of recruited neutrophils during the resolution of inflammation has received little attention until relatively recently (Haslett *et al.*, 1989). It had generally been assumed that neutrophils had a short tissue life and that they inevitably underwent necrosis and disintegrated *in situ* before their fragments were cleared by local phagocytes (Hurley, 1983). However, in view of the capacity of many of the granule contents of neutrophils to cause tissue damage (Weiss, 1989) and cleave tissue matrix proteins into chemotactic fragments (Vitario *et al.*, 1981) and evidence that neutrophil

accumulation plays a role in other inflammatory events such as generation of oedema (Wedmore and Williams, 1981) and influx of monocytes (Doherty *et al.*, 1988), necrotic neutrophil cell death would tend to damage healthy tissues and exacerbate the inflammatory process. Therefore, the clearance of the large number of neutrophils recruited to damaged tissues may be a prerequisite for the resolution of acute inflammation.

There is now evidence for an alternative fate of neutrophils whereby macrophages phagocytose intact neutrophils that have undergone apoptosis.

## 1.4. CELL DEATH: NECROSIS AND APOPTOSIS.

There are two main mechanisms of death in nucleated eukaryotic cells, characterised by both morphologic and biochemical differences.

### 1.4.1. Necrosis.

Necrosis is a pathological response to gross tissue insult such as severe hypoxia, ischemia or exposure to toxins and typically affects a number of contiguous cells. Necrosis is characterised by disruption of membrane integrity, including plasma, organelle and nuclear membranes, resulting in lysis of the cell. It is thought that the critical event leading to the development of necrosis is the loss of the properties of the membrane that are involved in regulation of the cell volume; a fall in membrane potential, loss of potassium from the cell and increased sodium entry. The final critical event of necrosis is a gross change in membrane permeability as demonstrated by the failure of the cell to exclude vital

dyes. A fall in intracellular ATP levels may relate to the loss of membrane function. The release of intracellular components as a result of the rupture of the cells often induces an inflammatory response that causes secondary tissue damage. Further aspects of necrosis have been considered in greater detail by Wyllie *et al.* (1980).

### 1.4.2. Apoptosis.

In contrast to necrosis, apoptosis is characterised by retention of cell membrane integrity and shrinkage of the cell. Its role in physiological and pathological events has been extensively reviewed (Wyllie *et al.*, 1980; Raff, 1992; Cohen and Duke, 1992; Cohen, 1993; Krammer *et al.*, 1994; Ashwell *et al.*, 1994). However, an overview of the main features will be given here. This form of programmed cell death is morphologically distinct from necrosis. The plasma membrane remains intact but becomes ruffled and blebbed while the cytoplasm condenses and becomes vacuolated. Apoptotic cells, in contrast to necrotic cells, do not show evidence of increased membrane permeability and exclude vital dyes. The most characteristic feature of apoptosis is the collapse of the nucleus while all other organelles remain intact. The nuclear membrane usually convolutes and the chromatin becomes condensed. Ultimately, the nucleus breaks up into one or several condensed fragments. Finally, the cell breaks up into membrane-bound apoptotic bodies which are dispersed into the intercellular tissue space and are either extruded into an adjacent lumen or, more commonly, phagocytosed by other cells.

Apoptosis is a gene directed process. Evidence for this has come from work carried out on the nematode *C. elegans* in which, at specific stages of development, a number of cells die by apoptosis. The activity of two

genes, *ced-3* and *ced-4*, is necessary for apoptosis to occur (Ellis and Horvitz, 1986). In contrast, activation of the gene *ced-9* suppresses apoptotic cell death (Hengartner *et al.*, 1992). A homologue of *ced-9* is *bcl-2* which has been shown to spur apoptosis in mammalian cells (Vaux *et al.*, 1988: Hengartner and Horvitz, 1994). The effect of Bcl-2 may be regulated by a related gene product, Bax (Oltvai *et al.*, 1993). The mammalian homologue of *ced-3* and *ced-4* that induces apoptosis is IL-1β converting enzyme (ICE: Lazebnik *et al.*, 1994). In addition, *c-myc* has also been shown to induce apoptosis in mammalian cells (Evans *et al.*, 1992: Shi *et al.*, 1992). Like *bcl-2*, *c-myc* is regulated by dimerisation with *max* (Blackwood and Eisenman, 1991). The nuclear collapse in apoptosis is associated with degradation of cellular DNA into single and multiple nucleosomes that results in the characteristic "ladder" pattern electrophoresis. This degradation of DNA appears to be a result of an enzymatic process, although the properties of the endogenous endonuclease remain to be determined.

Apoptosis is a physiological mode of cell death implicated in the steady-state kinetics of healthy tissues. Apoptosis characteristically affects single cells within tissues but during events such as embryological remodelling, large fields of cells undergo apoptosis. Even under these circumstances, apoptotic cell death does not induce an inflammatory response due to preservation of membrane integrity and rapid phagocytosis of apoptotic bodies.

### 1.4.3. Apoptosis in neutrophils.

Neutrophils constitutively undergo apoptosis during ageing *in vitro* (Savill *et al.*, 1989a) however, the rate of apoptosis can be modulated by inflammatory mediators such as GM-CSF, C5a and LPS (Brach *et al.*, 1992: Lee *et al.*, 1993). Whyte *et al.* (1993a) also report inhibition of neutrophil apoptosis upon elevation of intracellular free calcium, an event that has been shown to trigger apoptosis in other cells (McConkey *et al.*, 1989a: McConkey *et al.*, 1989b: Allbritton *et al.*, 1988). In addition to the characteristic morphological and nuclear changes associated with apoptosis (Wyllie *et al.*, 1980), Dransfield *et al.* (1994) describe altered CD16 expression on the surface of apoptotic neutrophils. During apoptosis, neutrophil membrane integrity is preserved and granule contents are retained within the cell (Savill *et al.*, 1989a) and a number of neutrophil functions such as chemotaxis, phagocytosis and granule release (Whyte *et al.*, 1993b) are down regulated. Phagocytosis of neutrophils by macrophages is also determined by apoptosis (Savill *et al.*, 1989a).

## 1.5. MACROPHAGE RECOGNITION OF APOPTOTIC CELLS.

### 1.5.1. Overview.

The rapid phagocytosis of apoptotic cells may prevent potential tissue damage as a result of lysis of these cells *in situ*. A number of apoptotic cell types have been identified as being recognised and phagocytosed by macrophages. Recognition of apoptotic cells is thought to involve multiple receptor-ligand interactions and current findings are summarised in Table 1.1. It is not known if recognition of apoptotic thymocytes by the sugar-lectin mechanism, described by Duvall *et al.*,

| APOPTOTIC CELL | RECOGNITION MECHANISM | REFERENCE |
|---|---|---|
| Thymocyte | Interaction between a thymocyte carbohydrate moiety and a lectin-like murine macrophage receptor * | Duval *et al.* (1985) |
| Neutrophil | Macrophage vitronectin receptor and CD36 act in concert to bind thrombospondin which acts as a molecular bridge between the macrophage and a negatively charged structure on the neutrophil | Savill *et al.* (1989a,b), (1990), (1992) |
| | Monocyte/macrophage-associated antigen specified by the mAb 61D3 functions as a receptor for apoptotic cells (also applicable for recognition of apoptotic lymphocytes). | Flora and Gregory, (1994) |
| Eosinophil | Unknown | Stern *et al.* (1992) |
| Lymphocyte | Loss of membrane phospholipid symmetry on lymphocyte membrane resulting in exposure of phosphatidylserine which is consequently recognised by an unknown ligand on the macrophage * | Fadok *et al.* (1992a) |

**Table 1.1. Summary of recognition mechanisms involved in macrophage recognition of apoptotic cells.** * murine model

(1985) also mediates phagocytosis of the apoptotic cell as this was a rosetting assay performed at 4°C. However, a role for a lectin in the uptake of apoptotic hepatocytes in rats has been indicated (Dini *et al.*, 1992). Fadok *et al.* (1992b) have also suggested that the mechanism by which apoptotic cells are recognised and phagocytosed by macrophages may be determined by the subpopulation of macrophages being examined. In a study of four different macrophage populations, murine peritoneal macrophages were found to bind apoptotic cells via phosphatidylserine while murine bone marrow macrophages use both the vitronectin receptor, αvβ3, (like human monocyte-derived macrophages) and phosphatidylserine receptor-dependent pathways (Fadok *et al.*, 1992b). Recently, another macrophage surface antigen has been identified as a mediator of apoptotic cell recognition (Flora and Gregory, 1994). Recognition of apoptotic neutrophils and lymphocytes was inhibited by the mAb 61D3, a widely used marker of monocyte/macrophage lineage cells. Furthermore, this recognition pathway is distinguishable from the αvβ3 mechanism involved in the recognition of apoptotic neutrophils. Thus, the mechanisms underlying rapid clearance of apoptotic cells may involve multiple phagocytic receptors.

**1.5.2. Macrophage phagocytosis of apoptotic neutrophils.**

Phagocytosis of neutrophils by macrophages has been described as far back as the turn of the century by Metchnikoff, providing evidence of an alternative neutrophil disposal route. A proportion of human monocyte-derived macrophages (mø) constitutively recognise and phagocytose aged apoptotic, but not freshly isolated, neutrophils (Newman *et al.*, 1982; Savill *et al.*, 1989a) indicating that the apoptotic cell provides a phagocytic

signal to the mø. In contrast to macrophage phagocytosis of particles opsonised with immunoglobulin, phagocytosis of apoptotic neutrophils does not evoke the release of pro-inflammatory macrophage mediators such as thromboxane B2 or N-acetyl-β-D-glucosamine (Meagher *et al.*, 1992). Such a mechanism for neutrophil clearance would tend therefore to limit tissue injury and promote resolution of inflammation and there is now clear histological evidence for the involvement of this process in acute inflammation (Savill *et al.*, 1989a; Jones *et al.*, 1993).

Two distinct recognition mechanisms have been described for human monocyte-derived macrophage phagocytosis of apoptotic neutrophils (Savill et *al.*, 1992; Flora and Gregory, 1994). Work presented in this thesis is based on the model for human macrophage recognition of apoptotic neutrophils proposed by Savill and colleagues in which three different molecules are thought to be involved: (1) the mø integrin αvβ3 (CD51/CD61) (Savill *et al.*, 1990), (2) mø CD36 and (3) the extracellular matrix molecule thrombospondin (Savill *et al.*, 1992). Savill and co-workers have proposed a model in which αvβ3 and CD36 on the mø surface act in concert to bind thrombospondin, thus forming a molecular bridge between the mø and an as yet unknown moiety on the apoptotic neutrophil surface (Fig. 1.1).

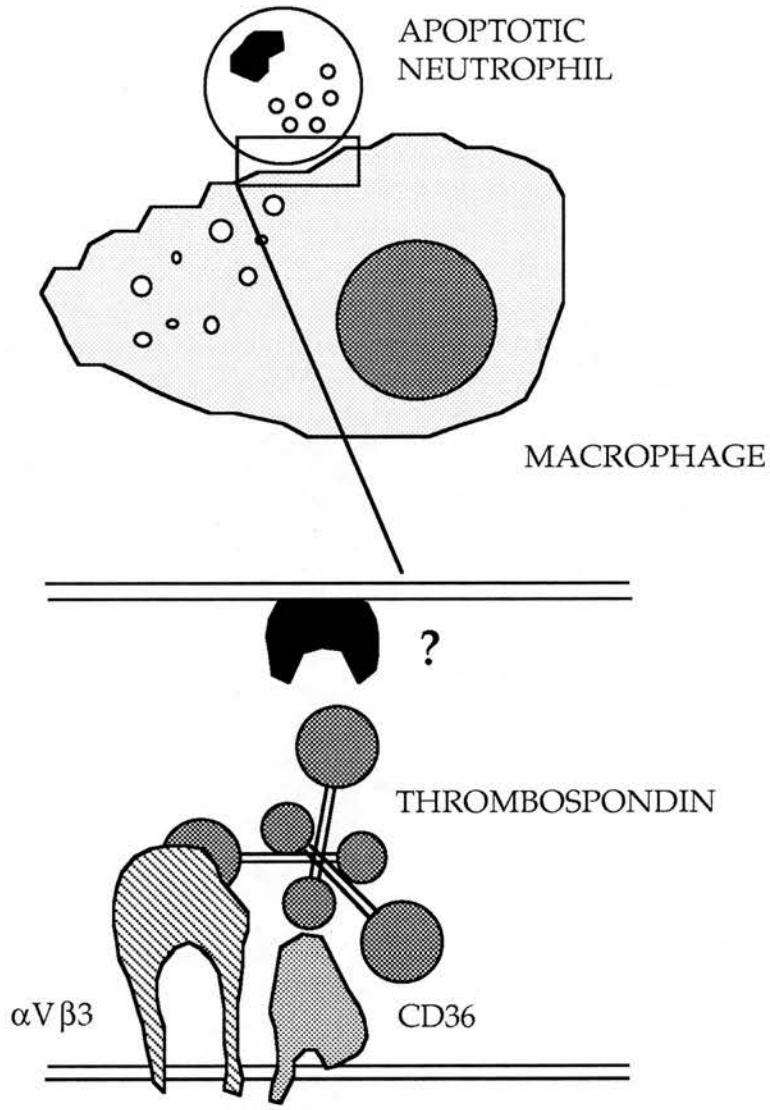


**Figure 1.1. Proposed mechanism by which macrophages recognise apoptotic neutrophils.**

Mø αvβ3 and CD36 act in concert to bind thrombospondin which acts as a molecular bridge between the mø and an unknown moiety (?) on the surface of the apoptotic cell (Savill *et al,* 1992). Reproduced with permission from Dr. I. Dransfield.

## 1.6. MOLECULAR MECHANISM OF MACROPHAGE PHAGOCYTOSIS OF APOPTOTIC NEUTROPHILS.

### 1.6.1. The αvβ3 vitronectin receptor.

The vitronectin receptor, αvβ3, is a member of the integrin family of adhesion molecules which are the major receptors mediating adhesion of cells to the extracellular matrix (ECM) and some important cell-cell adhesion events. Integrin expression is universal, at least one member of the family has been found on every cell/tissue studied. All integrins are heterodimers of a 120-180kD α–subunits noncovalently linked to smaller β-subunits (90-110kD). At least sixteen α-subunits and eight β-subunits have been identified. Many α-subunits associate with only one β-subunit, but several can associate with more than one β-subunit and, to date, twenty distinct integrin receptors are recognised (Hynes, 1992). Both subunits are transmembrane glycoproteins with short carboxyl terminal cytoplasmic domains, a single hydrophobic transmembrane segment and large globular amino terminal extracellular domains that associate to form the heterodimer. The subunits are products of different genes and have been highly conserved in evolution, the β-subunits show 37-45% homology while the α-subunits are 25-30% homologous.

Integrins are a phylogenetically ancient family of molecules. The term "integrin" was first coined as these receptors provide a transmembrane link between the outside of the cell and the cytoskeleton and were found to be homologous to the chicken fibroblast fibronectin receptor (the chicken integrin) (Tamkun *et al.*, 1986). The extracellular domains of integrins interact with a wide variety of ligands including ECM glycoproteins, complement components and cell surface glycoproteins. It is likely that the ligand binding site is contributed to by both subunits.

Some integrins bind different ligands while others can bind only one. Furthermore, several different integrins can bind to the same ligand but the same sequence is not always recognised. The binding of integrin and ligand requires divalent cations and is of low affinity ($10^{-6}$M). Putative divalent cation binding sites may be present on both subunits. Integrin β-subunit cytoplasmic domains have been shown to indirectly associate with the actin skeleton via cytoskeletal proteins such as talin (Horwitz *et al.*, 1986) and α-actinin (Otey *et al.*, 1990). There is less evidence for interaction of the α-subunit cytoplasmic tail with intracellular proteins although Leung-Hagesteijn and co-workers (1994) have demonstrated that calreticulin, an intracellular calcium-binding protein binds to an integrin α-subunit and can affect cell attachment to ECM substrates.

As well as providing a physical link between the cell and the ECM, it is clear that integrins are capable of transducing signals that affect a range of cellular events. The avidity of integrin complexes for their ligand can be modulated by inside-out signals from other receptors or accessory molecules (Ginsberg *et al.*, 1992) while outside-in signals from integrin-ECM contacts promotes phosphorylation of intracellular components despite the cytoplasmic domains having no intrinsic kinase activity (Damsky and Werb, 1992). Cellular responses to extracellular information is tightly regulated. Integrin-related signals are transduced in co-operation with other classes of adhesion receptors and/or growth factor receptors to ensure cells respond in the appropriate environmental context. Dysregulation of such integrated responses appear to be fundamental to the aetiology of disease.

The αvβ3 receptor is a member of the "cytoadhesin" subfamily of integrins and is expressed on a number of cell types. In terms of ligand binding, it is a highly promiscuous receptor, binding vitronectin, fibronectin, thrombospondin and fibrinogen via the RGD recognition sequence present in these molecules. αvβ3 is involved in a variety of biological activities including cell spreading and migration, tumour metastasis, bone resorption and cell signalling (see Felding-Habermann and Cheresh, 1993 and references therein).

**1.6.2. Thrombospondin and the glycoprotein CD36.**

The thrombospondin family comprises of five homologous proteins, thrombospondin 1-4 and cartilage oligomeric matrix protein, all of which are products of separate genes (Adams and Lawler, 1993). Initially isolated from α-granules of thrombin-stimulated platelets, thrombospondin-1 is secreted by a number of cell types including macrophages (Jaffe *et al.*, 1985; Savill *et al.*, 1992) and inflammatory neutrophils (Kries *et al.*, 1989). Thrombospondin has been implicated in a number of cellular processes including angiogenesis, inflammation, cell development and homeostasis. Of most relevance for this project however, is its involvement in cell-cell adhesion. Thrombospondin has been described as a molecular bridge for a number of cell-cell interactions including platelet-platelet aggregation (Silverstein *et al.*, 1986), platelet-monocyte and platelet-macrophage adhesion (Silverstein and Nachman, 1987) as well as between macrophages and apoptotic neutrophils in the process of phagocytosis of the apoptotic cell (Savill *et al.*, 1992). Also of relevance is the presence of thrombospondin in the extracellular matrix during early stages of wound repair (Raugi *et al.*, 1987) at a time when neutrophils would be removed from the tissue. Other functions of thrombospondin

and its involvement in physiology and pathophysiology has been reviewed recently (Lahav, 1993).

Thrombospondin comprises three identical subunits made up of several discrete structural domains (Fig. 1.2). The heparin binding domain is a small globular domain at the amino terminus of the peptide that binds to soluble heparin, cell-surface heparin sulphate proteoglycans and to sulfatides. Adjacent to the heparin binding domain are two cysteine residues one of which is involved in the formation of the thrombospondin trimer. The central core segment contains three types of repeating sequences (type I, II and III repeats) accounting for about half of the thrombospondin subunit. The second and third of the type I repeats have been shown to contain a major cell binding site centred around the Cys-Ser-Val-Thr-Cys-Gly sequence. The type II repeats contain sequences that may bind to soluble macromolecules, while a third cell binding sequence, Arg-Gly-Asp (RGD), is found in the last of the type III repeats. The carboxy-terminal domain is capable of binding a number of cell types by novel sequences that bear no homology to other adhesive proteins. Therefore, each subunit has at least four direct cell binding sites (reviewed in detail by Frazier, 1991) as well as sequences for the interaction of thrombospondin with other macromolecules.

Five distinct receptors have been identified for thrombospondin (reviewed in Frazier, 1991) including the integrin $\alpha v\beta 3$ which recognises the Agr-Gly-Asp sequence in the type III repeats of thrombospondin (Lawler and Hynes, 1989) and the glycoprotein CD36 that binds to the type I repeats containing the Cys-Ser-Val-Thr-Cys-Gly motif (Li *et al.*, 1994). CD36 (also known as glycoprotein IV or glycoprotein IIIb) is a single chain

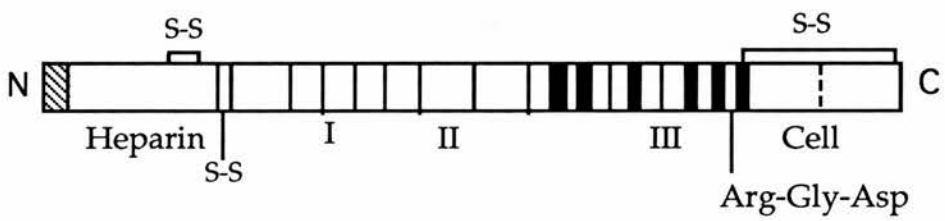


**Figure 1.2. Schematic representation of a subunit of thrombospondin.** Location of the type I, II and III repeats are shown as well as the positions of the heparin binding domain, the RGD binding sequence and the cell binding domain (Adapted from Frazier, 1991).

glycoprotein of molecular mass 88kD. It is expressed on a number of cell types including platelets, endothelial cells, monocytes and a variety of tumour cell lines (Asch *et al.*, 1987) as well as fibroblasts (Stomski *et al.*, 1992) and macrophages (Silverstein *et al.*, 1989) and has been identified as the receptor for thrombospondin in these cells. It has been shown to mediate platelet-monocyte and platelet-macrophage thrombospondin-dependent cell adhesion (Silverstein *et al.*, 1989) and is involved in the adhesion of malaria parasitized erythrocytes to microvasacular endothelium (Johnson *et al.*, 1993).

## 1.7. MODULATION OF MACROPHAGE PHAGOCYTOSIS OF APOPTOTIC NEUTROPHILS.

While much is understood about the mechanism of mø recognition of apoptotic neutrophils, little is known about the modulation of this system which would have important implications for regulating the resolution process of inflammation. There are several potential control mechanisms for the recognition and phagocytic pathways by which apoptotic cells are removed.

Necrotic neutrophils are also found at inflamed sites, thus the balance between apoptosis and necrosis could represent a pivotal point in the control of inflammation. Inflammatory mediators and growth factors prolong the lifespan of myeloid cells by inhibition of the rate of apoptosis in these cell populations (Brach *et al.*, 1992; Lee *et al.*, 1993). It has been shown that for neutrophil granulocytes, inhibition of apoptosis confers longevity of a range of cellular functions including phagocytic and secretory responses (Lee *et al.*, 1993). The rate at which neutrophils

undergo apoptosis at inflamed sites would serve a dual purpose of regulating the potential for neutrophil-mediated damage at these sites and the numbers of neutrophils that are available for recognition by mø.

Another level of control is regulation of the phagocytic potential of the macrophage. The novel receptors mediating macrophage recognition of apoptotic cells are critical determinants of the lack of macrophage secretory response (Meagher *et al.*, 1992). Alteration of mø phenotype may result in expression of the receptors that are involved in the phagocytic process. During the process of human monocyte differentiation to macrophages in vitro, expression of αvβ3 is induced (Andreesen *et al.*, 1990; Krissansen *et al.*, 1990) concomitantly with acquisition of the capacity to phagocytose apoptotic cells (Savill *et al.*, 1990). For murine macrophages, different phenotypes may be induced by local micro-environmental stimuli. In contrast to macrophages isolated from the peritoneal cavity that employ phosphatidylserine receptors, bone marrow-derived macrophages utilise an αvβ3 integrin-based recognition mechanism (Fadok *et al.*, 1992b). Thus, modulation of receptor expression during monocyte differentiation may determine the phagocytic ability of macrophage populations at different anatomic sites.

In addition to alteration of cell phenotype (rate of neutrophil apoptosis and monocyte-macrophage differentiation) which may occur over a period of hours, rapid control may be achieved by regulation of the function of receptors already present upon the mø surface. Receptor function may be rapidly turned on and off in response to environmental signals. Integrins are used by cells to sense and respond to micro-environmental stimuli and functional activity can be regulated from

within the cell, either as a result of altered cell surface distribution (Tang *et al.*, 1993), or conformational changes that affect ligand binding affinity (Ginsberg *et al.*, 1990; Frelinger III *et al.*, 1991). Cells are therefore able to dynamically modulate their repertoire of possible adhesive interactions mediated by these receptors in response to extracellular signals (Damsky and Werb, 1992; Ginsberg *et al.*, 1992). Thus the integrin αvβ3 represents a potential regulatory element in macrophage recognition of apoptotic neutrophils.

## 1.8. AIMS.

The aim of this thesis is to investigate mechanisms involved in modulating macrophage recognition of apoptotic neutrophils. Initial experiments sought to investigate whether regulation of the functional properties of the molecules implicated in the recognition process, notably the macrophage αvβ3 receptor, served to impart control over this phagocytic system. β1, β2 and β3 integrin function has been shown to be modulated by activation of intracellular protein kinases (Dustin and Springer, 1989; Shimizu *et al.*, 1990). αvβ3-mediated adhesion is induced in a number of β1-negative B cell lines following activation of protein kinase C (Stupack *et al.*, 1992). Therefore, the effect of short term pretreatment of monocyte-derived macrophages with activators of protein kinase on phagocytosis of apoptotic neutrophils was assessed. As integrins provide a transmembrane link between the interior of the cell and the extracellular environment, the relationship of macrophage αvβ3 receptor and the actin cytoskeleton was also examined. Subsequently, the importance of the adhesion state of the macrophage during phagocytosis of apoptotic neutrophils was investigated.

# CHAPTER 2. MATERIALS AND METHODS.

## 2.1. MATERIALS

### 2.1.1. Reagents.

All chemicals were obtained from Sigma Chemical Co., Poole, U.K. unless otherwise stated. Iscove's Dulbeco's modified Eagles medium (Iscove's DMEM), Hanks balanced salt solutions (HBSS) and culture supplements (Penicillin/Streptomycin) were from Gibco Laboratories, Paisley, Scotland and sterile tissue culture ware from Falcon Plastics, Becton Dickinson, Oxford, U.K. Phorbol 12,13-dibutyrate (PdBu) was purchased from Calbiochem, Nottingham, U.K. and a stock solution prepared in dimethylsulphoxide (DMSO) at 1mg/ml. 20mM stock solution of dibutyryladenosine 3'.5'-cyclic monophosphate (dbcAMP; Sigma) was prepared in Iscove's DMEM. Prostaglandin $E_2$ ($PGE_2$; Sigma) was prepared in ethanol at a concentration of 3mM while 50mM isobutyl methyl xanthine (IBMX; Sigma) was prepared in DMSO and isoquinolinylsulfonyl methylpiperazine (H-7; Sigma) was prepared in DMSO at a concentration of 0.23M. All stock solutions were stored at -20°C and diluted as required in Iscove's DMEM. Stock solutions of the ECM glycoproteins fibronectin (Fn; Sigma), vitronectin (Vn; Calbiochem) and collagen type I and type VI (Col I and Col VI; Gibco) were prepared in PBS at 1mg/ml, stored at -80°C and used in experiments at 10μg/ml PBS. Thrombospondin was purified by Dr. Ian Dransfield (Rayne Laboratory, University of Edinburgh, Scotland) from thrombin-activated platelets as described (Lawler *et al.*, 1985).

### 2.1.2. Antibodies.

Antibodies used in this study were rabbit immunoglobulins (Ig) to human erythrocyte membrane antigens, fluorescein isothiocyanate (FITC)-

conjugated F(ab')$_2$ fragment of rabbit Ig to mouse Ig, peroxidase-conjugated rabbit Ig to mouse Ig, biotinylated rabbit Ig to mouse Ig and ABComplex/AP, all from DAKO, Denmark. Monoclonal antibodies (mAb) are of $IgG_1$ isotype unless otherwise stated. 23C6 (CD51/CD61 [$\alpha v\beta 3$] complex) and 13C2 (CD51 [$\alpha v$]) were provided by Dr. Mike Horton, ICRF, London (Davis *et al.*, 1989). Purified PM6/13 (CD61 [$\beta 3$]; Shaw, 1994) was generously supplied by Dr. Mike Wilkinson, Royal College of Surgeons, London while SMØ (CD36; IgM; Hogg *et al.*, 1984) and mAb29 (CD15; IgM) were provided by Dr. Nancy Hogg, ICRF, London. FA6-152 (CD36) was selected from antibody panels obtained from the 5th International Workshop on leukocyte differentiation antigens (Shaw, 1994). mAb specific for thrombospondin, MA-I (type III repeats; Lawler *et al.*, 1985) and MA-IV (heparin binding domain) were the gift of Dr. Jack Lawler, Harvard Medical School, Boston. The hybridoma MOPC-21C (non-binding $IgG_1$ control mAb) was purchased from the European Animal Cell Culture collection, Porton Down, U.K. LIBS1 (activated CD61; Frelinger III *et al.*, 1990) was generously provided by Dr. Mark Ginsberg, Research Institute of Scripps Clinic, La Jolla, U.S.A. Mouse monoclonal antibody to human talin was purchased from Serotec Ltd, Oxford, U.K. TS2/16 (a CD29 [$\beta 1$] stimulatory mAb; Arroyo *et al.*, 1992), mAb13 (a CD29 [$\beta 1$] inhibitory mAb; Berdichevsky *et al.*, 1992) and BIIG2 (CD49e [$\alpha 5$]; Werb *et al.*, 1989) were donated by Dr. Don Salter, Department of Pathology, University of Edinburgh, Scotland. SAM-1 (CD49e [$\alpha 5$]) was supplied by Carl Figdor, University Hospital Nijmegan, Holland. mAb Bob 75 (CD15; IgM) was supplied by Dr. Jim Ross, Lister Laboratories, University of Edinburgh, Scotland and the UCHM1 clone (CD14; IgG2a; Hogg *et al.*, 1984) was originally from Peter Beverley, University College London.

## 2.2 CELL ISOLATION.

### 2.2.1. Cell separation using a Percoll density gradient centrifugation technique.

Mononuclear cells (MNC) and polymorphonuclear granulocytes (PMN) were isolated from peripheral blood using a modification of the method previously described by Savill *et al.*, 1989a. 40ml of freshly drawn citrated blood was added to 50ml Falcon tubes (Falcon Plastics) containing 4ml of 3.8% sodium citrate and centrifuged in an MSE Mistral 3000 centrifuge (Sanyo Gallenkamp, Loughborough, U.K.) at 300g for 20 minutes. Autologous serum was prepared at 37°C in glass tubes by recalcification of 10ml of the platelet-rich plasma layer using 220µl of 0.32% calcium chloride. Platelet-poor plasma (PPP) was also prepared by centrifugation of the platelet-rich plasma layer at 2500g for 20 minutes. Leukocytes were enriched from the cell pellet by sedimentation of erythrocytes for 30 minutes with 6% dextran T500 (1ml dextran/4ml blood cell pellet). The cells, spun out of the leukocyte-rich layer, were then resuspended in 3ml of 55% isotonic Percoll (9:1 vol./vol. Percoll:10x PBS) in HBSS without $Ca^{2+}/Mg^{2+}$. The cell fraction was then overlaid in 15ml Falcon tubes onto 3ml of 70% isotonic Percoll, previously overlaid on 3ml of 81% isotonic Percoll, and spun at 700g for 20 min (Dooley *et al.*, 1982). MNC were aspirated from the 55%/70% interface and PMN from the 70%/81% interface and washed initially in PPP, then HBSS without $Ca^{2+}/Mg^{2+}$ and finally HBSS with $Ca^{2+}/Mg^{2+}$ (1000rpm, 6 minutes). MNC were, on average, >95% pure and PMN >98% pure as assessed by cytospin preparations.

### 2.2.2. Cell separation by counterflow centrifugation elutriation.

A J-21M/E Beckman centrifuge (Beckman Instruments (U.K.) Ltd, High Wycombe, U.K.) fitted with a JE-6 elutriator rotor, was used in conjunction with a Masterflex peristaltic pump (supplied by Beckman) to regulate the flow rate of the medium through the system (Fig. 2.1). After purging with alcohol and distilled water, the circuit was thoroughly flushed through with HBSS with $Ca^{2+}/Mg^{2+}$ containing 0.2% PPP (elutriation medium) taking care to remove any air trapped within the tubing. Cells, pelleted from the leukocyte-rich layer (2.2.1), were pooled in a 10ml syringe and loaded into the sample chamber of the elutriator, the 3-way taps set to bypass the sample chamber. The cells were then loaded into the separation chamber by redirecting the elutriation medium through the sample chamber. Separation of the leukocytes was achieved by increasing the flow rate of the elutriation medium through the system. Cells were collected in 50ml fractions and the purity of each fraction assessed using an EPICS Profile II flow cytometer (Coulter Electronics, Luton, U.K.). Monocytes were pooled and cultured as described in 2.3.1(ii). Neutrophils fractions were cultured as described in 2.3.2.

## 2.3. CELL CULTURE.

### 2.3.1. Macrophages.

Monocytes were cultured either adherent to tissue culture plastic or in suspension in Teflon foils.

i) For adherent cell culture, MNC were resuspended at $4x10^6$/ml in Iscove's DMEM, allowed to adhere to 24 well tissue culture plates for one hour at 37°C, non-adherent cells removed and the monocytes washed twice in HBSS with $Ca^{2+}/Mg^{2+}$. Monocytes were then cultured for six days in Iscove's

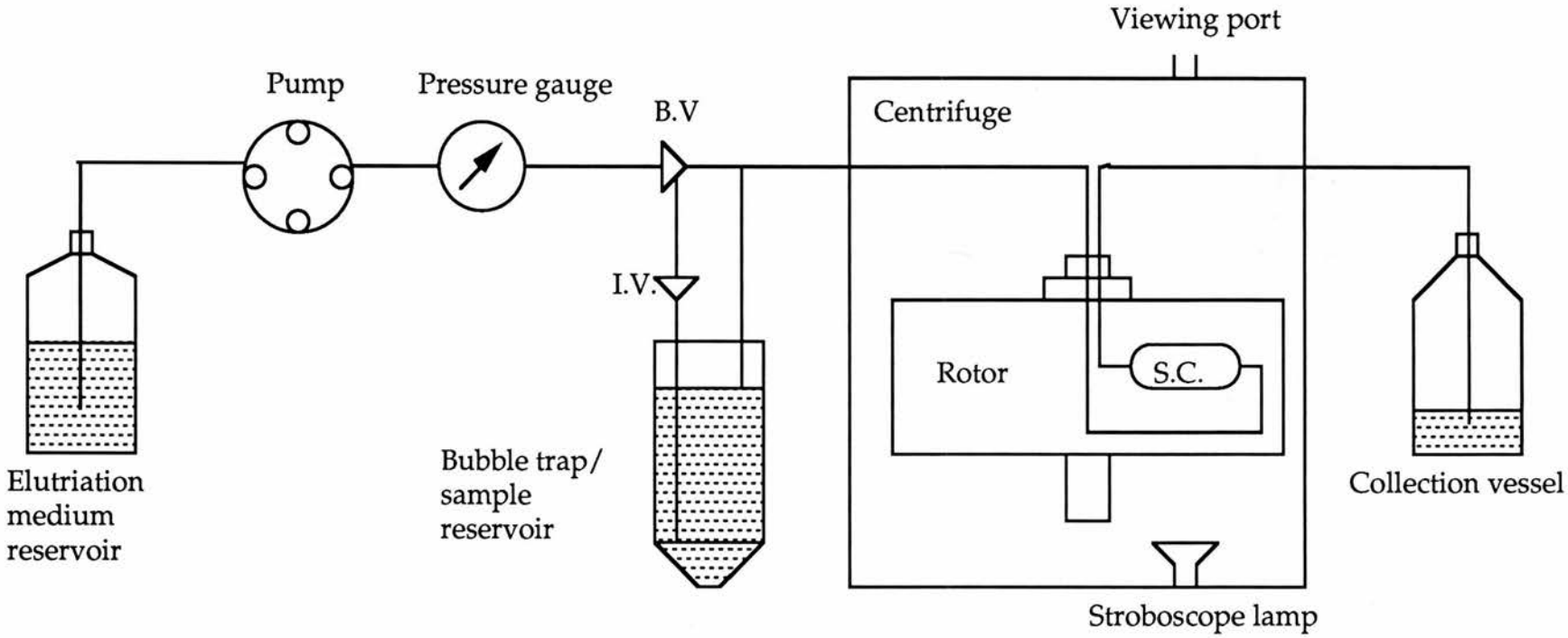


**Figure 2.1. Diagram of elutriation system.**

B.V. - bypass valve: I.V. - sample injection valve: S. C. - separation chamber.

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DMEM containing 10% autologous serum, with a change of medium on day 3 as previously described (Savill *et al.*, 1989a).

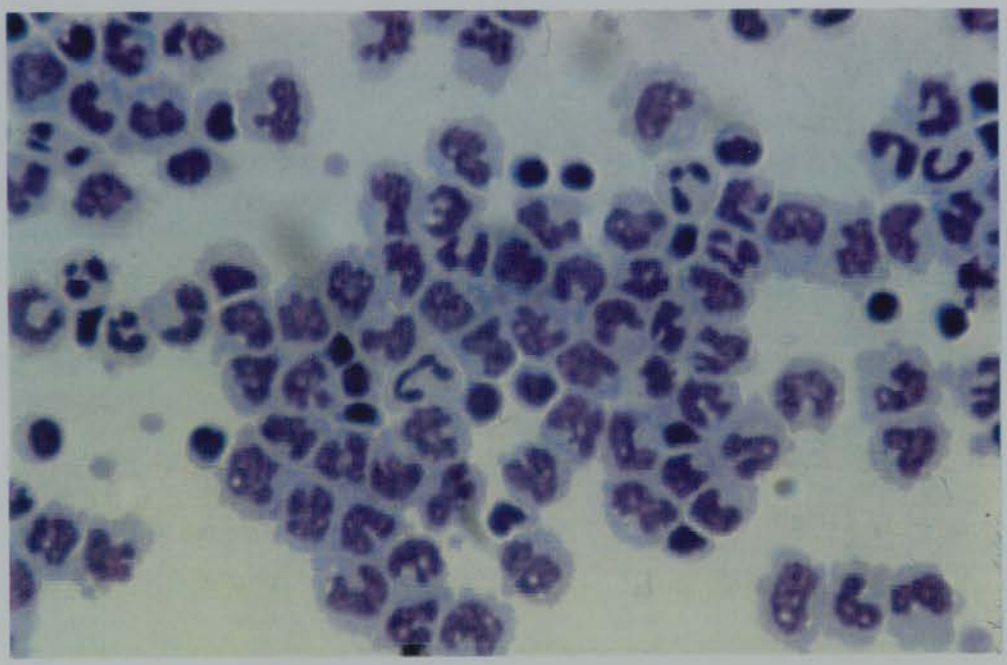
ii) For suspension culture, monocytes were separated from non-adherent lymphocytes by adherence of MNC to plasma-coated tissue culture dishes (Ackerman and Douglas, 1978). 140mm tissue culture dishes (Falcon Plastics) were incubated for 1 hour at 37°C with 3ml PPP, diluted 1:10 with PBS, per plate. The plates were then washed twice with PBS and $1.4x10^7$ MNC, in 7ml Iscove's DMEM containing 10% autologous serum, were added to each dish and incubated for 1 hour at 37°C. Non-adherent cells were removed by two washes in HBSS with $Ca^{2+}/Mg^{2+}$. Adherent cells were then detached by incubation with warm (37°C) PBS containing 5mM EDTA (BDH, Glasgow, U.K.) and 1% autologous serum for 10 minutes at 37°C. Monocytes obtained by this method (>90% pure by cytospin preparation; Fig 2.2) or by elutriation were then washed and resuspended at $1x10^6$/ml Iscove's DMEM containing 10% autologous serum and cultured for six days at 37°C in a 5% $CO_2$ atmosphere in sterile hydrophobic Teflon (ChemFab, Handforth, Cheshire, U.K.) foils. The foils were prepared by sealing the corners of Teflon squares with an HM3000 Impulse heat sealer (Hume Martin Ltd, London, U.K.) to form trays of approximately 1cm deep.

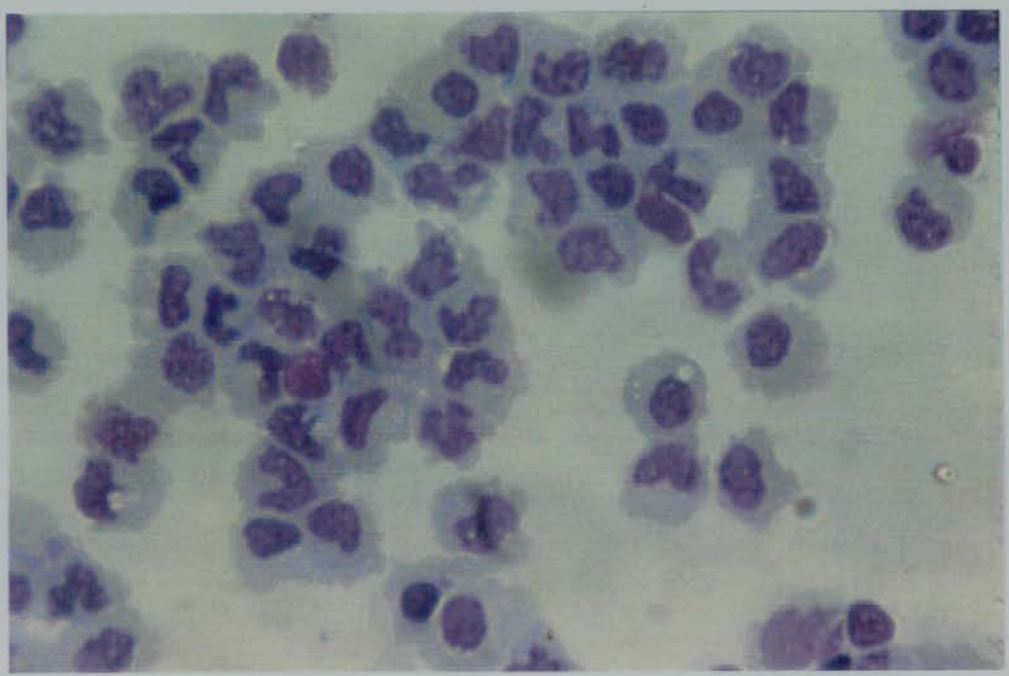
**2.3.2. Neutrophils.**

Neutrophils were cultured at $4x10^6$/ml in Iscove's DMEM containing 10% autologous serum in 5ml hydrophobic Teflon bags or tissue culture flasks at 37°C in a 5% $CO_2$ atmosphere for 18-24 hours. During this time a proportion of the cells underwent spontaneous apoptosis (Savill *et al.*, 1989a), as determined by morphological appearance.

**Figure 2.2. Isolation of monocytes from mononuclear cells.**
**Top panel:** Photomicrograph of mononuclear cells isolated from peripheral blood using a discontinuous Percoll density gradient. Magnification x400.

**Lower panel:** Photomicrograph of monocytes purified from mononuclear cells by adhesion to plasma-coated tissue culture plates. Magnification x400.





## 2.4. ASSESSMENT OF APOPTOSIS.

After 18-24 hours in culture, apoptosis of the neutrophils was quantified microscopically by morphological appearance, nuclear condensation and cytoplasmic vacuolation (Wyllie *et al.*, 1980; Fig. 2.3). That the cells were "viable" rather than necrotic was determined by trypan blue exclusion. For assessment of macrophage phagocytosis of apoptotic cells, neutrophils were >50% apoptotic and <2% necrotic (i.e. >98% excluding trypan blue).

## 2.5. STANDARD PHAGOCYTOSIS ASSAY FOR APOPTOTIC NEUTROPHILS.

### 2.5.1. Macrophages cultured in tissue culture plates.

Phagocytosis of apoptotic neutrophils was assayed by minor modifications of previously described methods (Newman *et al.*, 1982). Monocyte-derived macrophages (mø), cultured for six days in 24 well tissue culture plates, were washed once with Iscove's DMEM and $4x10^6$ apoptotic neutrophils, resuspended in Iscove's DMEM, were added to each well. After 30 minutes incubation at 37°C, wells were washed 3-5 times with ice cold PBS, fixed in PBS containing 2.5% glutaraldehyde for 10 minutes and stained for myleoperoxidase (MPO) at 37°C for 30 minutes using hydrogen peroxide and 0.1μM dimethoxybenzidine (o-diansidine) as substrate. Apoptotic neutrophils are 100% MPO-positive while six day mø are 100% MPO-negative (Fig. 2.4). The percentage of mø phagocytosing MPO-positive apoptotic neutrophils was quantified microscopically by examination of 4-5 randomly selected fields (a minimum of 500 cells). Experiments were carried out in duplicate or triplicate wells.

**Figure 2.3. Morphological appearance of apoptotic neutrophils.**
Neutrophils, aged for 18-24 hours in culture, which displayed nuclear condensation and cytoplasmic vacuolation (arrows) were considered to be apoptotic. Magnification x1000.

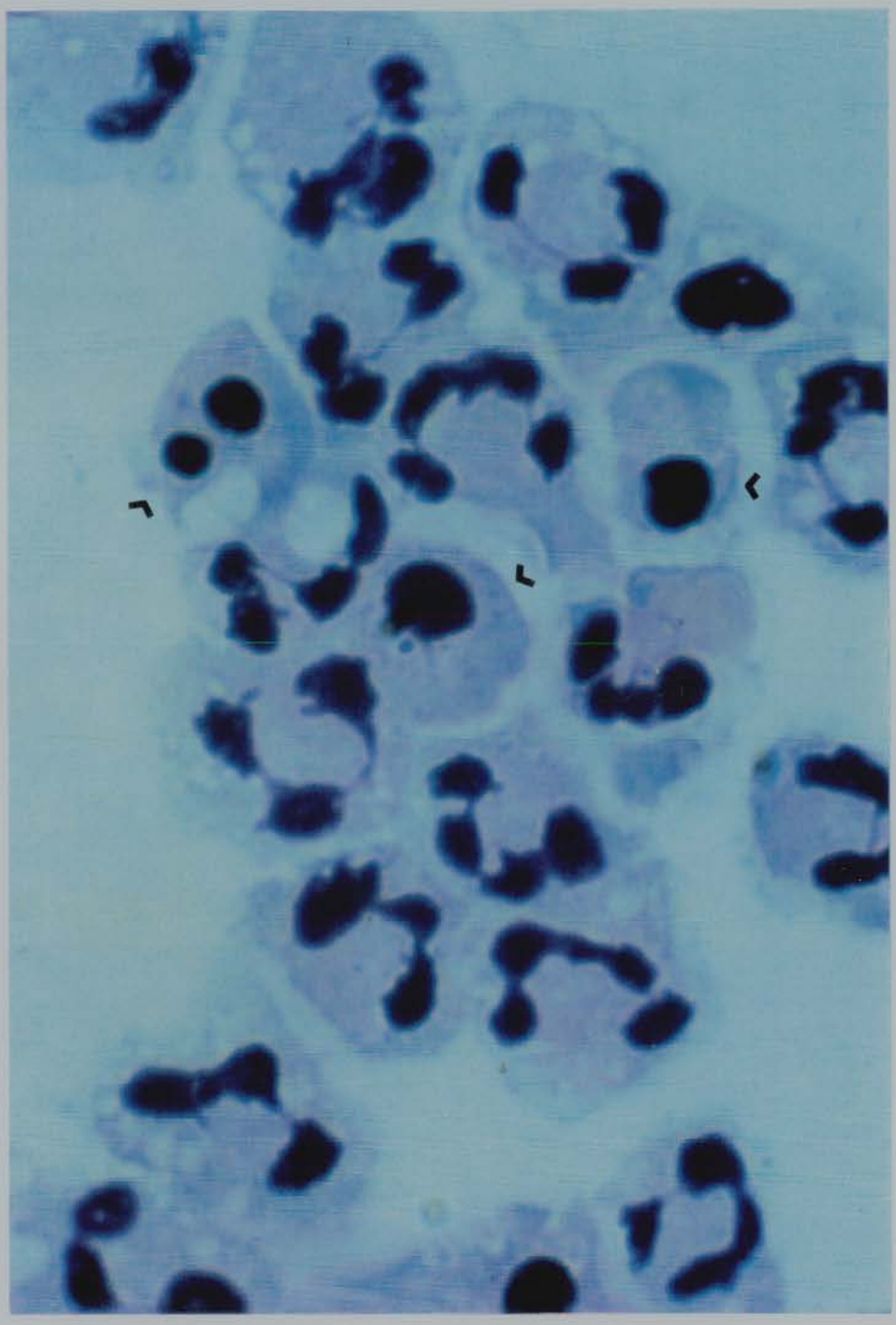
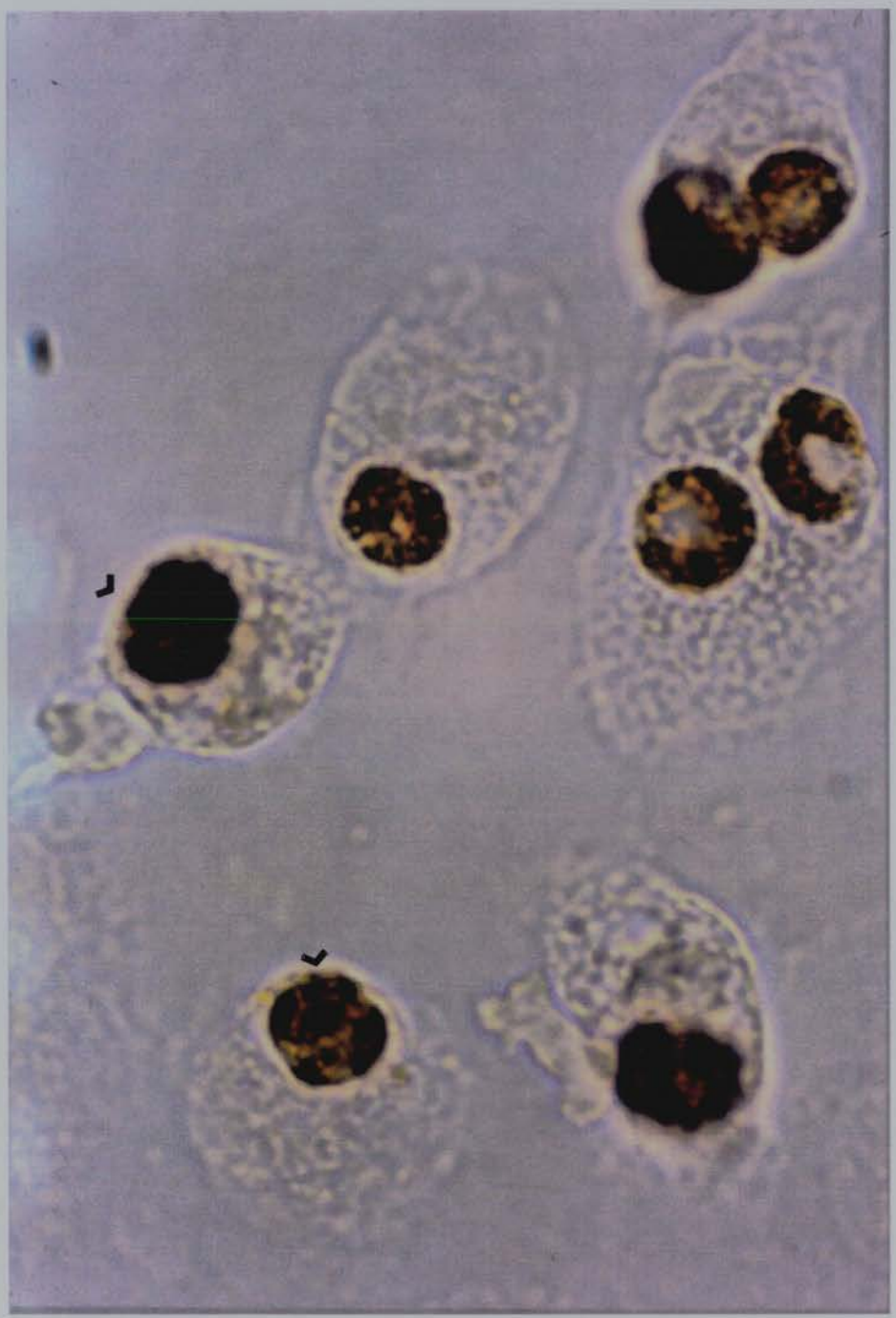


**Figure 2.4. Myleoperoxidase staining of apoptotic neutrophils phagocytosed by macrophages.**

Neutrophils, phagocytosed by mø, are readily identifiable by the orange/brown staining of MPO (arrows). Magnification x1000.



### 2.5.2. Macrophages cultured in Teflon foils.

Mø, recovered from Teflon foils by aspiration, were resuspended at $1x10^6$/ml Iscove's DMEM and either $10^5$/well added to 96 well tissue culture plates or multispot microscope slides (C.A. Hendley (Essex) Ltd, Essex, UK.) or $1.5x10^5$/well added to 8 well chamber slides (Gibco) and allowed to adhere by incubation for 1h at 37°C. After rinsing with Iscove's DMEM, $1x10^6$/well of apoptotic neutrophils were added to the 96 well plates or multispot slides for 30 minutes at 37°C ($1.5x10^6$/well to the chamber slides). Wells were then washed, fixed with glutaraldehyde and stained for MPO as described in 2.5.1.

## 2.6. PHAGOCYTIC ASSAY FOR IgG OPSONISED ERYTHROCYTES.

Erythrocytes were obtained from the red blood cell pellet following dextran sedimentation of the whole blood, washed in HBSS without $Ca^{2+}/Mg^{2+}$, resuspended at $25x10^6$/ml HBSS without $Ca^{2+}/Mg^{2+}$ and incubated at 4°C with rabbit IgG to human erythrocyte membrane (1:100) for 30 minutes. Opsonised erythrocytes were then washed, resuspended at $4x10^6$/ml HBSS with $Ca^{2+}/Mg^{2+}$ and incubated with mø on 24 well tissue culture plates as described for apoptotic neutrophils (2.5.1). After 30 minutes, the wells were washed once with PBS and non-phagocytosed erythrocytes were lysed with one drop of water per well for approximately 30 seconds after which time PBS was added to the wells and two further PBS washes carried out. The cells were then fixed with glutaraldehyde and stained for MPO as described in 2.5.1., erythrocytes staining MPO-positive.

## 2.7. MODIFICATIONS OF THE STANDARD PHAGOCYTOSIS ASSAY.

### 2.7.1. Pretreatment of macrophages with activators of protein kinase C and protein kinase A.

Mø were washed once with Iscove's DMEM and incubated for 15 minutes at 37°C with either 50nM PdBu (protein kinase C [PKC] activator) using DMSO, diluted by the same amount as a control, 2mM dbcAMP (protein kinase A [PKA] activator) or 3μM, 1.5μM or 0.3μM $PGE_2$ plus 0.5mM IBMX (PKA activation) or 6μM H-7 (PKC inhibitor). Iscove's DMEM was the control for the dbcAMP and $PGE_2$ treatments. The treated cells were then washed once with Iscove's DMEM prior to assessment of phagocytosis of apoptotic neutrophils or opsonised erythrocytes as previously described (2.5.1 and 2.6). There was no significant difference in the amount of phagocytosis between the two control conditions and for subsequent experiments using PdBu, Iscove's DMEM was used as the control medium.

### 2.7.2. Pretreatment of macrophages with antibody.

i) Mø, cultured in suspension and adhered to 96 well tissue culture plates or multispot microscope slides (2.5.2), were washed once with Iscove's DMEM and incubated for 15-20 minutes at room temperature with 50μl of relevant mAb optimally diluted in Iscove's DMEM. Apoptotic neutrophils were added to each well with the mAb still present and the assay carried out as described in 2.5.2.

ii) Mø, cultured in suspension, were resuspended in Iscove's DMEM and $1.5x10^6$ cells incubated at 4°C for 30 minutes with mAb in 96 flexiwell plates (Becton and Dickinson, Oxford, U.K.). Cells were then adhered to wells of chamber slides in the presence of the mAb for 1 hour after which time the

cells were washed and the phagocytosis assay carried out as described (2.5.2).

### 2.7.3. Plating of macrophages on different matrix substrates.

8 well chamber slides were coated overnight at 4°C with 10µg/ml of Fn, Vn, Col I and Col VI. Mø, cultured in Teflon foils, were spun out of the culture medium with 10mM EDTA. The wells were washed twice with PBS and $1.5x10^6$ mø, resuspended in Iscove's DMEM, were added to each well and adhered for 1 hour at 37°C and the phagocytic assay carried out as previously described (2.5.2).

## 2.8. MEASUREMENT OF cAMP LEVELS IN MACROPHAGES TREATED WITH $PGE_2$.

### 2.8.1. Preparation of cell samples.

Mø were cultured over six days from MNC adherent to 6 well tissue culture plates as described in 2.3.1. The cells were washed once with Iscove's DMEM and incubated with Iscove's DMEM or Iscove's DMEM containing 0.5mM IBMX and 3µM $PGE_2$ for 0.5, 2, 5, 10, 15 or 45 minutes. The reactions were terminated by rapid aspiration of the medium followed by the addition of 600µl ice cold 0.5M trichloroacetic acid. The cells were left to extract on ice for 20 minutes then scraped off and transferred to eppendorf tubes. The samples were then vortexed and centrifuged (10000g, 5 minutes) and 500µl of the supernatant added to 125µl of 10mM EDTA (pH 7.0) and 500µl of freshly prepared 1,1,2-trichlorotrifluroethane (BDH)/tri-n-octylamine (50:50, v/v; Downes *et al.*, 1986). After further vigorous mixing the samples were centrifuged (10000g, 2 minutes) and 500µl of the aqueous layer removed and neutralised with 100µl 6M sodium hydrogencarbonate. Three 50µl aliquots

from each sample were then used in the cyclic AMP (cAMP) assay (section 2.8.2).

### 2.8.2. cAMP radioreceptor assay.

Assays were performed in triplicate at 4°C in a final volume of 300µl. Each tube contained 50µl buffer (50mM Tris-HCl, 4M EDTA, pH 7.5), 50µl sample or standard containing known amounts of cAMP (doubling dilutions from 0.125-16pmol with 250pmol cAMP used to define non-specific binding) and 100µl [$^3$H]-cAMP (88000 dpm; DuPont (U.K.) Ltd, Stevenage, U.K.). The reaction was initiated by addition of 150µl cAMP binding protein (final concentration, 0.85µg protein/ml; prepared by Dr. Edwin Chilvers, Rayne Laboratory, University of Edinburgh, Scotland, as described in Brown *et al.*, 1971) and incubated overnight at 4°C. The reaction was terminated by the addition of 250µl 0.5% activated charcoal (BDH). After 3 minutes, the samples were centrifuged (10000g, 4 minutes) and 200µl of the supernatant transferred to 5ml scintillation vials (Canberra Packard, Pangbourne, U.K.). 5ml of Flo-Scint™ IV scintillation fluid (Canberra Packard) was added to each vial and the samples analysed on a liquid scintillation counter (Canberra Packard). Figure 2.5 shows a typical standard curve representative of four experiments.

## 2.9. FLOW CYTOMETRY.

Mø, cultured in suspension, were resuspended at 1x10$^6$/ml Iscove's DMEM. 10$^5$ mø were incubated in a 96 well multititre plate (Becton Dickinson) for 15 minutes at 37°C with either 100µl 50nm PdBu, 2mM dbcAMP or Iscove's DMEM as control. Cells were washed twice with PBS containing 0.2% BSA and 0.1% sodium azide and incubated at 4°C the relevant primary

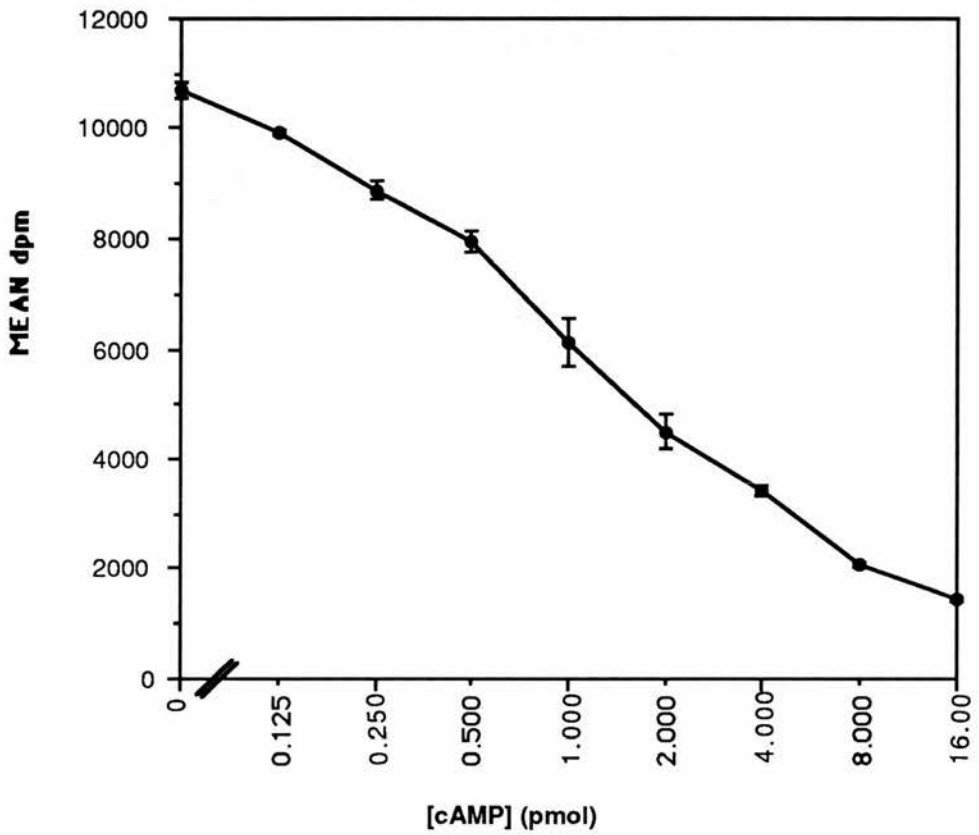



**Figure 2.5. Typical standard curve obtained from cAMP radioreceptor assay.**

This is the standard curve obtained for the experiment presented in chapter 3 (section 3.3 and Fig. 3.6) and represents one of four experiments. Points represent dpm values (mean ± S.D.) of triplicate samples.

antibody. After 30 minutes, the cells were washed twice as before and incubated for a further 30 minutes at 4°C with FITC-conjugated goat anti-mouse Ig (1:25). After two further washes, cells were fixed in PBS containing 5% formaldehyde prior to flow cytometric analysis using an EPICS Profile II flow cytometer (Coulter Electronics).

## 2.10. IMMUNODETECTION OF THROMBOSPONDIN.

Mø, cultured on 24 well tissue culture plates, were washed thoroughly and treated with 0.5ml PdBu, dbcAMP or Iscove's DMEM for 15 minutes as previously described (section 2.5.1). The cells were then washed once in Iscove's DMEM and 200µl of Iscove's DMEM added to each well for 30 minutes at 37°C after which time the supernatant from each well was collected. The supernatants were then concentrated approximately twenty-fold using Centron 3 microconcentrators (Amicon Ltd., Stonehouse, U.K.) by centrifugation in a J2-MC centrifuge (Beckman) at 7500g for one hour. Doubling dilutions of the mø supernatants or other samples were made in PBS and 3µl of each dilution were applied to a nitrocellulose membrane (Anderman, Kingston-upon-Thames, U.K.) using a 20µl Gilson pipette. The membrane was left to air dry and incubated overnight at 4°C in PBS containing 0.2% Tween 20 (PBS-T) containing 5% non-fat dried milk. The membrane was then washed twice in PBS-T and incubated at room temperature with mAb against thrombospondin. After 30 minutes, unbound mAb was removed by washing twice in PBS-T and the membrane incubated for a further 30 minutes with peroxidase-conjugated rabbit anti-mouse Ig (1:1000) before being washed twice more in PBS-T. The membrane was then incubated for approximately 3 minutes in enhanced chemiluminescence detecting reagents (Amersham International plc, Buckinghamshire, U.K.),

excess liquid absorbed onto tissue and the nitrocellulose wrapped in 'clingfilm'. The nitrocellulose was then exposed to Hyperfilm-ECL (Amersham) in an autoradiography cassette for 5-30 seconds prior to development using an AGFA 242S automatic processor.

A technical complication was that using this technique, it was found that the peroxidase-conjugated rabbit anti-mouse Ig cross reacted with serum (Fig. 2.6a.). Hence, prior to use, the antibody was preabsorbed for 60 minutes against a piece of nitrocellulose that had been soaked in serum autologous to the mø donor and left to air dry. As seen in figure 2.6b, this eliminated the cross reactivity of the peroxidase-conjugated antibody.

Thrombospondin could be detected in the culture medium as shown in figure 2.7b. Autologous serum was found to be the source of thrombospondin in the culture medium (Fig. 2.7c). Since this might contribute to levels of thrombospondin present in the supernatants and thus an overestimation of the level of thrombospondin secreted, the mø were washed thoroughly at the start of the assay with Iscove's DMEM to remove any residual culture medium.

Serial dilutions of purified thrombospondin were also applied to the nitrocellulose to determine the sensitivity of the assay.

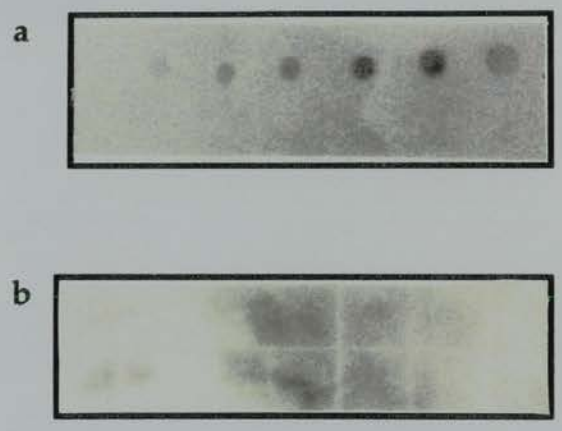
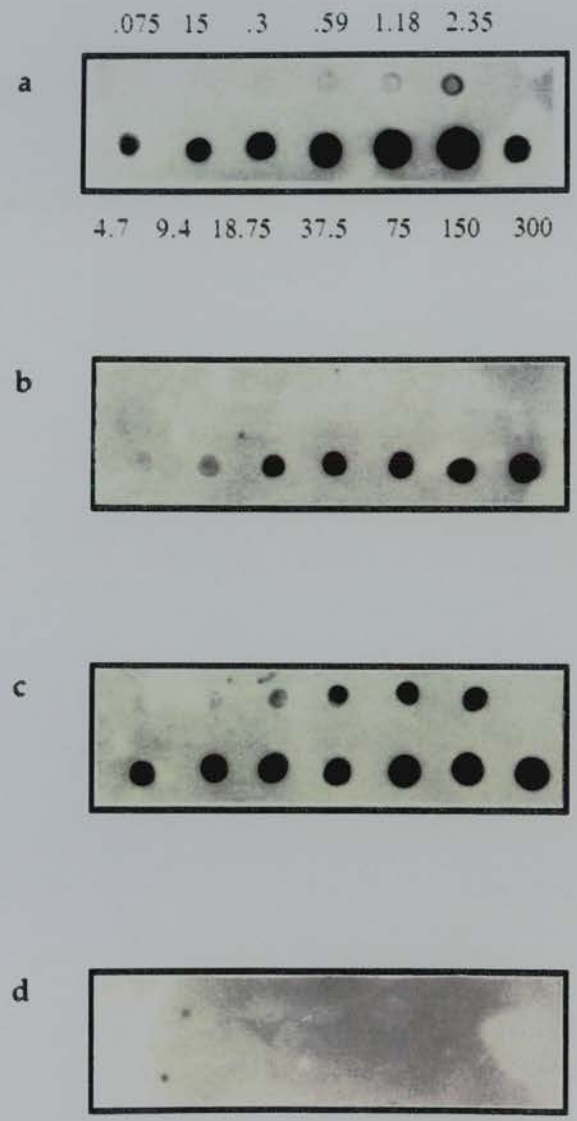



**Figure 2.6. Cross reaction of peroxidase-conjugated antibody with serum.**
a) Peroxidase-conjugated antibody to mouse immunoglobulin cross reaction with serum.
b) Cross reaction of peroxidase-conjugated antibody to mouse immunoglobulin was eliminated by exposing the antibody to serum prior to its use in the assay.

**Figure 2.7. Thrombospondin detection in macrophage culture medium.** Thrombospondin was detected in the macrophage culture medium. The two constituents of the culture medium, serum and Iscove's DMEM, were tested to determine the source of thrombospondin. Serum was found to contain thrombospondin. a) thrombospondin (μg/ml), b) macrophage culture medium, c) serum, d) Iscove's DMEM.


.075 15 .3 .59 1.18 2.35
a
4.7 9.4 18.75 37.5 75 150 300
b
c
d

## 2.11. IMMUNOCYTOCHEMISTRY.

### 2.11.1. Receptor distribution on macrophages treated with protein kinase activators.

Mø, recovered from Teflon foils by aspiration, were resuspended at $1x10^6$/ml Iscove's DMEM and $10^5$ cells/well adhered to 2 wells of multispot microscope slides for 1 hour at 37°C. Mø were treated with protein kinase activators or control media for 15 minutes at 37°C then washed with Iscove's DMEM, left to dry and fixed in acetone for 10 minutes. At this stage the slides could be frozen and stained at a later date having been allowed initially to defrost in acetone. Slides were then washed twice in Tris buffered saline (TBS) and incubated at room temperature in a humid chamber with TBS containing 20% normal rabbit serum (NRS; Scottish Antibody Production Unit, Lanarkshire, U.K). After 10 minutes, the serum was drained off and cells incubated for 30 minutes with primary antibody optimally diluted in 20% NRS. The cells were then washed and blocked with 20% NRS as before and incubated for another 30 minutes with biotinylated rabbit Ig to mouse Ig (1:1600). After two further washes in TBS, cells were incubated for a further 30 minutes with an avidin-biotinylated alkaline phosphate complex. Finally, cells were incubated with the alkaline phosphate substrate Vector Red (Vector Laboratories, Peterborough, U.K.) for 15 minutes, washed for 5 minutes in water and dehydrated through 85%, 95% and 100% ethanol. After clearing with xylene, slides were mounted in DPX (BDH). Samples were viewed with an Olympus BH-2 RFCA fluorescent microscope (Olympus Optical Co. (U.K.) Ltd, London, U.K.) and photographed with an Olympus OM-4Ti camera using Kodak EKTAChrome 64Ti colour film.

### 2.11.2. Staining of actin on treated macrophages.

Mø adherent to multispot slides (described in section 2.11.1) were washed with Iscove's DMEM, fixed for 5 minutes in ice-cold acetone washed in PBS and incubated in a humid chamber for 10 minutes with PBS containing 20% NRS. The serum was then drained off and the cells incubated with 0.15μM rhodamine-conjugated phalloidin (Cambridge Bioscience, Cambridge, U.K.) in PBS containing 0.2% BSA and 0.1% sodium azide. After 20 minutes at room temperature, the slides were washed with PBS and mounted with PBS containing 50% glycerol.

### 2.11.3. Dual immunofluorescent techniques.

i) Antibody/phalloidin staining - fixed adherent mø were washed and blocked as described (2.11.1). Cells were then incubated for 30 minutes in primary antibody optimally diluted in 20% NRS in PBS. The slides were then washed in PBS, blocked again with 20% NRS and incubated with FITC-conjugated anti-mouse Ig diluted 1:50 in 20% NRS in PBS. After 30 minutes the cells were washed in PBS then incubated with rhodamine-conjugated phalloidin for a further 20 minutes. Slides were washed a final time in PBS and mounted in 50% glycerol.

ii) Receptor distribution associated with mø phagocytosis of apoptotic neutrophils - the standard apoptotic neutrophil phagocytosis assay was carried out as described for the multispot slides (2.5.2). Slides were fixed for 10 minutes in acetone, washed twice with TBS and then incubated for 10 minutes with 20%NRS in TBS. Immunodetection of neutrophils was performed as follows: slides were incubated for 30 minutes with either Bob 75 or mAb29 (CD15; both IgM). These antibodies are neutrophil specific and did not label the mø. Slides were then washed twice in TBS, blocked with

20% NRS and incubated for a further 30 minutes with FITC-labelled affinity purified antibody to mouse IgM (Kirkgaard and Perry Laboratories Inc., Maryland, U.S.A.). Slides were then labelled as described in 2.11.1. except a goat anti-mouse $IgG_1$ biotinylated whole antibody (Amersham) was used instead of the biotinylated rabbit Ig to mouse Ig and streptavidin-biotynlated phycoerythrin complex (Amersham) used instead of the avidin-biotinylated alkaline phosphate complex and Vector Red substrate.

An exception to this protocol was when LIBS1 was used as the mø primary antibody. This mAb preferentially reacts with a site on the β3 subunit that is expressed only after ligand binding causes the receptor to change shape (Frelinger III *et al.*, 1990). Therefore, the LIBS1 was present during the phagocytosis assay and after the apoptotic neutrophils were labelled with CD15 and FITC, the second part of the staining technique was begun with the biotinylated $IgG_1$.

## 2.12. PHAGOCYTOSIS ASSAY IN SUSPENSION.

### 2.12.1. Standard suspension assay.

Mø, cultured in suspension, were spun out of culture medium and incubated at 4°C at $2x10^6$/ml of phycoerythrin-conjugated CD14 (PE-CD14; DAKO) diluted 1:8 in PBS. 20 hour cultured neutrophils were spun out of the culture medium and incubated at 37°C at $2x10^6$/ml of carboxyfluorescein diacetate (CFDA: diluted 1:40000 in PBS: provided by Dr. Andrew Jackson, Department of Surgery, University of Edinburgh, Scotland). After 30 minutes the cells were washed and resuspended in Iscove's DMEM: mø at $1x10^6$/ml and cultured neutrophils at $10x10^6$/ml. 100µl of each cell type were added to the wells of a 96 well flexiwell plate. The cells were then

either fixed immediately to prevent any phagocytosis occurring or incubated for 30 minutes at 4°C or 37°C ± 5mM EDTA after which time the cells were fixed in 5% formaldehyde and duplicate samples were analysed on an EPICS Profile II flow cytometer. Signals from the cytometer which were red positive (fluorescent mø) were simultaneously analysed for green fluorescence (cultured neutrophils). For two colour work, electronic compensation was adjusted using red only (mø) and green only (cultured neutrophils) to ensure no false double positives were observed. Cytospins were also made of each sample. The standard adherent phagocytosis assay was carried out in parallel to the assay in suspension using the same labelled cells to verify the system.

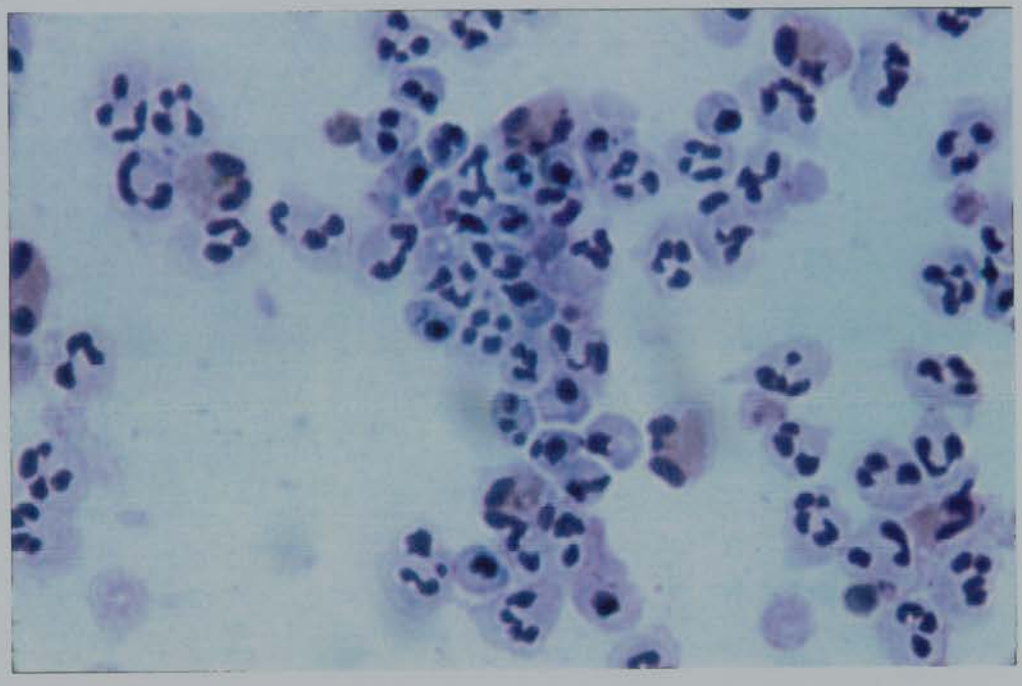
**2.12.2. Isolation of apoptotic neutrophils from a mixed aged population.**

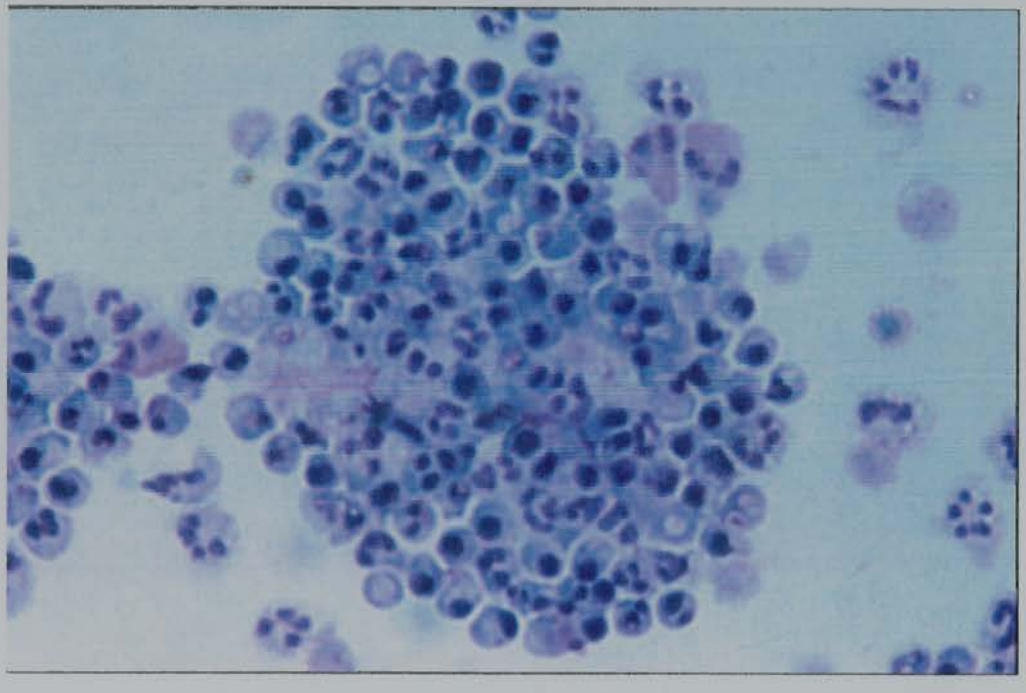
For some suspension assays, the apoptotic neutrophil population was enriched by removing the non-apoptotic aged cells. Neutrophils, cultured overnight, were labelled with CFDA as described in 2.12 then incubated with the antibody leu8 (CD62L [L-selectin]; IgG2b; clone SK11, Becton Dickinson) for 30 minutes at 4°C. The cells were then washed out of the CFDA and incubated at 4°C in Iscove's DMEM with DYNABEADS® (Dynal (U.K) Ltd, Wirral, U.K.), previously washed twice in Iscove's DMEM at a ratio of 3 beads to every cell. After 30 minutes of continual gentle mixing, the cells were depleted twice using a Magnetic Particle Concentrator® (Dynal (U.K.) Ltd.). This removed the majority of non-apoptotic aged cells, increasing the percent of apoptotic neutrophils (Fig. 2.8). The enriched apoptotic cell population (>70% apoptotic) was then washed, recounted, resuspended at $10x10^6$/ml Iscove's DMEM and the suspension assay carried out as described above (section 2.12.1). As before, a standard phagocytic assay was

**Figure 2.8. Isolation of apoptotic neutrophils from purified neutrophils aged in culture.**

**Top panel:** Photomicrograph of purified neutrophils aged overnight in culture. Cultures contain a proportion of non-apoptotic cells which do not exhibit condensed nuclei and cytoplasmic vacuoles, features characteristic of apoptosis. Proportion of apoptotic cells prior to depletion = 47.5%. Magnification x400.

**Lower panel:** Apoptotic neutrophils were enriched from the cultured population above, by incubation of the cells with leu8 (CD62L) antibody and subsequent removal of the non-apoptotic aged cells using DYNABEADS®. Proportion of apoptotic cells after depletion = 73.8%. Magnification x400.





run in parallel using the mixed aged population of neutrophils and the enriched apoptotic population.

**2.12.3. Separation of the two cell types after the suspension assay.**

i) A FACStar PLUS (Becton Dickinson, Oxford, U.K.) cell sorter was used in an attempt to separate the distinct peaks that were observed on the EPICS profiles that might have represent phagocytic/nonphagocytic fractions. However, because of the relatively small number of mø involved initially, insufficient cells were obtained at the end of the sort to be conclusive.

ii) Cells were separated using a magnetic cell sorting method. Cells were labelled with PE-CD14 and CFDA as previously described (section 2.12.1) and incubated together at 37°C. After 30 minutes, the cells were spun down and incubated for a further 30 minutes at 4°C in 0.5ml PBS with 75µl MACS goat-anti-mouse IgG Microbeads (Eurogenetics U.K. Ltd., Teddington, U.K.) which bind to the PE-CD14 labelled mø. The cell suspension was then loaded onto a MiniMACS separation column, previously washed through with PBS and placed in a MiniMACS Separation Unit (both from Eurogenetics U.K. Ltd.). The eluant (fraction 1) was collected, and the column washed through with 1ml and 0.5ml PBS (fractions 2 and 3 respectively). The column was then removed from the separation unit and washed a final time with 1ml of PBS (fraction 4). Cytospins and flow cytometer samples were made of each eluant fraction, of the cell suspensions initially loaded onto the column and of the individual cell types involved.

To determine if the in vitro manipulations were affecting the levels of phagocytosis in suspension, mø were labelled for 30 minutes at 4°C with UCHM1 (CD14; 1:2) in the Teflon foils in which they were cultured. The mø

were then gently recovered from the Teflon, spun out of the culture medium and antibody, counted and suspended at $1x10^6$/ml Iscove's DMEM. Apoptotic neutrophils were not labelled but resuspended at $10x10^6$/ml Iscove's DMEM and added to Teflon foils in a ratio with the mø of 10:1. The cell suspensions were then incubated at 37°C for 30 minutes after which time, magnetic separation was performed. The standard phagocytosis assay was run in parallel using the same labelled/unlabelled cells as appropriate.

## 2.13. STATISTICAL ANALYSIS.

All graphs display the mean ± the standard error of the mean. The Students t test was used unless otherwise indicated.

# CHAPTER 3.

## 3.1. INTRODUCTION.

There is much evidence that the molecules αvβ3, CD36 and thrombospondin are involved in the recognition of apoptotic neutrophils, as described in chapter one. However, little is known about the underlying regulatory mechanisms of this phagocytic system. Protein kinase C and protein kinase A have been implicated in modulating β1, β2 and β3 integrin function (Shimizu *et al.*, 1990; Dustin and Springer, 1989; Stupack *et al.*, 1992 respectively). Protein kinase C (PKC) is a calcium- and phospholipid-dependent kinase implicated in a variety of cellular responses. Diacylglycerol, a product of phosphatidylinositol 4,5-bisphosphate hydrolysis, activates PKC which in turn phosphorylates a range of cellular proteins (Nishizuka, 1986). To date, nine distinct isoforms of PKC have been identified which may have distinct biological roles (for review article, see Hug and Sarre, 1993). PKC has been shown to be the receptor for phorbol esters (Kikkawa *et al.*, 1983), tumour promoters that activate PKC by mimicking the action of diacylglycerol. The enzyme protein kinase A (PKA) occurs as a tetramer consisting of two regulatory subunits and two catalytic subunits bound together. The regulatory subunits each have two cAMP binding sites and PKA has been shown to be the major intracellular receptor for cAMP. Adenylyl cyclase catalyses the formation of cAMP from ATP. In the absence of cAMP, the PKA complex has low activity, however, when cAMP binds to the regulatory subunits of PKA, it promotes dissociation of the tetramer and the enzymatic activity of the catalytic subunit markedly increases. PKA catalyses the transfer of the terminal phosphate of ATP to specific substrate proteins. For reviews see Soderling (1990), Taylor *et al.*, (1990) and Walaas and Greengard (1991). I therefore sought to assess the

effect of short term pretreatment of mø with activators of PKC and PKA to determine the effect on mø recognition of apoptotic neutrophils.

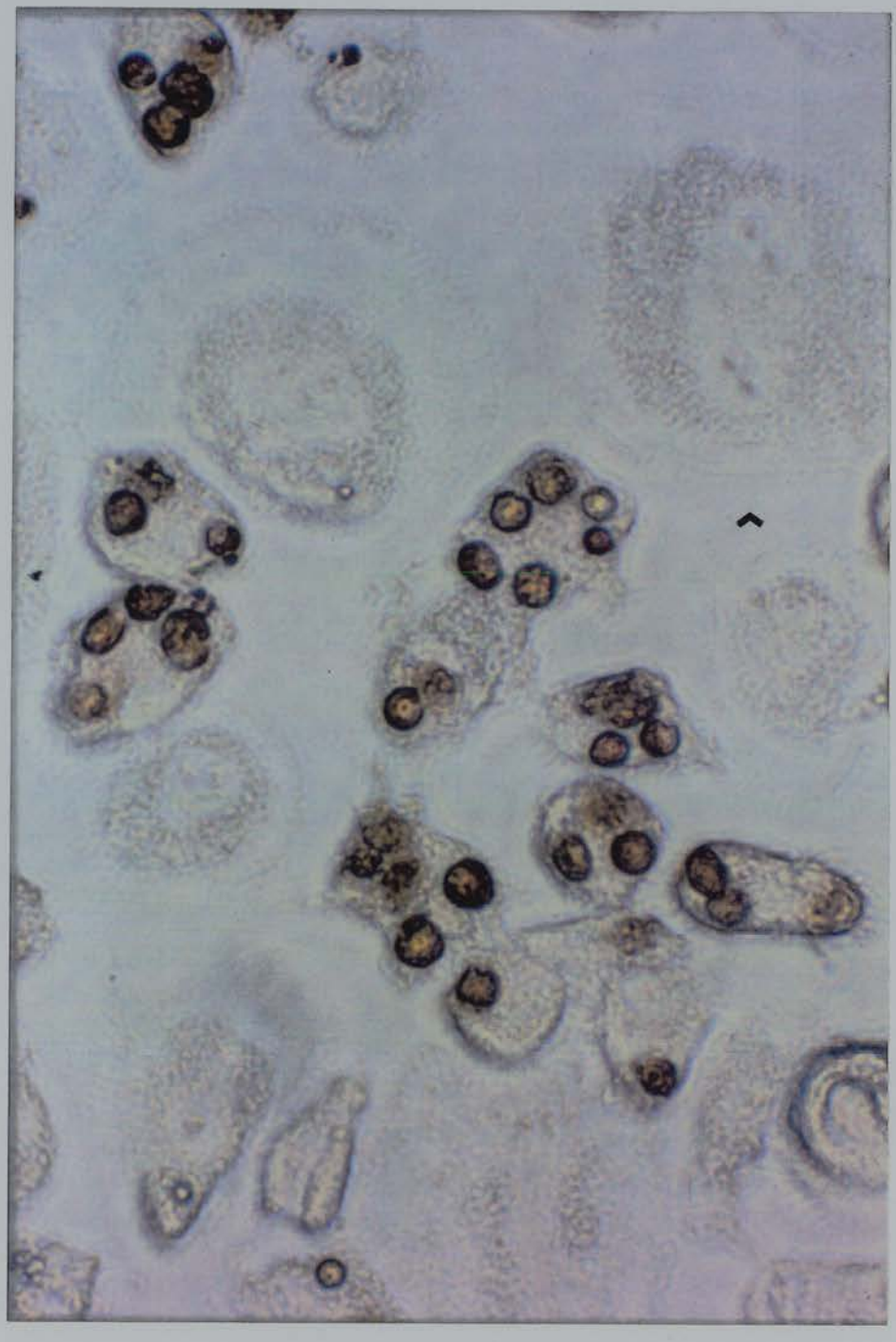
## 3.2. EFFECT OF SHORT TERM PRE-TREATMENT OF MACROPHAGES WITH PdBu AND dbcAMP ON RECOGNITION OF APOPTOTIC NEUTROPHILS.

A typical light microscopy field of the phagocytosis assay performed under control conditions is shown in figure 3.1. While most MPO-positive cells within mø appeared intact after the standard 30 minute assay, some partially degraded MPO-positive material was usually apparent. It was observed that phenotypically small mø regularly phagocytosed apoptotic neutrophils whereas there was a sub-population of giant multi-nucleated cells that consistently did not. Levels of phagocytosis within an experiment were reproducible between the duplicate or triplicate wells. However, because of the variation of the levels of phagocytosis observed between experiments (ranging from 4.7% - 59.3%, a consequence of mø donor variability), results are expressed as a percent of the phagocytosis levels observed under control conditions in order to allow comparison of data from different experiments.

In initial experiments, the effect of 15 minutes pre-treatment of mø with the membrane permeable phorbol ester PdBu and the membrane permeable analogue of cAMP, dbcAMP (activators of PKC and PKA respectively) upon mø recognition of apoptotic neutrophils was assessed. When compared with mø phagocytosis of apoptotic neutrophils under control conditions, pre-treatment of mø with 50nM PdBu increased the percent of mø ingesting apoptotic neutrophils. In contrast, 2mM dbcAMP pre-treatment of mø decreased the percent of cells phagocytosing apoptotic neutrophils (Fig. 3.2).

**Figure 3.1. Typical appearance of the apoptotic neutrophil phagocytosis assay performed under control conditions.**

Mø that have phagocytosed aged neutrophils can be seen to contain one or more orange/brown MPO-positive neutrophils. Both intact and partially degraded neutrophils are visable within the mø. A giant multinuclear mø, typical of a sub-population that was consistently observed not phagocytose apoptotic neutrophils, is indicated (arrow). Magnification x400.



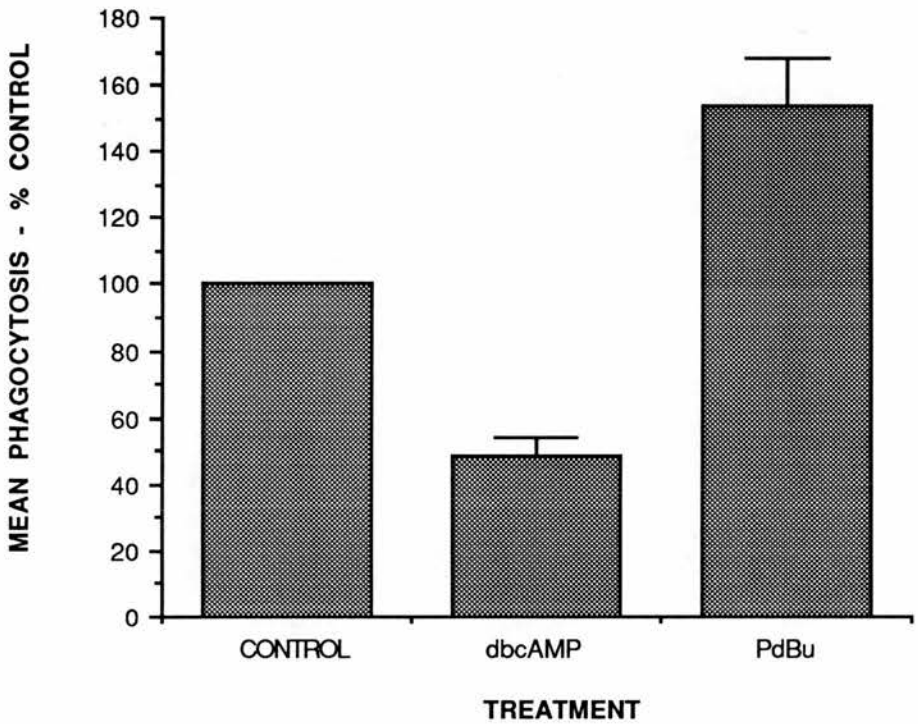



**Figure 3.2. Modulation of macrophage recognition of apoptotic neutrophils by protein kinase activation.**

Mø were pre-treated with either dbcAMP (2mM) or PdBu (50nM) for 15 minutes and then assessed for their ability to phagocytose aged apoptotic neutrophils. In this series of experiments, 26.84 ± 4.2% (mean ± S.E.) of macrophages phagocytosed apoptotic neutrophils under control conditions. Activation of PKC with PdBu significantly increased the percentage of macrophages phagocytosing apoptotic neutrophils (154% of control levels; $p<0.002$; $n=17$) while PKA activation with dbcAMP reduced the phagocytic response to 49% of control levels ($p<0.001$; $n=18$).

There was no significant difference between treatments in the number of cells counted per microscope field (data not shown) indicating that the changes seen are not due to loss of cells as a result of incubation with PdBu or dbcAMP.

Within individual experiments, the change in percent of phagocytosis relative to control values as a result of treatment with PdBu or dbcAMP was found not to be of the same magnitude (Fig. 3.3). One possibility considered was that the level of phagocytosis under control conditions determined the extent to which these agents exerted their effects. The results were therefore further analysed to see if PdBu and dbcAMP were exerting their effects to a greater extent on experiments with low or high control values respectively (Fig. 3.4). There appears to be no correlation between the extent of inhibition observed with dbcAMP and the level of phagocytosis occurring under control conditions. A trend does appear to exist between PdBu induced increase of phagocytosis and control levels. Additional experiments may resolve this but, due to the variation in the percentage of phagocytosis observed under control conditions as a consequence of mø donor variability, results may not prove to be conclusive. An alternative method may be to investigate the effects of PdBu and dbcAMP in experiments in which different concentrations of neutrophils are added to mø monolayers. Newman *et al.*, (1982) have shown that the level of phagocytosis of apoptotic cells by macrophages is dependent on the concentration of neutrophils. If the effects of PdBu are dependent upon the strength of the phagocytic stimulus, the relative increase in the proportion of mø that phagocytose apoptotic neutrophils at low cell concentrations should be greater than that at high cell concentration.

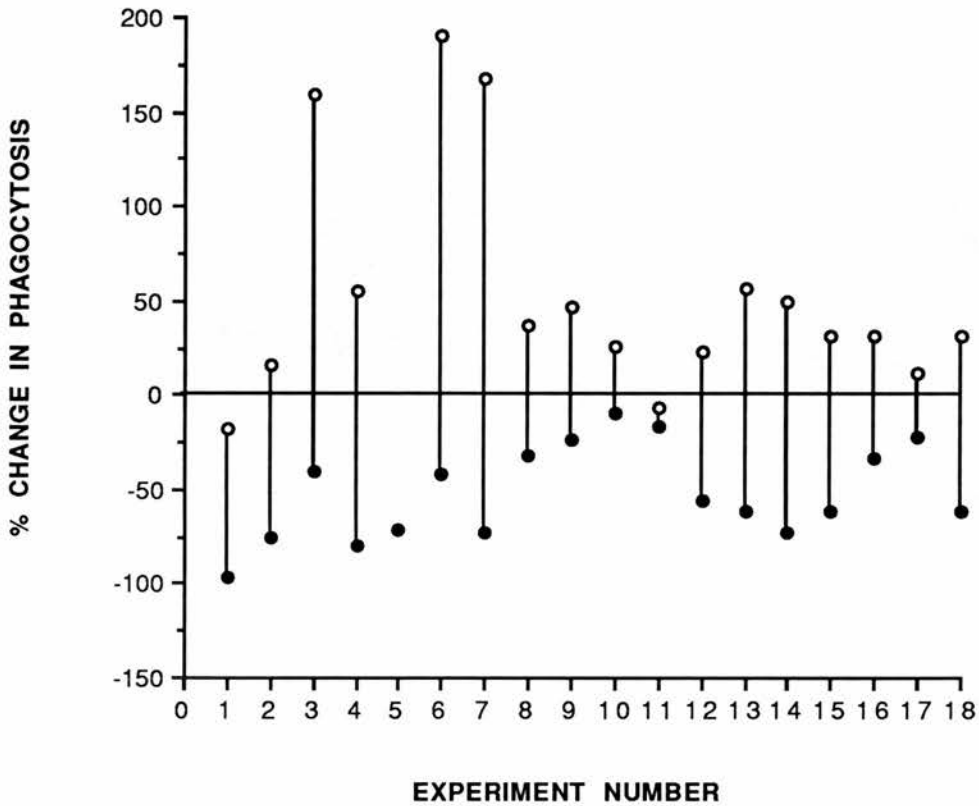



**Figure 3.3. Change in phagocytosis relative to control levels within individual experiments.**

Within an experiment, the percent change in phagocytosis observed upon treatment with PdBu or dbcAMP is not necessarily the same with respect to the relevant controls (o PdBu, • dbcAMP).

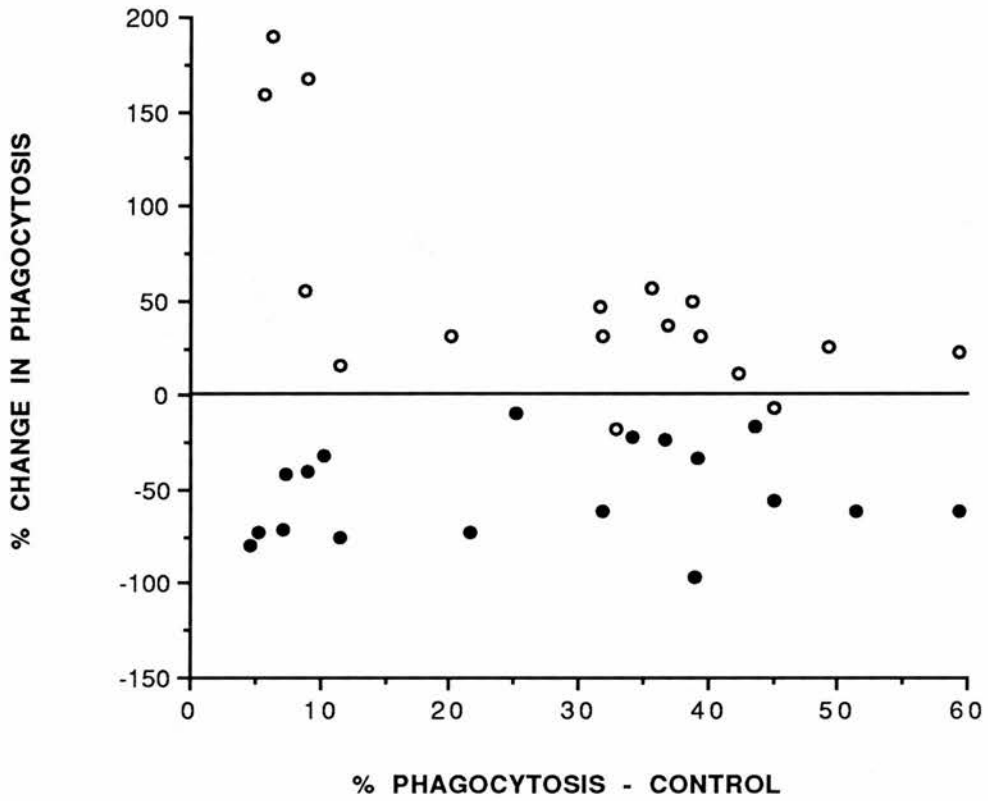



**Figure 3.4. Correlation between the percent change in phagocytosis upon treatment with the percent of phagocytosis under control conditions.**
PdBu may enhance phagocytosis levels more in experiments with low counts of phagocytosis. dbcAMP appears to inhibit mø phagocytosis of apoptotic neutrophils to a similar degree across the range of phagocytosis levels under control conditions (o PdBu, • dbcAMP).

## 3.3. EFFECT OF THE INFLAMMATORY MEDIATOR $PGE_2$ ON MACROPHAGE RECOGNITION OF APOPTOTIC NEUTROPHILS.

Arachidonic acid metabolites of the prostaglandin series, in particular $PGE_2$, are key inflammatory mediators acting via adenylate cyclase activation and thus elevation of intracellular cAMP. It was therefore tested whether $PGE_2$, like dbcAMP, would inhibit mø recognition of apoptotic cells. Treatment of mø for 15 minutes with $PGE_2$ plus the phosphodiesterase inhibitor IBMX resulted in a dose-dependent inhibition of phagocytosis of apoptotic neutrophils comparable with the levels of inhibition observed after dbcAMP treatment (Fig. 3.5).

$PGE_2$ has been shown to suppress macrophage function by increasing intracellular cAMP levels (Hutchison and Myers, 1987; Bonta *et al.*, 1981; Bonta and Parnham, 1982). However, it has been suggested that inhibition of macrophage phagocytosis by E-type prostaglandins is the result of a direct effect on the cell membrane (Oropeza-Rendon *et al.*, 1980). A cAMP radioreceptor assay was used to determine if there was a rise in the concentration of intracellular mø cAMP following treatment with $PGE_2$. Figure 3.6 shows that in this system, $PGE_2$ causes a rise in intracellular mø cAMP to a maximum by 2 minutes and while the concentration falls, it remains elevated above control conditions throughout the time course of the assay. This would suggest that $PGE_2$ is exerting its inhibitory effect on mø phagocytosis of apoptotic neutrophils by elevation of intracellular levels of mø cAMP.

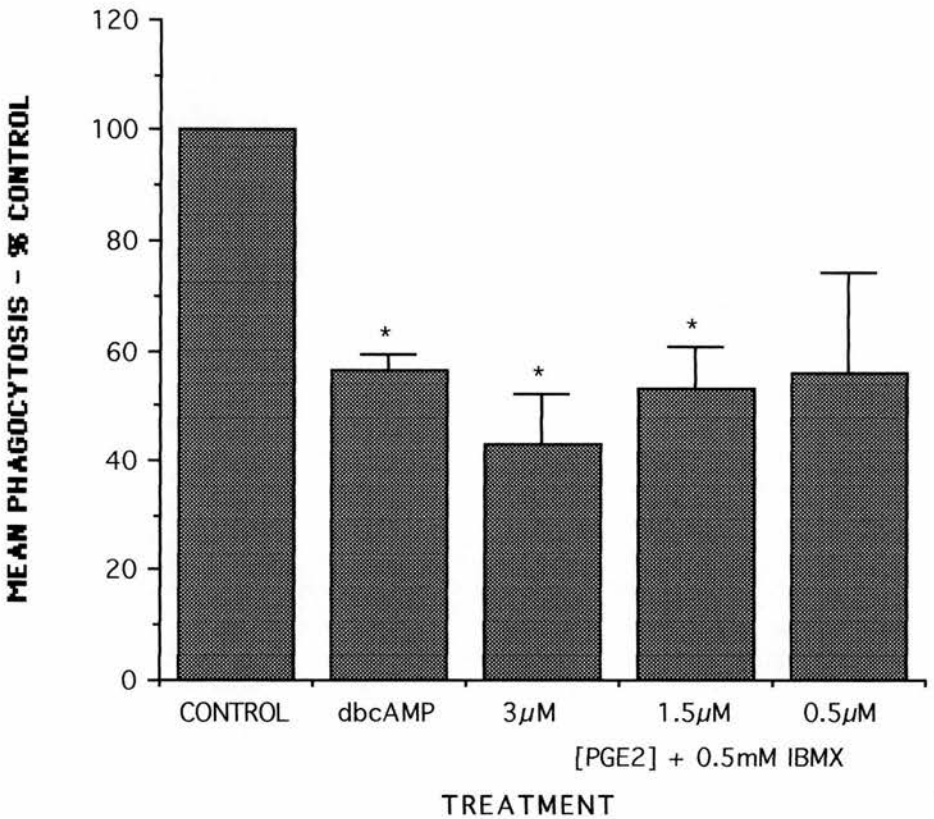



**Figure 3.5. Effect of prostaglandin $E_2$ on macrophage recognition of apoptotic neutrophils.**

Monocyte-derived macrophages were pre-treated with different concentrations of $PGE_2$ and 0.5mM IBMX and then assessed for their ability to recognise apoptotic neutrophils. Phagocytosis under control conditions was 16.8 ± 1.3% (mean ± S.E.; n=3). Inhibition was dose-dependent with maximum observed inhibition (43% of control levels) seen at 3μM $PGE_2$ (* $p < 0.05$).

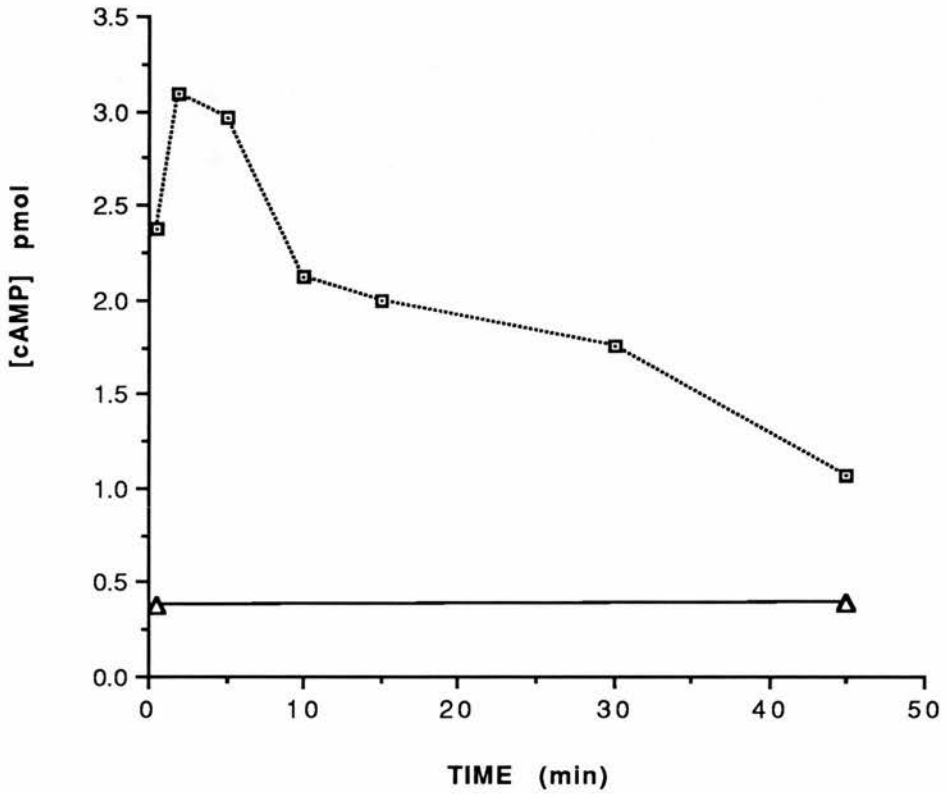



**Figure 3.6. Effect on intracellular levels of cAMP in monocyte-derived macrophages treated with $PGE_2$.**

Mø were treated for 0.5, 2, 5, 10, 15, 30 and 45 minutes with 3μM $PGE_2$ and 0.5mM IBMX and assayed for cAMP as described in section 2.8. Points are the mean ± S.D. of triplicate samples and the figure represents one of four assays. The standard curve obtained with this experiment is shown in figure 2.5.Δ control,□PGE $_2$ + IBMX.

## 3.4. EFFECT OF PKC INHIBITION AND DOWN-REGULATION ON MACROPHAGE RECOGNITION OF APOPTOTIC NEUTROPHILS.

To assess the effect that PKC inhibition would have on mø recognition of apoptotic neutrophils, mø were treated for 15 minutes with the PKC inhibitor H-7 (6μM; Hidaka *et al.*, 1984). Mø were also treated for 5 minutes with H-7 prior to being treated for 15 minutes with PdBu to determine if PKC inhibition altered the enhanced effect that PdBu has on this phagocytic system. Pretreatment of mø with H-7 alone had little effect on recognition of apoptotic neutrophils when compared to control conditions. However, H-7 inhibited the 40% increase in mø recognising apoptotic neutrophils induced by PdBu, the percent of mø recognising apoptotic neutrophils remaining unchanged from the control value (Fig. 3.7) indicating that while activation of PKC augments mø phagocytosis of apoptotic neutrophils, mø recognition under standard assay conditions does not appear to be PKC dependent.

In a number of cell types, prolonged treatment with phorbol esters results in reduced intracellular PKC concentrations (Rodriguez-Pena and Rozengurt, 1984; Stabel *et al.*, 1987; Adams and Gullick, 1989). 50nM PdBu was prepared in Iscove's DMEM plus 10% serum and mø were treated for 48 hours, 24 hours or 15 minutes prior to addition of apoptotic neutrophils. Mø treated for 15 minutes with 50nM PdBu showed the characteristic increase in phagocytosis of apoptotic neutrophils whereas treatment of mø for 24 hours with PdBu resulted in a decrease in the percent of mø phagocytosing apoptotic cells to 56.73% of control levels. Surprisingly, 48 hour treatment of mø caused the percent of cells that phagocytosed apoptotic neutrophils to rise to 147.18% of control levels (Fig 3.8).

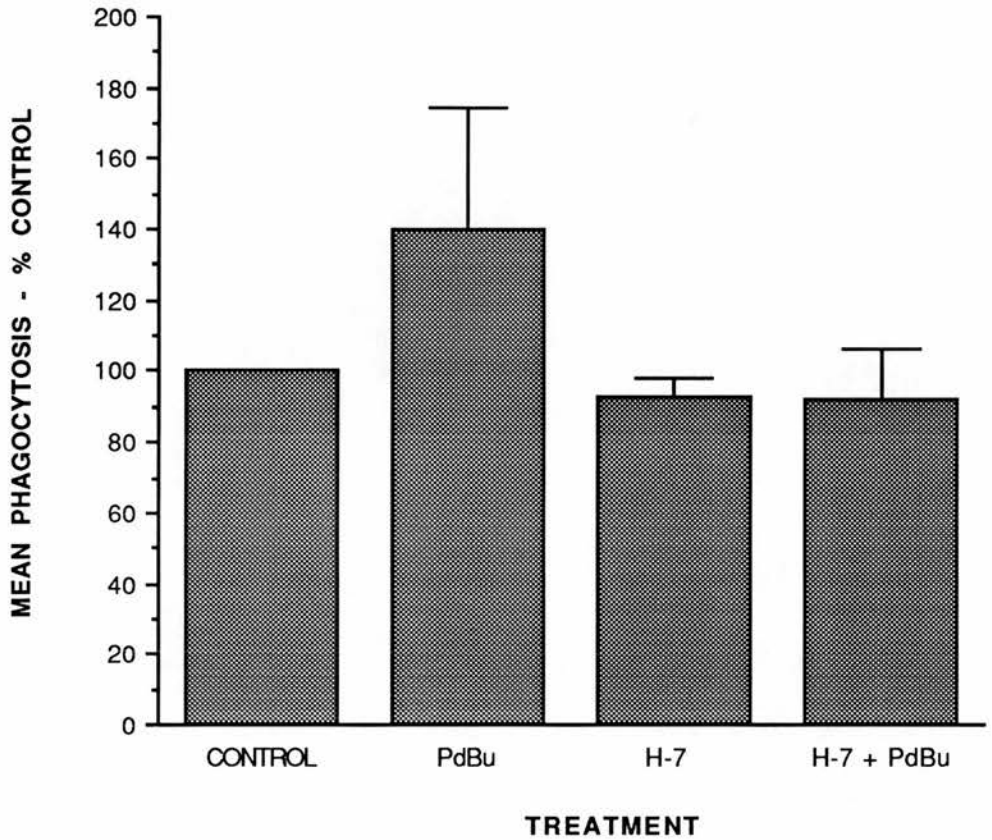



**Figure 3.7. Effect of PKC inhibition on macrophage recognition of apoptotic neutrophils.**

Mø were treated for 15 minutes with either PdBu (50nM) or H-7 (6μM) or for 5 minutes with H-7 followed by 15 minutes with PdBu. When compared to control conditions, treatment of mø with H-7 alone has no effect on the percent of mø recognising apoptotic neutrophils. However, the 40% increase induced by PdBu is abolished when mø are treated initially for 5 minutes with H-7. In this series of experiments, 34.79 ± 3.51% (mean ± S.E.) of mø phagocytosed apoptotic neutrophils under control conditions (n=3).

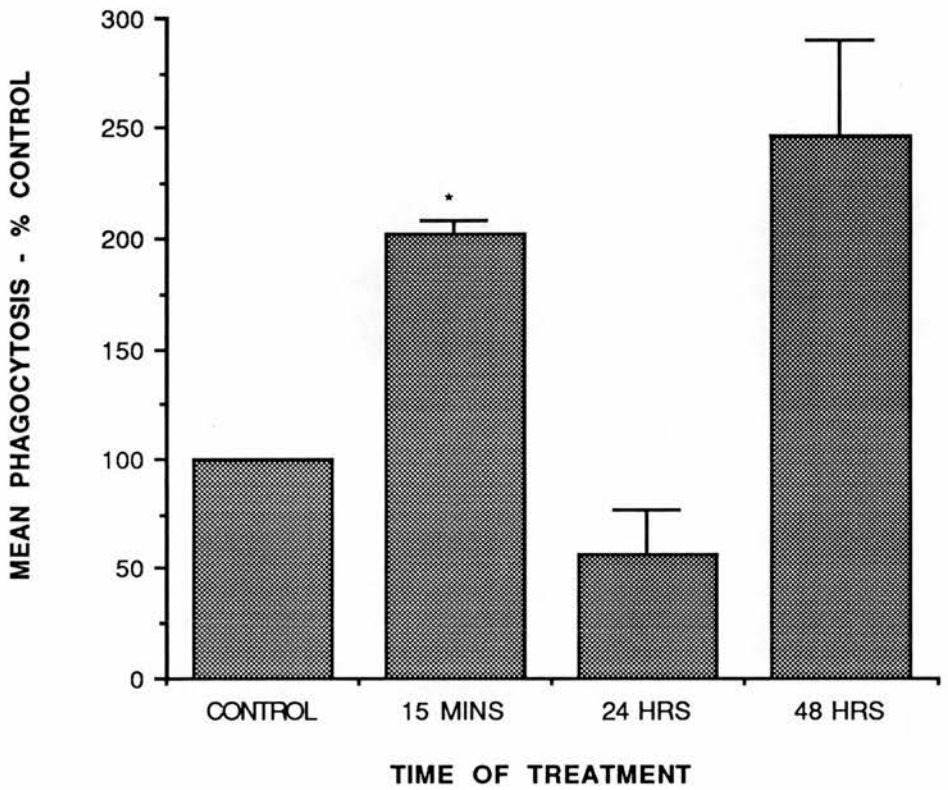



**Figure 3.8. Effect of long term pretreatment of macrophages with PdBu on phagocytosis of apoptotic neutrophils.**

Treatment of mø for 15 minutes increased the percent of mø phagocytosing apoptotic neutrophils (202.4% of control levels; *$p<0.005$). Prolonged treatment of mø with PdBu for 24 hours took phagocytic levels down to 56.73% of control values. Surprisingly, 48 hour treatment raised the percent of mø phagocytosing apoptotic neutrophils to a level comprable with the 15 minute treatment (n=3).

Together, these results suggest that the potential of monocyte-derived macrophages to phagocytose apoptotic cells can be rapidly and dynamically regulated and that modulation of protein kinase activity is associated with regulation of mø recognition of apoptotic neutrophils.

### 3.5. EFFECT OF SHORT TERM PRETREATMENT OF MACROPHAGES WITH PdBu AND dbcAMP ON RECOGNITION OF IG-OPSONISED ERYTHROCYTES.

It was possible that protein kinase activation was exerting effects down-stream of the initial events in mø recognition of apoptotic neutrophils resulting in functional alterations that would affect all mø phagocytic processes. To investigate whether PdBu and dbcAMP had similar effects on Ig receptor-mediated phagocytosis, similar treatment protocols were used and the ability of mø to phagocytose Ig-opsonised erythrocytes was assessed. In contrast to the effect of PdBu or dbcAMP on mø recognition of apoptotic neutrophils, no enhancement or reduction of phagocytosis of Ig-opsonised erythrocytes was observed (Fig. 3.9) suggesting that activators of protein kinases are not acting non-specifically.

### 3.6. EFFECT OF PdBu AND dbcAMP ON MACROPHAGE SURFACE EXPRESSION OF αvβ3 AND CD36.

All mø express αvβ3 and CD36 (Fig. 3.10). Using flow cytometry I next examined whether altered mø functional capacity in response to activating stimuli involved altered expression of these receptors. Although control and treated mø expressed CD51 (αv), CD61 (β3) and CD36 (Fig. 3.10), no significant alteration in surface expression, as assessed by changes in mean

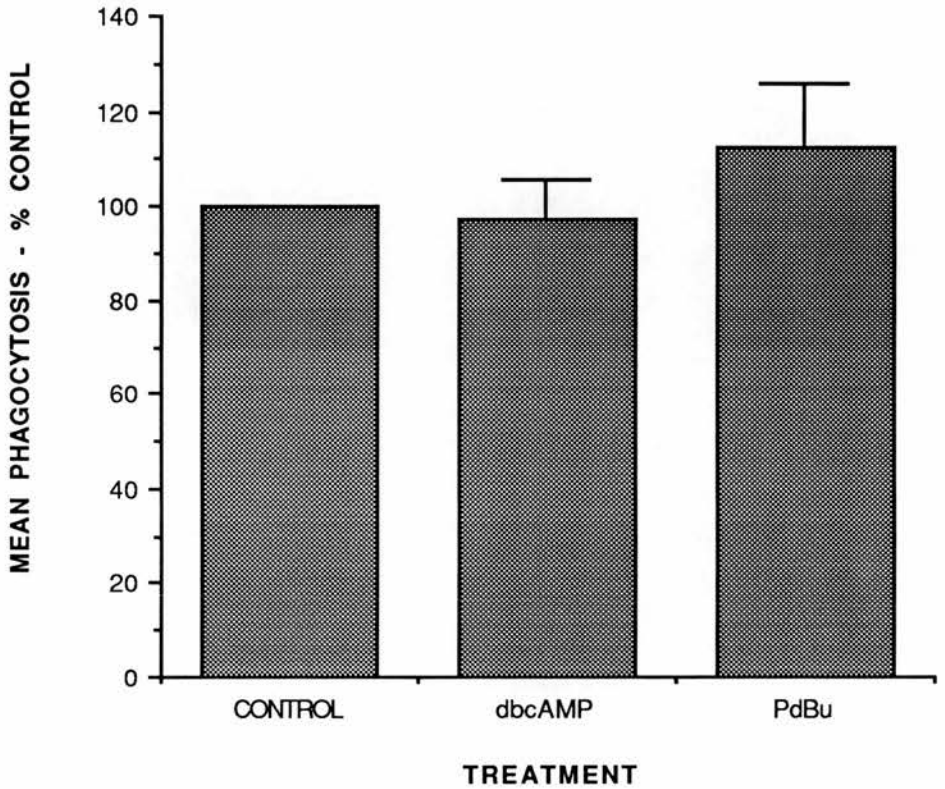
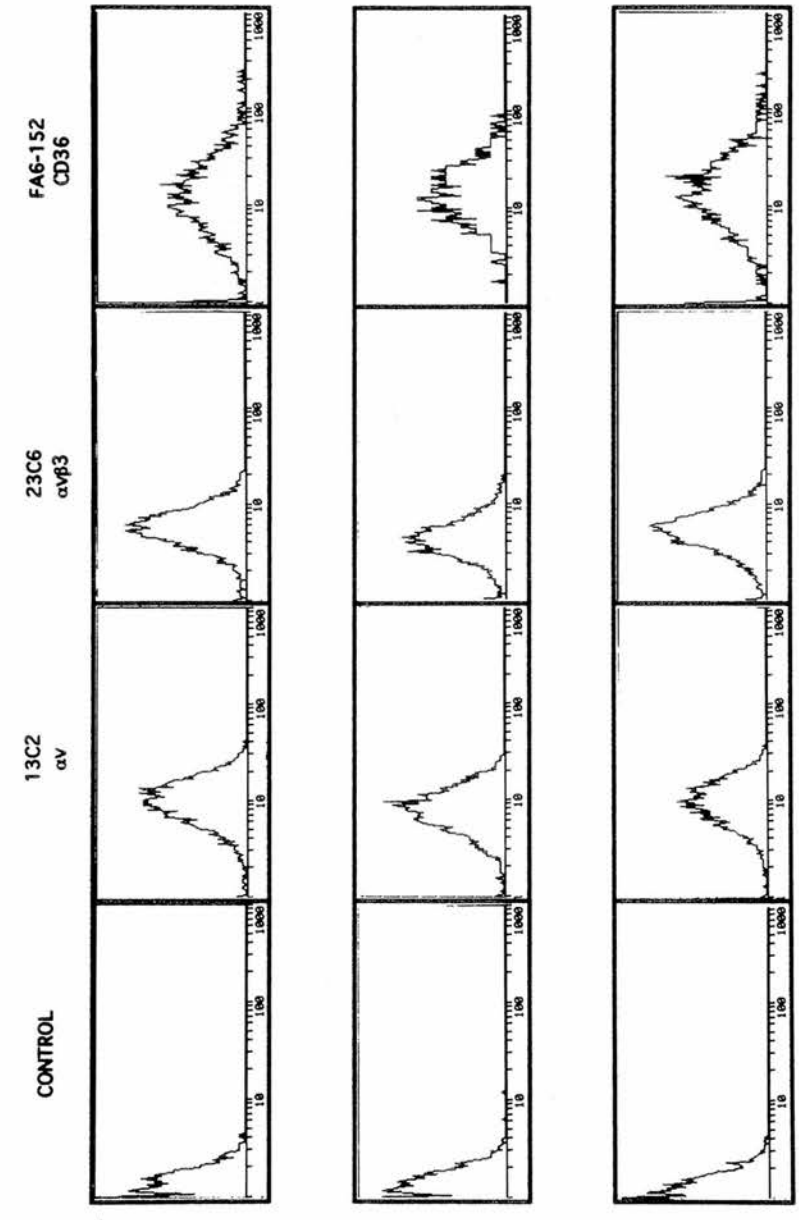



**Figure 3.9. Effect of dbcAMP and PdBu on macrophage phagocytosis of Ig-opsonised erythrocytes.**

Mø were treated with 2mM dbcAMP or 50nM PdBu for 15 minutes prior to assessment of their ability to phagocytose Ig-opsonised erythrocytes. In contrast to the effects of these reagents on apoptotic neutrophil phagocytosis, no significant change in Fc-mediated phagocytosis was observed (PdBu, n = 3; dbcAMP, n = 5). Under control conditions, 34.15 ± 11.55% (mean ± S.E.) of the mø recognised Ig-opsonised erythrocytes.

**Figure 3.10. Expression of αvβ3 and CD36 on treated and untreated macrophages.**

Monocyte-derived macrophages were pre-treated with protein kinase activators dbcAMP and PdBu or Iscove's DMEM as control for 15 minutes and examined for expression of αvβ3 and CD36 expression by flow cytometry. Representative fluorescence histograms from one of three experiments using mAb for αv (13C2), αvβ3 (23C6) and CD36 (SMØ) are shown.


CONTROL
13C2
αv
23C6
αvβ3
FA6-152
CD36
MEDIUM
PdBu
dbcAMP

fluorescence intensity, was observed following pre-treatment with protein kinase activators (Table 3.1). These data suggest that the effect of protein kinase activators on mø recognition of apoptotic cells does not occur as a result of modulation of the surface expression of the receptors involved in the recognition process.

## 3.7. ROLE OF THROMBOSPONDIN IN MACROPHAGE PHAGOCYTOSIS OF APOPTOTIC NEUTROPHILS.

As the levels of expression of αvβ3 and CD36 were not affected I next considered whether alteration in PKC or A activity affected the extracellular matrix molecule that has been suggested to act as a bridge between the αvβ3 and CD36 present on the mø surface and the apoptotic cell, mø secreted thrombospondin (Savill *et al.*, 1992).

### 3.7.1. Effect of PdBu and dbcAMP on the levels of thrombospondin secreted by macrophages.

Thrombospondin has been shown to be present in the assay medium after mø have been co-incubated with apoptotic neutrophils (Savill *et al.*, 1992). A sensitive dot blot assay using mAb to two distinct epitopes of thrombospondin (detection limit ≤ 0.05μg/ml of thrombospondin) was used to measure thrombospondin present in supernatants obtained from treated mø. It was found that extensive washing of mø was necessary to remove thrombospondin present in the culture medium (Fig. 2.7). The supernatants, isolated from untreated mø or mø pre-treated with either PdBu or dbcAMP for 15 minutes, washed and cultured for 30 minutes in medium alone, were tested for the presence of thrombospondin. However, somewhat surprisingly, thrombospondin could not be detected in supernatants from

| Experiment | Antibody | Control | PdBu | dbcAMP |
|---|---|---|---|---|
| 1 | | 3.2 | 3.8 | 4.4 |
| 2 | 13C2 | 3.9 | 3.9 | 3.8 |
| 3 | | 9.6 | 7.8 | 9.4 |
| 1 | | 3.6 | 4.1 | 3.8 |
| 2 | 23C6 | 3.3 | 4.9 | 5.0 |
| 3 | | 5.8 | 4.3 | 5.3 |
| 1 | | 6.2 | 4.8 | 5.6 |
| 2 | SMØ | 3.8 | 5.1 | 8.9 |
| 3 | | 3.1 | 3.3 | 3.7 |

**Table 3.1. Mean fluorescent values for expression of the vitronectin receptor and CD36 on the surface of control and treated macrophages.**
Values are the mean of duplicate samples and comparison of the mean fluorescence of each molecule following PdBu or dbcAMP treatment reveals no significant change in receptor expression from control levels in any of the experiments performed. Mean ± S.D. of mean fluorescent values for control mAb : experiment 1 = 1.9± 0.1, experiment 2 = 2.5 ± 0.2, experiment 3 = 1.4 ± 0.1

either control or treated mø, even when concentrated 20-fold (Fig. 3.11). Similar results were obtained with another mAb, MA-IV, to a different domain of thrombospondin (data not shown). The fact that thrombospondin could not be detected in medium under control conditions and that protein kinase activation did not induce thrombospondin secretion questions the role of secreted thrombospondin in the phagocytosis of apoptotic neutrophils.

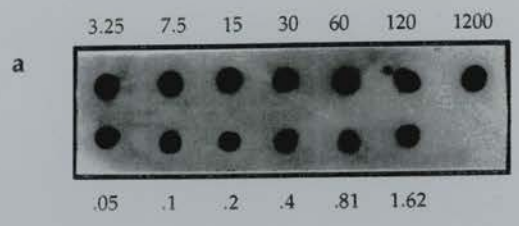
### 3.7.2. Effect of PdBu and dbcAMP on levels of macrophage surface-bound thrombospondin.

Since protein kinase activation does not cause alterations in the levels of surface expression of mø receptors or in the levels of thrombospondin secreted by mø, the altered functional activity caused by the protein kinase activators might alter levels of thrombospondin bound to the surface of the mø. Using indirect immunofluorescence flow cytometry, the level of surface-bound thrombospondin was analysed. In order to avoid problems of possible masking of epitopes on surface-bound thrombospondin, two mAb with distinct epitope specificites were used (MA-I binds to the type III repeat regions of thrombospondin and MA-IV binds to the heparin binding domain of thrombospondin). However, there was no significant change in the amount of thrombospondin bound to the surface of the mø as assessed by mean fluorescent values of treated and untreated mø (Fig. 3.12). These results demonstrate that protein kinase activation does not alter binding of thrombospondin to the mø surface and strongly suggest that modulation of mø recognition of apoptotic cells occurs independently of alterations of mø surface expression of $\alpha v\beta 3$ and CD36 and mø secretion and/or binding of thrombospondin.

**Figure 3.11. Effects of protein kinase activators on secretion of thrombospondin by macrophages.**

Supernatants obtained from mø pre-treated with PdBu or dbcAMP were concentrated 20-fold and serial dilutions dotted onto nitrocellulose. The membrane was then probed with mAb MA-I specific for thrombospondin and bound mAb visualized with peroxide-conjugated-anti-mouse Ig antibody using enhanced chemiluminescence detection reagents and autoradiography. Assay detection limit $\leq 0.05 \mu g/ml$ of thrombospondin.

a) thrombospondin ($\mu g/ml$) b) medium control c) PdBu d) dbcAMP.


a
3.25 7.5 15 30 60 120 1200
.05 .1 .2 .4 .81 1.62
b
c
d

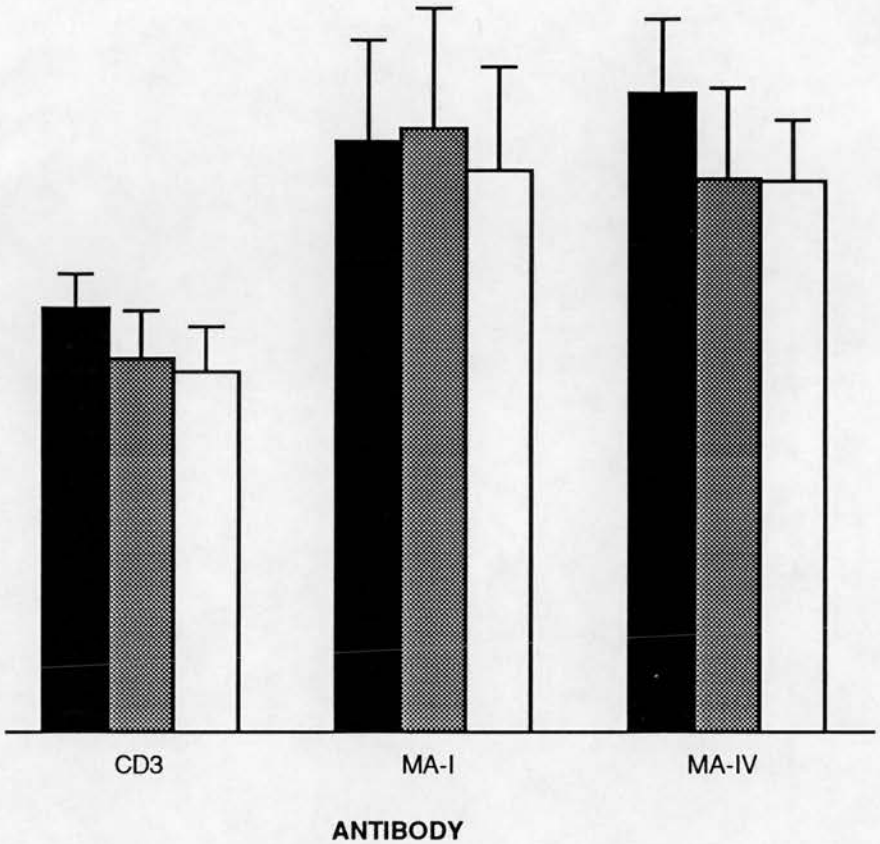



**Figure 3.12. Effects of protein kinase activation on levels of thrombospondin bound to the macrophage surface.**

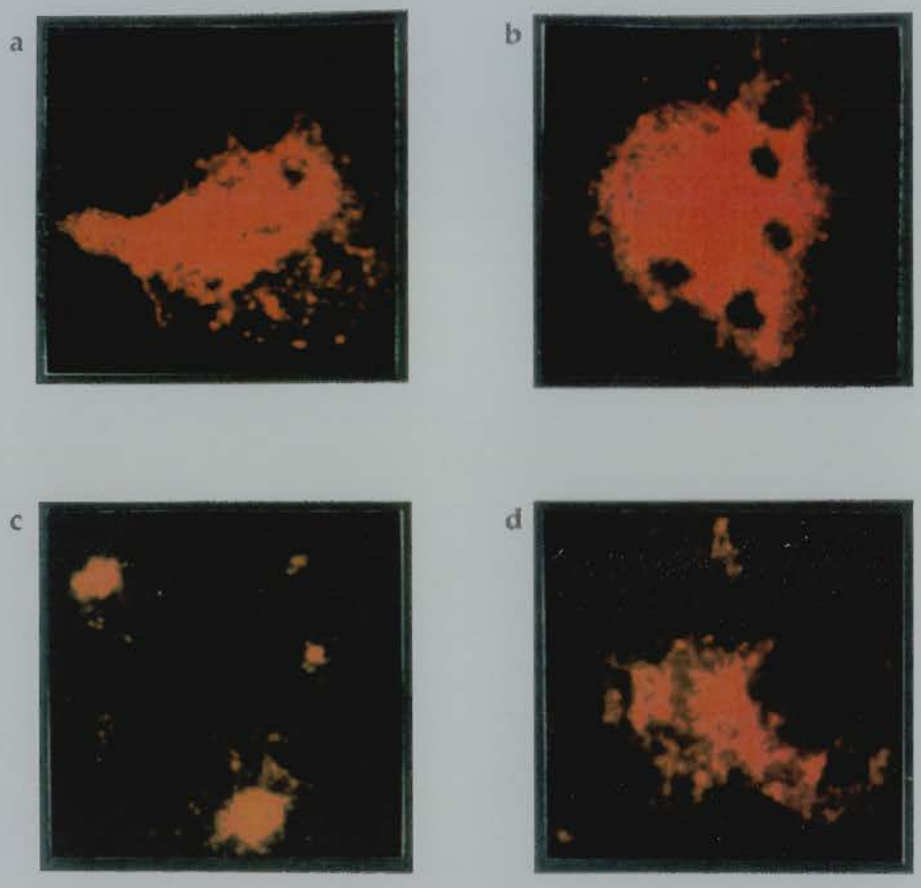
The levels of surface-bound thrombospondin on untreated mø or following pre-treatment with protein kinase activators was assessed by flow cytometry. Mø were pre-treated for 15 minutes with control media (black bars), PdBu (grey bars) or dbcAMP (white bars). Antibodies against the type III repeats, MA-I, and the heparin binding domain, MA-IV, of thrombospondin were used to detect any change in surface bound thrombospondin. No significant change in the amount of surface bound thrombospondin was observed upon activation of PKC or PKA (n=3).

## 3.8. EFFECT OF PROTEIN KINASE ACTIVATORS ON MACROPHAGE RECEPTOR DISTRIBUTION.

Another possible mechanism for the observed effects of protein kinase activators upon mø recognition of apoptotic neutrophils is that the membrane distribution of receptors involved in the recognition process could be altered without changes in the number or affinity of the receptors expressed on the mø surface. Using the mAbs PM6/13 (1:50) against the vitronectin receptor β3-subunit and FA6-125 (1:100) against the CD36 thrombospondin receptor, the cellular distribution of these receptors on mø pre-treated with dbcAMP or PdBu for 15 minutes was examined using indirect immunofluorescence microscopy. In keeping with data from flow cytometric analysis, all monocyte-derived macrophages were found to express β3 and CD36. The distribution of CD36 was found to be similar on mø treated with PdBu and dbcAMP (Fig. 3.13a and b respectively). However, heterogeneity in terms of the pattern of distribution of the β3 subunit was observed in both untreated and treated mø populations. For some mø, a distinct "punctate" distribution of β3 (Fig. 3.13c) was readily distinguished from a uniform distribution (Fig. 3.13d). It was therefore assessed whether pre-treatment of mø with either PdBu or dbcAMP altered the proportions of mø which had these staining patterns. Comparison of β3 distributions in PdBu-treated and cAMP-treated mø revealed that dbcAMP reduced the percentage of mø with a punctate β3 distribution (see Table 3.2). Protein kinase activation may therefore serve to modulate the mø phenotype in terms of cellular distribution of the integrin αvβ3, thereby regulating recognition of apoptotic neutrophils.

**Figure 3.13. Effect of protein kinase activation upon distribution of receptors on the macrophage surface.**

Mø, pretreated with PdBu and dbcAMP, were fixed and stained with mAb against CD36 or the β3 subunit to determine if activation of PKC or PKA altered the distribution of the receptors involved in mø recognition of apoptotic neutrophils. Typical distributions observed for CD36 on mø treated with either PdBu or dbcAMP are shown (a and b respectively). Two distinct patterns of β3 distribution were observed, with a distinct 'punctate' pattern (c) contrasting with a more homogenous staining pattern (d). Magnification x1000.


a
b
c
d

| Experiment | TREATMENT | | % change |
|---|---|---|---|
| number | PdBu | dbcAMP | dbcAMP/PdBu |
| 1 | 31.9 | 26.8 | 16.0 |
| 2 | 37.1 | 19.2 | 48.3 |
| 3 | 19.4 | 10.6 | 45.5 |

**Table 3.2. Percent of monocyte-derived macrophages with punctate PM6/13 (β3) staining pattern following pre-treatment with the protein kinase activators PdBu and dbcAMP.**

### 3.9. SUMMARY OF RESULTS.

Activation of PKC by treatment with PdBu up-regulates mø recognition of apoptotic neutrophils while PKA activation by dbcAMP causes down regulation of recognition. Within an experiment, the change in phagocytosis between PdBu and dbcAMP treatments, relative to controls, is not necessarily of the same magnitude and results suggest that PdBu may increase the percent of mø phagocytosing apoptotic neutrophils more in experiments in which there is a low level of phagocytosis in untreated cells. In contrast, dbcAMP appears to inhibit to a similar degree irrespective of the amount of phagocytosis in control conditions.

The inflammatory mediator $PGE_2$ reduces phagocytosis of apoptotic neutrophils by elevating intracellular mø cAMP. Inhibition of PKC with H-7 alone does not affect the percent of mø recognising apoptotic neutrophils. However, H7 does inhibit the enhanced phagocytosis induced by PdBu treatment. Prolonged treatment of mø for 24 hours with PdBu reduces the percent of mø recognising apoptotic neutrophils. Unexpectedly however, 48 hour treatment of mø with PdBu causes in an increase in the number of mø phagocytosing apoptotic neutrophils.

The effect of PKC and PKA activation appears to be specific for this phagocytic system as no change in phagocytosis of Ig-opsonised erythrocytes is observed upon pretreatment of mø with PdBu or dbcAMP and changes in recognition levels occur without altered $\alpha v\beta 3$ or CD36 expression on the surface of the treated mø. Thrombospondin could not be detected in the medium from treated or untreated mø and activation of PKC or PKA does not alter the amount of thrombospondin bound to the surface

of the mø. Finally, treatment with PdBu and dbcAMP altered the distribution of mø β3 but not CD36.

### 3.10. DISCUSSION

The control mechanisms that underlie mø recognition, phagocytosis and removal of apoptotic cells from inflamed sites are central to our understanding of the control of inflammation and the processes by which it resolves. Perturbation of the balance between neutrophil recruitment from the circulation, phagocytic removal of apoptotic neutrophils by macrophages (or other phagocytes) and neutrophil disintegration or necrosis may determine whether inflammation resolves or progresses to the chronic inflammatory states associated with most inflammatory diseases. However, since some macrophage populations (e.g. giant cells and alveolar macrophages) are relatively inefficient at phagocytosing apoptotic cells, microenvironment-dependent phenotypic alterations during differentiation may also regulate this process (Rutherford *et al.*, 1993). Evidence to date suggests that binding and subsequent phagocytosis of apoptotic neutrophils by mø requires both CD36 and αvβ3. However, although all mø express these molecules (Fig. 3.9), not all mø respond to the stimulus of apoptotic cells by phagocytic uptake. Therefore, expression is necessary but not sufficient for all mø to ingest apoptotic neutrophils. This observation implies that further regulatory mechanisms exist.

Here, it has been demonstrated that modulation of protein kinase activity has the potential to alter the ability of monocyte-derived macrophages to recognise and phagocytose apoptotic neutrophils thereby providing a rapid, subtle response to micro-environmental stimuli. Activators of PKA were

found to reduce the percentage of mø phagocytosing apoptotic cells, whereas activators of PKC increased the proportion of responding mø. Treatment with the PKC inhibitor H-7 caused no change in the level of phagocytosis when compared to control conditions. However the PdBu-induced response was ablated by H-7 , suggesting that mø phagocytosis of apoptotic neutrophils is not PKC dependent. Treatment of mø for 24 hours with PdBu (down-regulating PKC) reduced the number of mø phagocytosing apoptotic neutrophils seemingly contradicting the H-7 and PdBu results. However, total inhibition was not achieved following 24 hour treatment and 48 hour treatment of mø saw phagocytosis levels, relative to control, comparable with that seen after 15 minutes of PdBu treatment. With respect to PKC down-regulation, different PKC isoenzymes are reported to react differently within a single cell type (Ase *et al.*, 1988) and between cell types (Adams and Gullick, 1989). Knowledge of the PKC isoforms involved, use of more specific PKC inhibitors and quantification of PKC upon phorbol ester treatment (Stabel *et al.*, 1987) could help clarify the role of PKC in mø phagocytosis of apoptotic neutrophils.

Although it is possible that all monocyte-derived macrophages are capable of phagocytosing apoptotic cells providing that a certain activation "threshold" is reached, the increase in the percentage of mø phagocytosing apoptotic cells following phorbol ester treatment was ~150% of control levels, suggesting the system could be responsive to stimulants other than PKC activators. Alternatively, some mø may be unable to respond to the apoptotic cell stimulus. In support of this argument, it has been observed that some mø sub-populations (notably multi-nucleated giant cells, Fig. 3.1) may be particularly inefficient at phagocytosis of apoptotic neutrophils.

The above observations may be of great significance for removal of apoptotic neutrophils *in situ*. A variety of inflammatory mediators including TNF and many cytokines (IL8, C5a, fMLP) ultimately simulate PKC activity and may increase apoptotic cell removal, whereas agents which stimulate adenyl cyclase activity and activate PKA (Aderem, 1993) could possibly decrease apoptotic cell removal. Inflammatory mediators may have complex effects upon leukocyte function in terms of resolution of inflammation. Treatment of mø with $PGE_2$ inhibited subsequent recognition of apoptotic cells thereby reducing macrophage potential for phagocytic removal of apoptotic cells. One consequence of this might be that although $PGE_2$ acts as an "anti-inflammatory" agent in terms of neutrophil function at inflamed sites, an increased proportion of neutrophils may progress from apoptosis to necrosis which would result in an increased potential for local tissue damage and prolongation of the inflammatory response.

In keeping with previous observations (Newman *et al.*, 1991), phorbol ester and dbcAMP treatment of human monocyte-derived macrophages had no effect on Fc receptor-mediated phagocytosis suggesting the effects of PKC and PKA activation are acting specifically on the apoptotic neutrophil phagocytic system. Protein kinase activation did not alter mø expression of $\alpha v\beta 3$ or CD36 or mø surface bound thrombospondin. It is of interest that phorbol ester treatment induces CD36 expression on the U-937 macrophage cell line (Talle *et al.*, 1983). Thrombospondin (0.59μg/ml) has previously been detected in the assay medium following mø phagocytosis of apoptotic neutrophils (Savill *et al.*, 1992). Using a detection technique, 1000 times more sensitive than that previously used, thrombospondin could not be detected in concentrated supernatants from either control or treated mø. The supernatants tested for the presence of thrombospondin were obtained in the

absence of apoptotic neutrophils and it is possible that apoptotic neutrophils act as a stimulus to induce thrombospondin production by mø. Alternatively, results presented in this thesis suggest that thrombospondin detected by Savill and colleagues (1992) could be residual serum from the mø and neutrophil culture media (Fig. 2.7a) which I have found requires extensive washing to remove completely. That phagocytosis occurs in the absence of detectable amounts of secreted thrombospondin questions the bridging role suggested by Savill *et al.* (1992) for mø-synthesised thrombospondin in phagocytosis of apoptotic neutrophils.

The αvβ3 integrin mediates adhesion to substrates (Wayner *et al.*, 1991; Charo *et al.*, 1990; Smith *et al.*, 1990) and promotes migration on ECM components (Leavesley *et al.*, 1993). PKC regulates the function of αvβ3 independently of alterations of surface expression in some B cell lines (Stupack *et al.*, 1992). Regulation of focal contact formation and co-localisation of vinculin and talin with clusters of integrin receptors occurs in a PKC-dependent manner (Woods and Couchman, 1992), possibly as a result of direct phosphorylation of integrin subunits (Freed *et al.*, 1989). Interestingly, for cell lines that express both αvβ3 and αvβ5, only αvβ3 becomes associated with focal contacts and not αvβ5 (Woods and Couchman, 1992). The distribution of the αvβ3 integrin and CD36 on mø following pre-treatment with protein kinase activators was therefore examined.

In treated mø, no alterations in receptor distribution of either CD36 or a number of integrin α-subunits including αv, α5, and αp150,95 were noted (not shown). In contrast, for a subpopulation of mø, altered distribution of the integrin β3-subunit was observed. These changes occurred in parallel

with changes in the percentage of mø phagocytosing apoptotic cells following pre-treatment with these agents. Thus, homogeneous distribution of αvβ3 was associated with low levels of mø phagocytosis, whereas a punctate distribution was associated with higher levels of phagocytosis. Taken together, results presented in this chapter suggest that receptor redistribution may provide a mechanism for increased avidity of binding via αvβ3 which results in enhanced mø recognition of apoptotic cells.

In summary, I have shown that short term treatment of mø with protein kinase activators modulates mø recognition of apoptotic cells. These are observations which may have significant functional *in vivo* implications since a variety of mediators likely to be present at inflamed sites have proven capacity to influence these pathways. Modulation appears to be specific for this phagocytic system and is independent of changes in expression of the receptors implicated in the recognition process or modulation of thrombospondin production/binding by mø. A specific, rapid redistribution of αvβ3 was observed in treated mø which may be related to the alteration in the percentage of mø which are able to recognise apoptotic cells. It is reasonable to speculate that rapid redistribution of αvβ3 on the surface of the macrophage may play a role in regulatory mechanisms that underlie macrophage recognition of apoptotic cells.

# CHAPTER 4.

## 4.1. INTRODUCTION.

The cytoskeleton of the macrophage consists of a three-dimensional network of actin filaments and associated proteins (Yin and Hartwig, 1988: Baba *et al.*, 1991). Communication between the cytoskeleton and the ECM is via integrins which provide a trans-membrane link (Tamkun *et al.*, 1986). At sites of cell-ECM or cell-cell adhesion, the integrin β-subunit cytoplasmic domain has been shown to indirectly associate with the actin skeleton via cytoskeletal proteins such as talin (Horwitz *et al.*, 1986: Tapley *et al.*, 1989) and α-actinin (Otey *et al.*, 1990). Talin and α-actinin binding to integrin is a low affinity interaction ($10^{-6}$ to $10^{-7}$M). α-subunits have different cytoplasmic sequences and different integrin receptors for the same ligand can differ in apparent associations with the cytoskeleton (Wayner *et al.*, 1991: Elices *et al.*, 1991) suggesting that the α-subunit may regulate the types of interaction that the β-subunit cytoplasmic tail can have with its cytoskeletal binding sites. Evidence suggests ligand-induced regulation of focal adhesion localisation, the α-subunit serving to interact (directly/indirectly) with sites on the β-subunit (Ylanne *et al.*, 1993). Ligation would propagate a conformational change that allows the β-subunit cytoplasmic domain to interact with cytoplasmic molecules giving rise to discrete intracellular functions (for review see Sastry and Horwitz, 1993). Since results presented in the previous chapter demonstrated altered β3 distribution associated with modulation of mø recognition, dual immunofluorescence techniques were used to investigate if there was association of mø αvβ3 with mø cytoskeletal components and to assess the effect that PKC and PKA activation might have on such interactions.

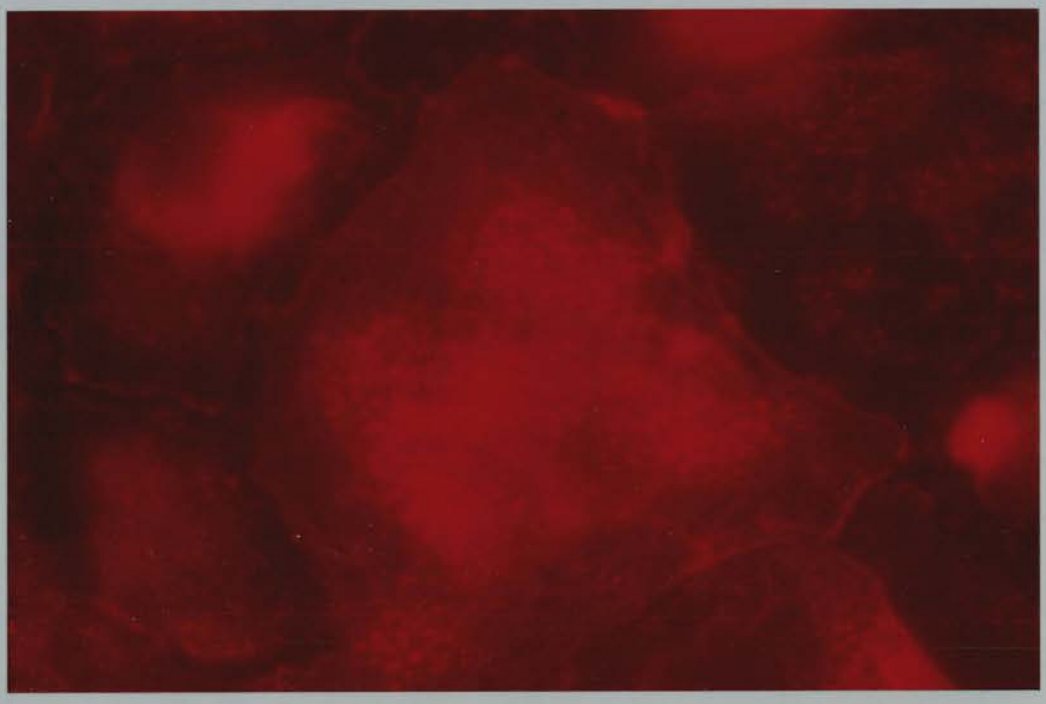
## 4.2. ASSOCIATION OF TALIN BUT NOT INTEGRIN β3 SUBUNIT WITH THE MACROPHAGE ACTIN CYTOSKELETON AND THE EFFECT OF PROTEIN KINASE ACTIVATION.

Mø, treated for 15 minutes with PdBu, dbcAMP or Iscove's DMEM as control, were fixed and double-stained for either the vitronectin receptor β subunit (β3), talin or vinculin (a cytoplasmic protein that interacts with talin) (Burridge and Mangeat, 1984), and for F-actin. The distribution of the β3 subunit, cytoskeletal proteins and actin was examined by indirect immunofluorescence microscopy and confocal microscopy.

Mø treated with Iscove's DMEM and PdBu exhibited a network of actin filaments often traversing the cell (Fig. 4.1 and 4.2 respectively) and terminating at the substrate-associated side of the cell at small focal adhesions, characterised by the presence of talin (Fig. 4.3) and vinculin (not shown). In mø treated with dbcAMP, most of the of longer actin filaments had disappeared and the actin was observed as short, irregular appearing aggregates, the majority concentrated within the body of the cell (Fig. 4.4). Confocal microscopy still showed association of talin (and vinculin) with actin however, dbcAMP treatment caused the discrete colocalisation seen in control and PdBu treated cells to disappear and a diffuse staining pattern to form (Fig.4.3). Thus, one effect of dbcAMP was to alter mø actin cytoskeletal architecture. However, αvβ3 is not specifically localised at sites of actin foci, which contain talin and vinculin. This suggests that αvβ3 is not involved in the regulation of mø cytoskeletal organisation.

**Figure 4.1. (Top panel) Actin distribution of untreated macrophages.**
Unterated mø, adhered for 75 minutes in Iscove's DMEM, were fixed and stained with rhodamine-conjugated phalloidin. A prominent system of actin filaments are seen to traverse the cell and terminate in punctate structures at the substrate-associated surface of the mø. Magnification x1000.

**Figure 4.2. (Lower panel) Actin distribution of macrophages treated with PdBu.**
Mø, adhered for 60 minutes in Iscove's DMEM, were treated for 15 minutes with PdBu (50nM), fixed and stained with rhodamine-conjugated phalloidin. Both linear and punctate structures are visible comparable to those observed in untreated mø (Fig. 4.1). Magnification x1000.



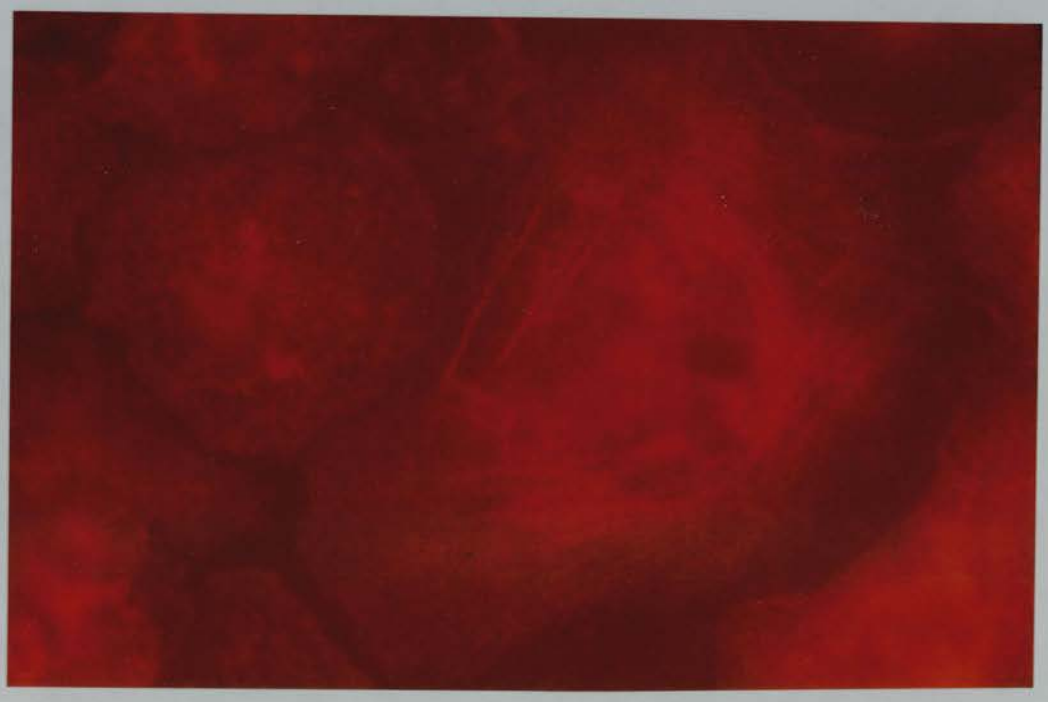
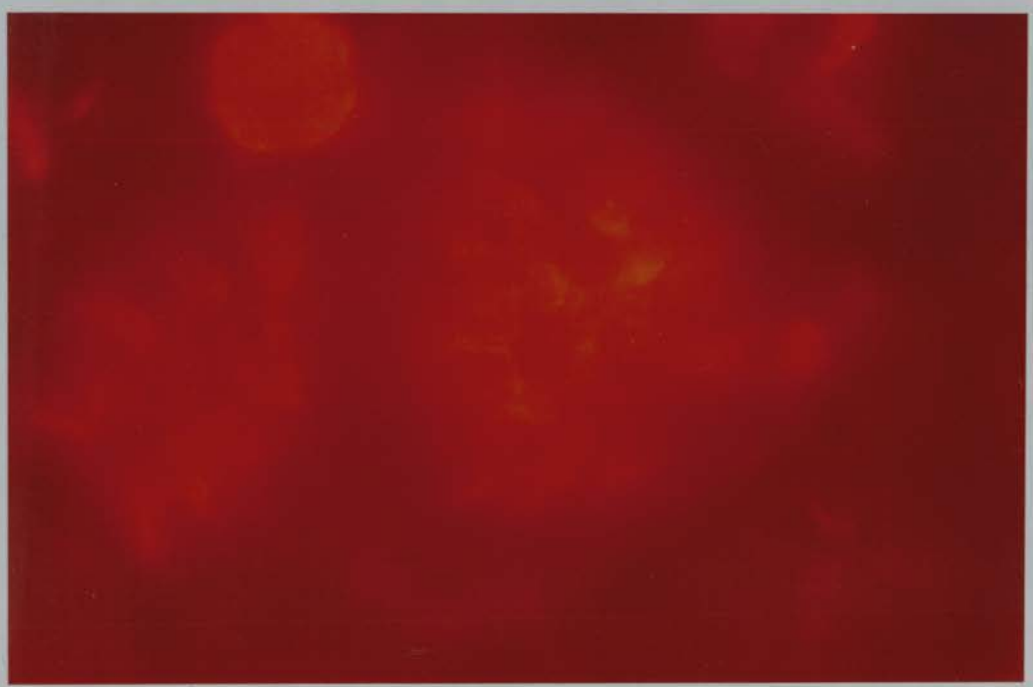


**Figure 4.3. Distribution of actin and talin of untreated macrophages and macrophages treated with dbcAMP.**

Dual fluorescence confocal microscopy shows the relationship between talin and the mø actin cytoskeletal framework. In control cells (and PdBu treated mø, not shown) actin is seen to terminate in focal adhesions, as determined by the presence of talin (and vinculin, not shown). Treatment with dbcAMP causes disruption of focal contacts resulting in actin and talin co-localising in a more diffuse staining pattern. Magnification x1600.

CONTROL
dbcAMP
ACTIN
TALIN

**Figure 4.4. Actin distribution of macrophages treated with dbcAMP.**
Actin filaments, observed in control and PdBu treated mø to terminate at the substrate-associated side of the cell, are not apparent in mø treated with dbcAMP (top panel). Instead, the majority of actin appears concentrated within the body of the cell in short irregular aggregates (lower panel). Magnification x1000.



## 4.3. ROLE OF THE INTEGRIN αvβ3 IN MACROPHAGE PHAGOCYTOSIS OF APOPTOTIC NEUTROPHILS.

As a consequence of the observed effects of protein kinase activators on the mø actin cytoskeleton and the fact that the vitronectin receptor was not seen to associate with actin, I re-examined the role of the vitronectin receptor in the phagocytosis of apoptotic neutrophils.

### 4.3.1. Antibodies against the integrin αvβ3 do not inhibit macrophage phagocytosis of apoptotic neutrophils.

Mø, cultured in suspension and adhered for 60 minutes to 96 well plates or multispot microscope slides, were treated with saturating concentrations of mAb against αv and αvβ3 for 20 minutes and assessed for phagocytosis of apoptotic neutrophils as described in 2.7.2i. A mAb against CD36 was also tested. These mAb have previously been shown to bind to mø (Fig. 3.9) however, preincubation of mø with mAb against the vitronectin receptor or CD36 caused no significant change in the levels of phagocytosis when compared to control levels (Fig. 4.5).

In addition to the mAb against αv (CD51) and αvβ3 (CD51/CD61), a panel of mAb against CD61 (the β3-subunit) was obtained from the Vth International Workshop on Leukocyte Differentiation Antigens and tested in the apoptotic neutrophil phagocytosis assay. All of these mAb were shown to bind to mø by flow cytometric analysis (Fig. 4.6) however, in keeping with previous data, little change was observed in phagocytosis relative to control levels (Fig. 4.7).

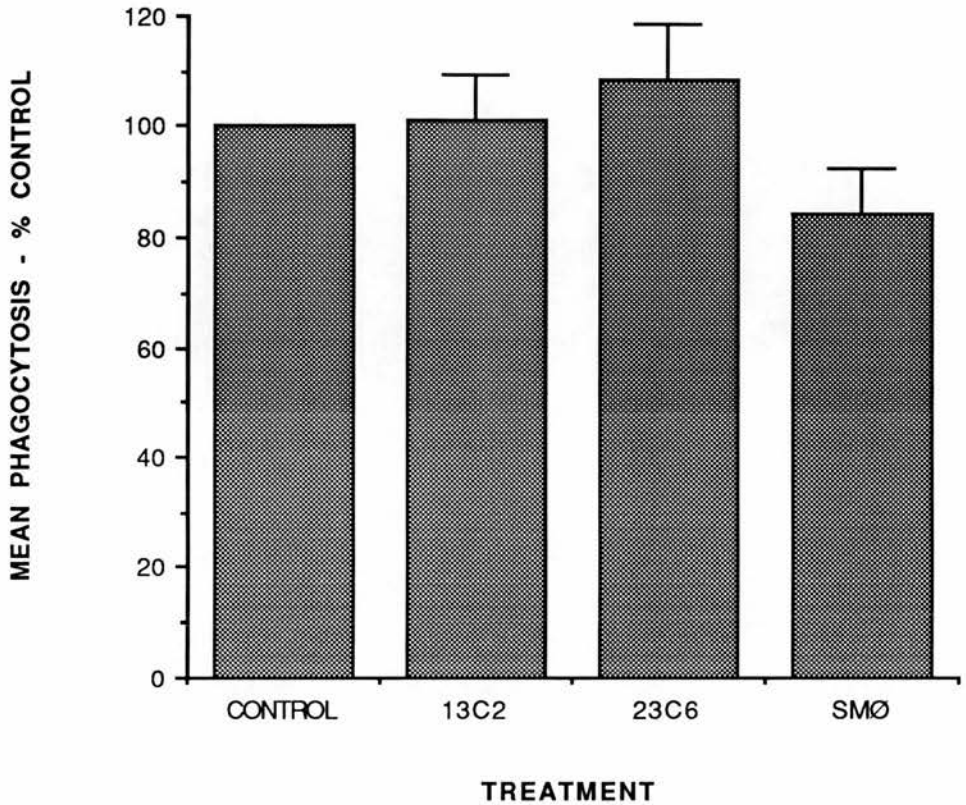



**Figure 4.5. Effect of monoclonal antibodies against the αvβ3 vitronectin receptor and CD36 on macrophage phagocytosis of apoptotic neutrophils.** Mø were treated for 20 minutes at room temperature with mAb against αv (13C2; 1:50; n= 5), αvβ3 (23C6; 1: 50; n=4) and CD36 (SMØ; 1:25; n=3) prior to assessment of their ability to phagocytose apoptotic neutrophils. Preincubation of mø with mAb caused no significant change in phagocytosis levels when compared to control. In this set of experiments, 27.6 ± 6.5% (mean ± S.E.) of mø phagocytosed apoptotic neutrophils under control conditions.

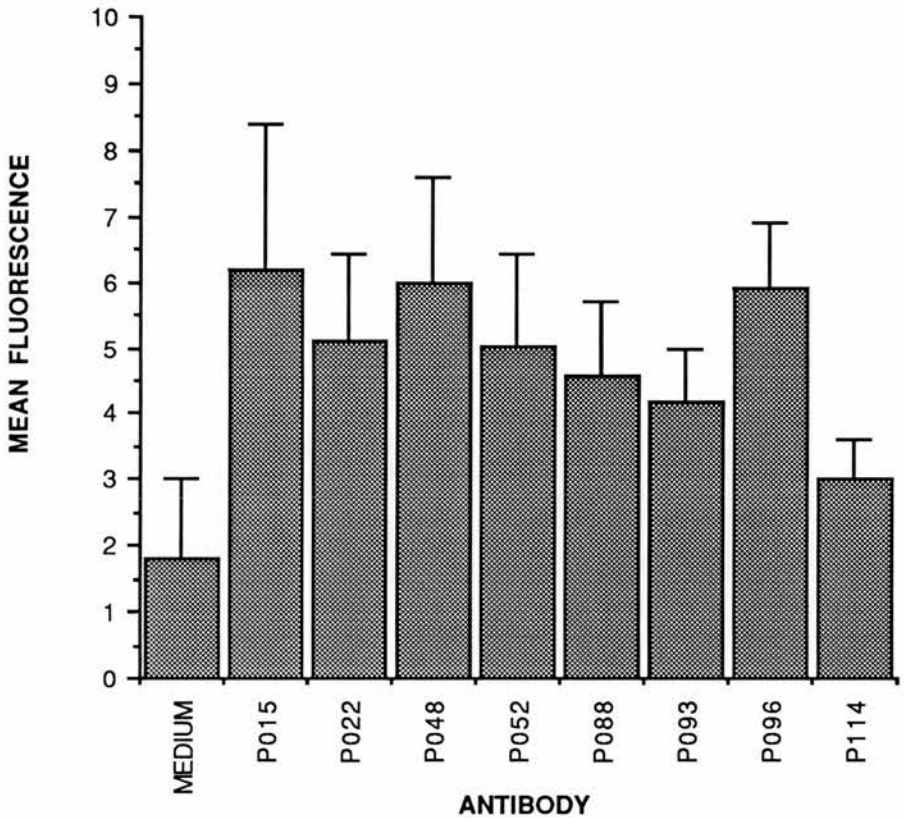



**Figure 4.6. Expression of CD61 (β3-subunit) on human monocyte-derived macrophages.**

The expression of the vitronectin receptor β3-subunit was assessed by flow cytometry using a panel of CD61 mAb. Results are expressed as the mean fluorescent value ± S.D. of two experiments.

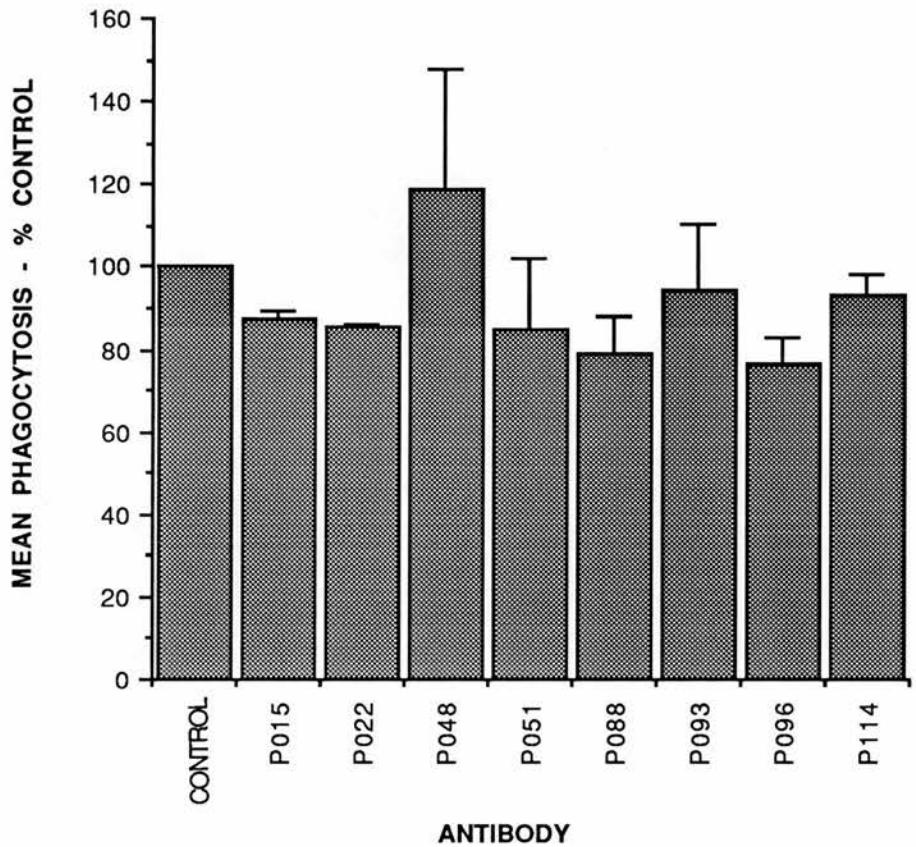



**Figure 4.7. Effect of a panel of CD61 monoclonal antibodies on macrophage phagocytosis of apoptotic neutrophils.**

Mø were incubated for 20 minutes with one of eight mAb against CD61 (the β3-subunit) and their ability to phagocytose apoptotic neutrophils assessed. Compared to control values, preincubation of mø with anti-CD61 mAb had no significant effect on phagocytosis levels. 20.22 ± 5.4% (mean ± S.E.) of mø in control conditions recognised apoptotic neutrophils.

### 4.3.2. Distribution of macrophage αvβ3 integrin during phagocytosis of apoptotic neutrophils.

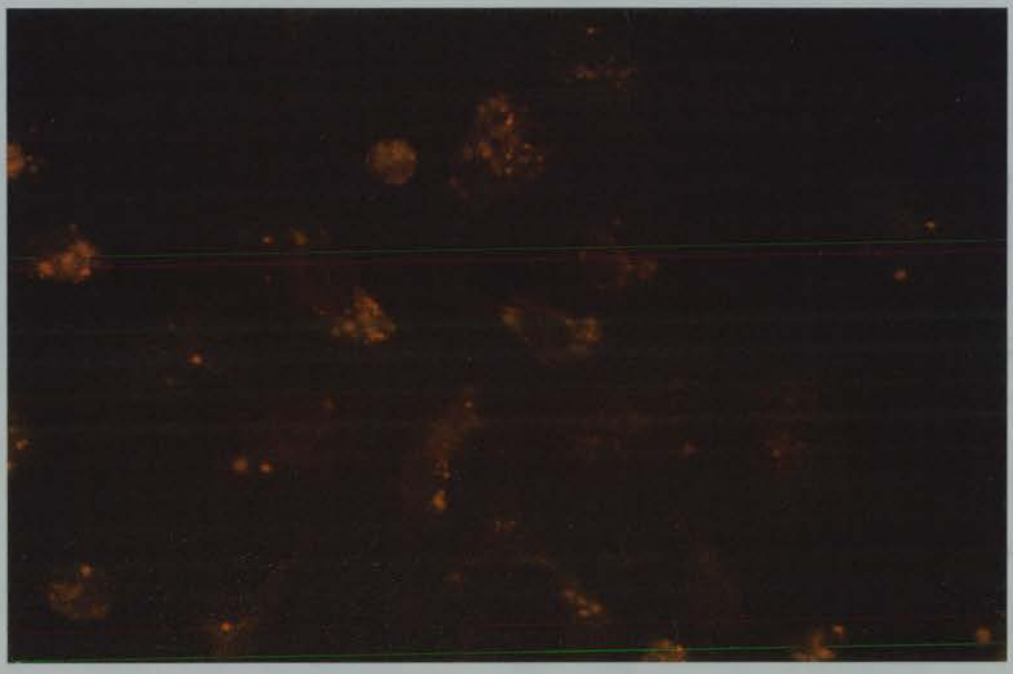
Following on from the observation that the β3-`subunit does not colocalise with actin and that mAb against the vitronectin receptor do not inhibit phagocytosis, the distribution of αvβ3 during phagocytosis of apoptotic neutrophils was investigated. The standard apoptotic neutrophil phagocytosis assay was carried out on multispot microscope slides, fixed and double stained for apoptotic neutrophils and mø CD36 (SMØ; 1:25), αvβ3 (LM609; 1:250) and β3 (PM6/13; 1:50) as described in 2.11.3ii. Distribution of mø receptors and apoptotic cells were then assessed by indirect immunofluorescent microscopy.

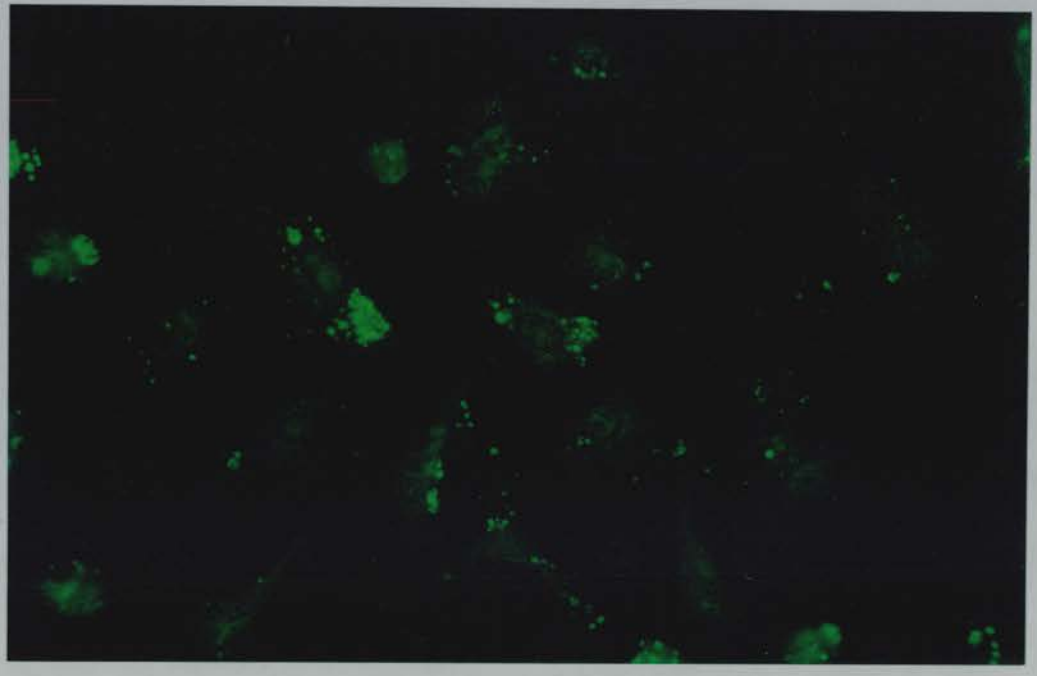
No co-localisation of mø receptors with the apoptotic neutrophils was observed using the above mAb. In addition, the mAb LIBS1, which preferentially binds to a site exposed on the β3 integrin subunit when the receptor has been bound by ligand (Frelinger III *et al.*, 1990), was used. As the binding site for this mAb is exposed upon ligand binding, it was considered necessary, in these experiments, to add LIBS1 during the phagocytosis assay. Figure 4.8 shows the LIBS1 staining pattern of mø involved in an apoptotic neutrophil phagocytosis assay and the same field of view stained for neutrophil location. Observations from repeated experiments failed to provide conclusive evidence for mø β3 co-localisation with apoptotic neutrophils.

These results, along with the data presented in chapter 3 (section 3.7.1.), question the molecular mechanism for recognition of apoptotic neutrophils by macrophages proposed by Savill and co-workers (Savill *et al.*, 1990 and 1992). Using a sensitive detection technique,

**Figure 4.8. Distribution of macrophage β3-subunit during phagocytosis of apoptotic neutrophils.**

Dual immunofluorescent staining was used to assess the distribution of mø β3-subunit during mø phagocytosis of apoptotic neutrophils. The mAb LIBS1 was used to show the location of the β3-subunit (top panel). The position of apoptotic neutrophils in the same field of view is also shown (lower panel). Magnification x400.





thrombospondin could not be detected in macrophage supernatants and several mAb against CD36 and the vitronectin receptor subunits do not affect the level of phagocytosis relative to control conditions. Finally, no co-localisation of αvβ3 and apoptotic neutrophils could be observed.

## 4.4. EFFECT OF OTHER INHIBITORS OF MACROPHAGE PHAGOCYTOSIS OF APOPTOTIC NEUTROPHILS ON THE MACROPHAGE CYTOSKELETON.

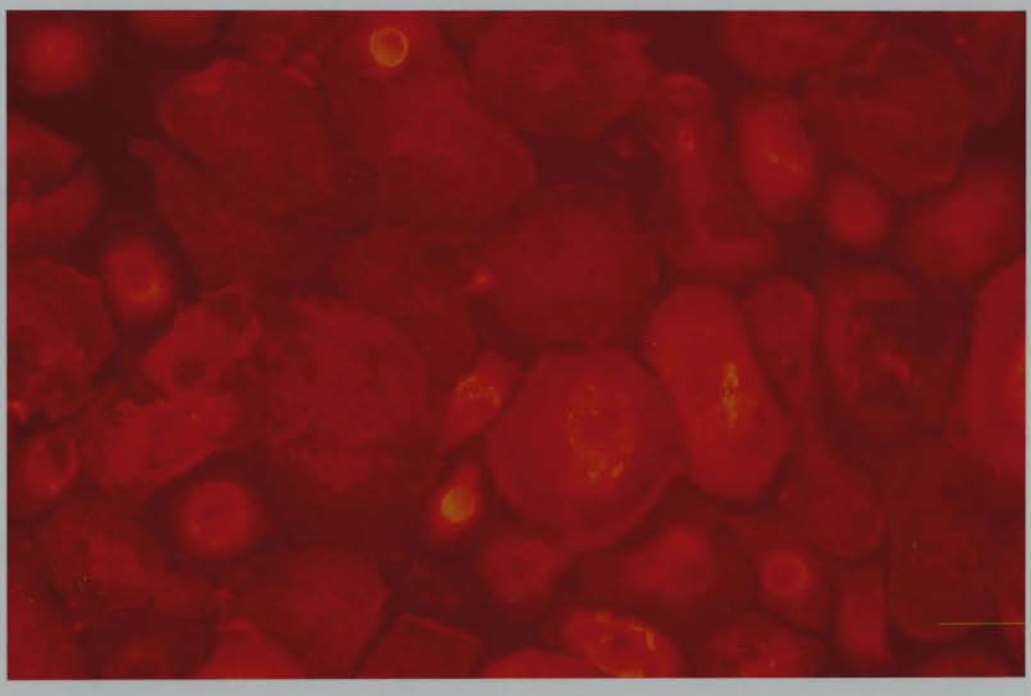
Since dbcAMP was found to affect the mø cytoskeleton, I next considered whether mø cytoskeletal structure was affected following treatment with other compounds that have, in this thesis and in previous studies (Savill *et al.*, 1989 and 1990), been reported to inhibit phagocytosis of apoptotic neutrophils.

As described in chapter 3, $PGE_2$ has been shown to reduce mø phagocytosis of apoptotic neutrophils to 49.57 ± 5.51% (mean ± S.E.) of control values. As seen in figure 4.9, treatment of mø with $PGE_2$ causes disruption of the actin cytoskeleton similar to that seen after dbcAMP treatment and is consistent with the observed elevation of intracellular cAMP following $PGE_2$ treatment.

Savill *et al.* (1990) reported specific inhibition of phagocytosis of apoptotic neutrophils by the tetrapeptide RGDS. No disruption of the actin cytoskeleton was observed in mø incubated with the RGDS peptide (Fig. 4.10) and RGDS did not inhibit phagocytosis when added to the apoptotic neutrophil assay (Fig. 4.11), supporting further my previous observations that question the direct involvement of the αvβ3 integrin receptor.

**Figure 4.9. Actin distribution of macrophages treated with $PGE_2$.**
**Top panel:** Untreated mø, adhered for 75 minutes in Iscove's DMEM, fixed and stained with rhodamine-conjugated phalloidin. Magnification x400.

**Lower panel:** Mø, adhered for 60 minutes in Iscove's DMEM were treated for 15 minutes with PGE2 (3μM), fixed and stained with rhodamine-conjugated phalloidin. Actin appears to have formed into aggregates in the centre of the cell. Magnification x400.



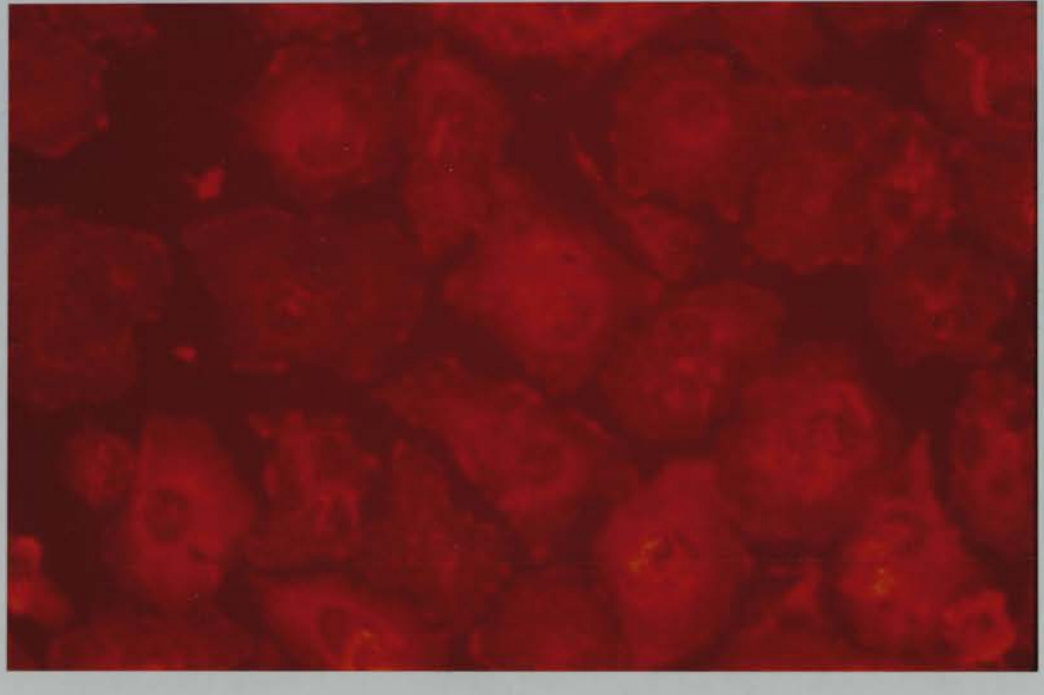
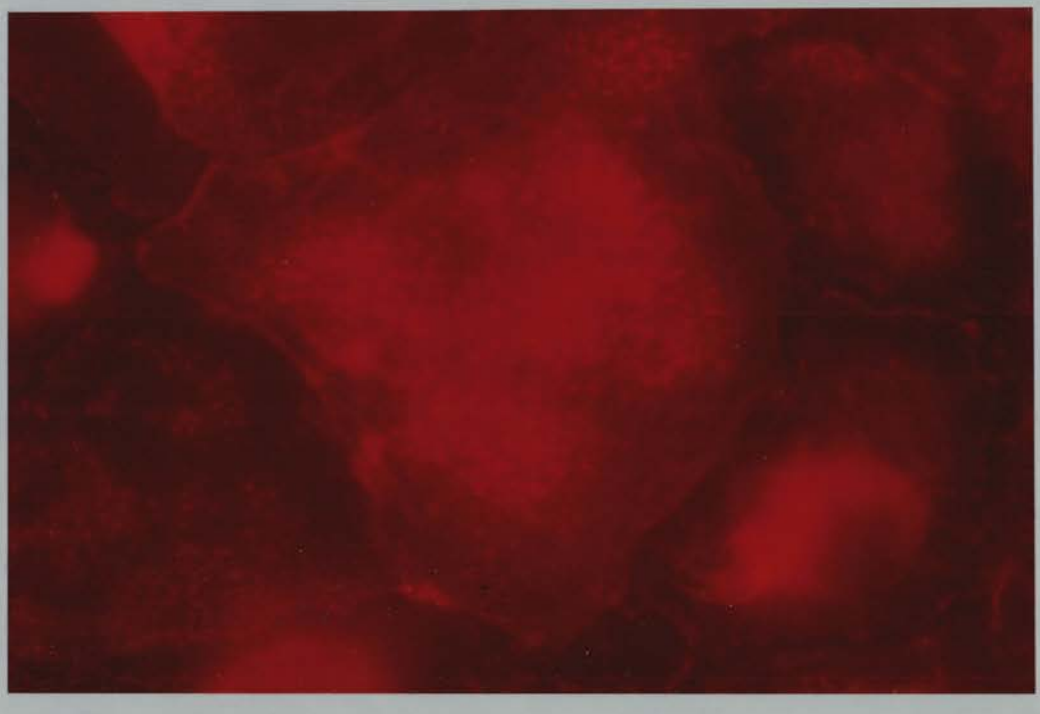
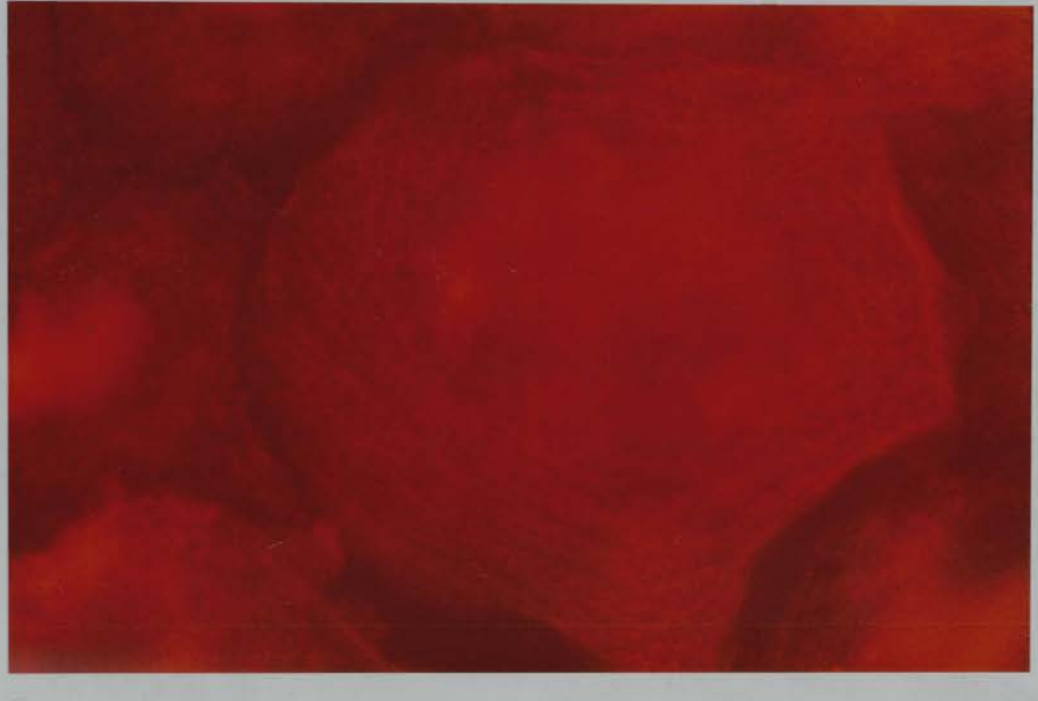


**Figure 4.10. Actin distribution of macrophages incubated with the tetrapeptide RGDS.**

**Top panel:** Untreated mø, adhered for 90 minutes in Iscove's DMEM, fixed and stained for F-actin. Magnification x1000.

**Lower panel:** Mø, adhered for 60 minutes in Iscove's DMEM and incubated a further 30 minutes with the tetrapeptide RGDS (1mM), were fixed and stained for F-actin. As seen in control cells, mø incubated with RGDS display a network of actin filaments terminating in punctate focal adhesions. Magnification x1000.





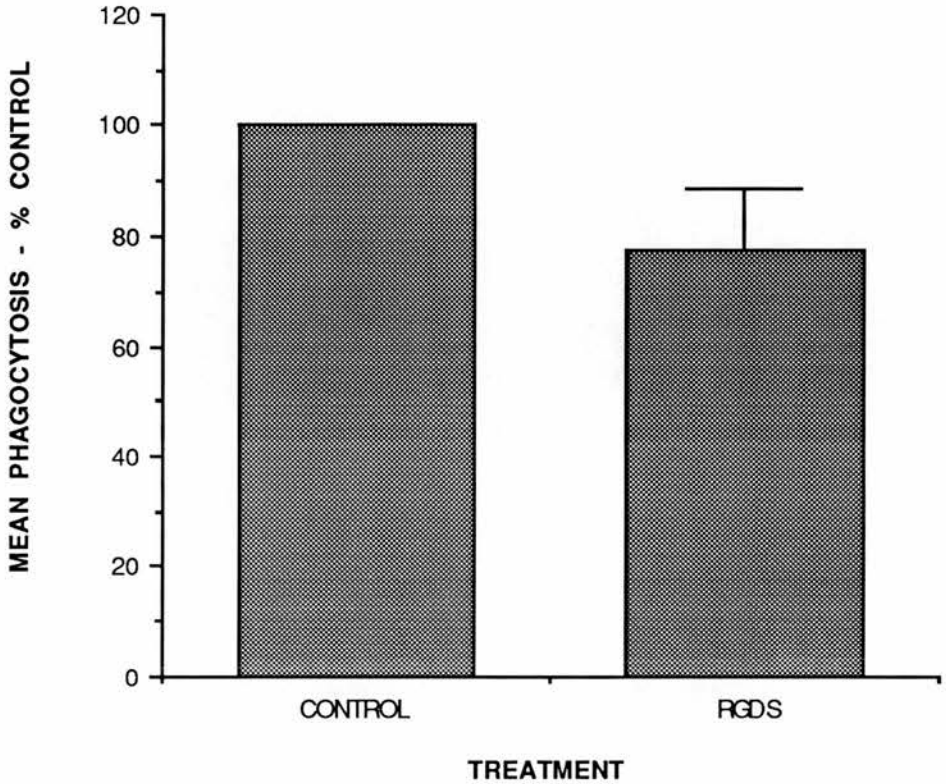



**Figure 4.11. Effect of the tetrapeptide RGDS on macrophage phagocytosis of apoptotic neutrophils.**

The presence of the RGDS peptide (1mM) during the phagocytic assay had no significant effect on the level of phagocytosis when compared to control conditions. 22.06 ± 4.5% (mean ± S.E.) of mø phagocytosed apoptotic neutrophils under control conditions. n=3.

Phagocytosis of apoptotic neutrophils is dependent on $Mg^{2+}$ and $Ca^{2+}$ (Savill *et al.*, 1990). Addition of EDTA (5mM) to the phagocytosis assay inhibits mø phagocytosis by almost 100% (Fig. 4.12) however, a change in the staining pattern of the actin cytoskeleton is observed after 30 minutes treatment of mø with EDTA (Fig. 4.13). Actin dense areas can be seen around the periphery of cells and cell contact appears to have been lost.

## 4.5. SUMMARY OF RESULTS.

In untreated and PdBu treated mø, the cytoskeletal proteins talin and vinculin colocalise with actin at the end of stress fibres in focal contacts. Treatment of mø with dbcAMP results in disruption of the mø stress fibre network although talin and vinculin are still seen to associate with the actin. No colocalisation of the β3-subunit is observed with the ends of the actin network and αvβ3 cannot be shown to localise with apoptotic neutrophils during the phagocytosis assay. Furthermore, mAb against αvβ3 and CD36 did not inhibit mø phagocytosis of apoptotic neutrophils. Other inhibitors of macrophage phagocytosis of apoptotic neutrophils ($PGE_2$ and EDTA) were shown to disrupt the macrophage actin framework. The tetrapeptide RGDS did not alter the percent of macrophages phagocytosing apoptotic neutrophils and did not cause disruption of the macrophage cytoskeleton.

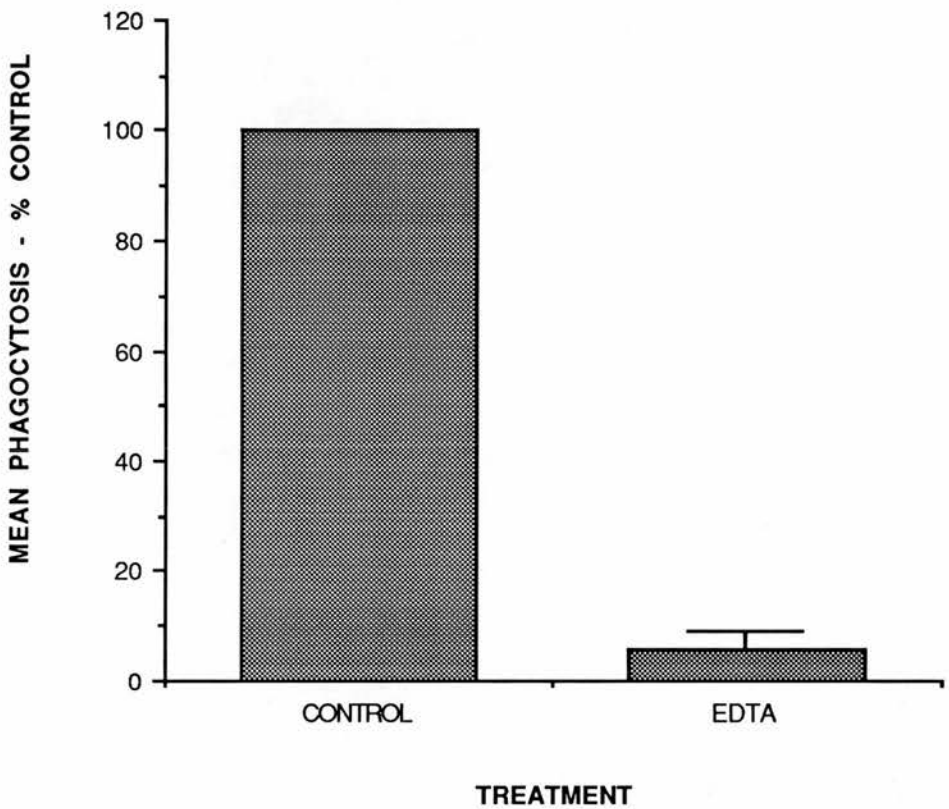
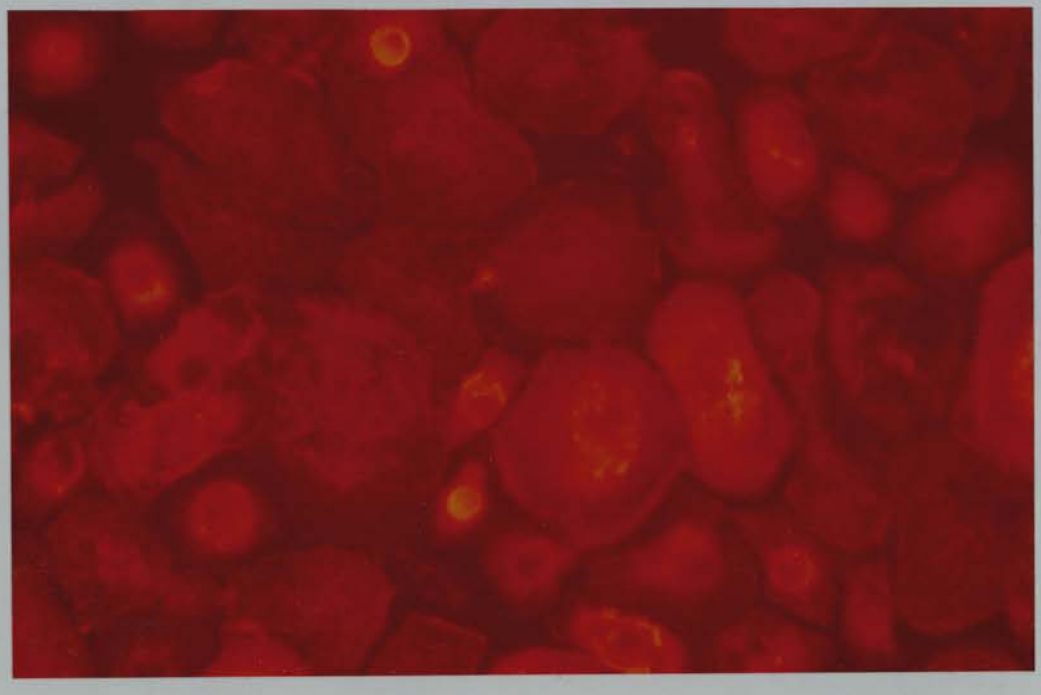



**Figure 4.12. Effect of EDTA on macrophage phagocytosis of apoptotic neutrophils.**

The standard phagocytosis assay was carried out in the presence of 5mM EDTA. Phagocytosis was inhibited to 5.96% of control levels. In this set of experiments, 21.36 ± 6.1% (mean ± S.E.) of mø phagocytosed apoptotic cells ($p<0.001$; $n = 3$).

**Figure 4.13. Actin distribution of macrophages treated with EDTA.**
**Top panel:** Untreated mø, adhered for 75 minutes in Iscove's DMEM, fixed and stained with rhodamine-conjugated phalloidin. Magnification x400.

**Lower panel:** Mø, adhered for 60 minutes in Iscove's DMEM and treated a further 30 minutes with EDTA (5mM) were fixed and stained for F-actin. Actin dense areas at the periphery of the cells are apparent when compared to untreated mø. Magnification x400.



## 4.6. DISCUSSION.

In this chapter, the effect of protein kinase activation on the actin cytoskeleton and associated cytoskeletal proteins of monocyte-derived macrophages has been investigated. Untreated control cells and mø treated for 15 minutes with PdBu contain a prominent system of actin filaments, crossing the main body of the mø in roughly parallel arrays, associated with the substrate side of the cell. The cytoskeletal proteins actin and vinculin were found to associate with the ends of these actin filaments at small focal contacts. Treatment of mø with dbcAMP resulted in the disruption of the actin filaments into smaller aggregates found more within the cell body and while talin and vinculin were still found to colocalise with the actin there was no sign of the focal adhesion points seen in control cells and those treated with PdBu.

The response to phorbol esters and dbcAMP tends to be dependent on the cell type being studied. Previous studies have reported that in certain cell types, PKC activators strongly promote cell spreading (Petty, 1989; Grant and Aunis, 1990) and adhesion (Danilov and Juliano, 1989; Gladwin *et al.*, 1990), actin assembly (Phaire-Washington *et al.*, 1980) and the development of stress fibres (Grant and Aunis, 1990) and focal adhesions (Woods and Couchman, 1992). However, these are in contrast to other reports demonstrating disruption of the actin cytoskeleton upon phorbol ester treatment (Schliwa *et al.*, 1984; Kellie *et al.*, 1985; Sobue *et al.*, 1988; Danowski *et al.*, 1988; Turner *et al.*, 1989; Ciesieski-Treska *et al.*, 1991). Likewise dbcAMP treatment is reported to have diverse effects depending on cell type, disrupting the actin cytoskeleton integrity and impairing adhesion of various cell types (Hamachi *et al.*, 1984; Lamb *et al.*, 1988, Petty and Martin, 1989; Turner *et al.*, 1989; Lampugnani *et al.*, 1990;

Goldman and Abramson, 1990; Valitutti *et al.*, 1993; Glass and Kreisberg, 1993) and yet promoting cell spreading and adhesion in others (Leader *et al.*, 1983; Cheung and Juliano, 1985).

In guinea-pig peritoneal macrophages, Hamachi *et al.* (1984) showed that dbcAMP promotes actin disassembly. Petty and Martin (1989) observed a decrease in cell spreading and rearrangement of the actin skeleton in the murine macrophage cell line RAW264 when treated with dbcAMP. Furthermore, Petty (1989) reports that treatment of RAW264 macrophages with activators of PKC increases cell spreading, probably mediated by the cytoskeleton, and that simultaneous dbcAMP treatment inhibits the PKC induced effects. These results lead Petty to suggest a bi-directional control system regulating macrophage spreading whereby PKA provides a negative regulatory signal to the PKC pathway. This would be in keeping with preliminary data obtained for simultaneous treatment of mø with PdBu and dbcAMP. In this case, phagocytosis levels were on a par with the separate dbcAMP treatment. As different cell types exhibit different functions and modes of interaction for PKC and PKA (Kikkawa and Nishizuka, 1986), it would be valuable to pursue this observation further to determine if such a control mechanism proposed by Petty exists for mø phagocytosis of apoptotic neutrophils.

Talin and vinculin have been shown to be phosphorylated (Litchfield and Ball, 1986; Werth *et al.*, 1983; Kawamoto and Hidaka, 1984) raising the possibility that phosphorylation status may regulate association with the cytoskeleton. However, in keeping with effects of protein kinase activators on actin organisation, talin phosphorylation by activation of PKC appears to be cell type specific. Turner *et al.* (1989) found that in BSC-

1 cells, talin phosphorylation accompanied stress fibre disruption whereas there was no change in the phosphorylation state of talin or disruption of stress fibres in REF-52 cells treated with phorbol ester. However, they did observe low levels of dephosphorylation of talin and disruption of stress fibres occurred in REF-52 cells when treated with dbcAMP but suspect that an alternative mechanism is responsible for cytoskeletal disruption. Lamb *et al.* (1988) demonstrate that dbcAMP treatment and microinjection of the catalytic subunit of A-kinase results in phosphorylation of myosin light chain kinase (MLCK), causing inactivation of MLCK, subsequent dephosphorylation of the myosin light chain and dissolution of actin microfilaments. Although the phosphorylation state of proteins following treatment of mø with PdBu or dbcAMP has not been examined in this thesis, data presented here suggest that this would provide useful information relating to the mechanism by which cytoskeletal architecture is controlled in mø and its relevance in mø recognition of apoptotic cells.

Specific inhibition of mø phagocytosis of apoptotic neutrophils by mAb against the αvβ3 vitronectin receptor (13C2 [αv] and 23C6 [αvβ3]) has been reported and a direct role for the vitronectin receptor in mø recognition of apoptotic neutrophils proposed (Savill *et al.*, 1990). This is in contrast to the findings reported here. It is of interest to note that high concentrations (1:25) of mAb ascites fluids were used in the inhibition experiments (Savill *et al.*, 1990) whereas in the experiments described here, purified mAb was used. It is possible that ascitic fluid contains other proteins that might affect mø recognition of apoptotic neutrophils (e.g. other antibodies) and account for these discrepancies. Alternatively, it may be that when used at high concentrations these mAb may inhibit

adhesion events which may, in turn, alter cytoskeletal structure. In addition, a panel of mAb specific for the β3-subunit of the vitronectin receptor were used in the phagocytosis assay and none of the eight antibodies had any significant effect on the level of mø phagocytosis of apoptotic neutrophils. One unlikely possibility is that none of these mAb bind to functional epitopes on CD61 and thus do not affect mø phagocytosis. This could be tested by use of these mAb in another assay of β3 function e.g. JY cell adhesion to vitronectin (Stupack et al., 1992). Furthermore, the tetrapeptide RGDS, shown previously to inhibit phagocytosis (Savill *et al.*, 1990), did not alter the amount of phagocytosis of apoptotic neutrophils in this study. Neither did it cause any change in the actin staining pattern. Although RGDS was found not to exert inhibitory effects in this thesis, it is of interest that Werb *et al.*, (1989) show substantial reorganisation of the actin cytoskeleton of fibroblasts upon treatment with an RGD containing peptide indicating that RGDS may also serve to promote de-adhesion events.

The effect of other proposed inhibitors of macrophage phagocytosis of apoptotic neutrophils on the actin cytoskeleton was also studied. $PGE_2$, shown in this thesis to reduce the level of phagocytosis, disrupted the mø actin framework in a manner similar to that seen in dbcAMP treated cells. Previously, macrophage phagocytosis of apoptotic neutrophils was described to be dependent on the divalent cations $Mg^{2+}$ and $Ca^{2+}$ (Savill *et al.*, 1990). In this project, EDTA was the most effective inhibitor of mø phagocytosis of apoptotic neutrophils when included in the phagocytosis assay, supporting this observation. However, incubation of macrophages with EDTA was found to have a dramatic effect on the mø actin cytoskeleton. Savill *et al.* (1989b), also report that the level of mø

phagocytosis of apoptotic cells is affected by the pH of the assay medium. Indeed, initial phagocytosis assays of this thesis were carried out in HBSS with $Ca^{2+}/Mg^{2+}$ but phagocytosis levels were low (<10%) and this was attributed to acidification of the HBSS during the course of the assay. To avoid this problem, the assay medium was changed to Iscove's DMEM. pH affects the function of gelsolin (Lamb *et al.*, 1993), a calcium-dependent actin binding protein that binds to the barbed end of actin filaments and severs actin-actin bonds (Yin *et al.*, 1981). Activation of gelsolin has been postulated to be involved in receptor-mediated reorganisation of the actin skeleton. One possibility is that activation of protein kinases, particularly PKA, alters the function of this actin binding protein and thus cytoskeletal organisation.

The necessity for cellular energy in mø phagocytosis of apoptotic neutrophils is demonstrated by complete inhibition when the phagocytic assay is performed at 4°C. This may demonstrate the requirement for microfilament structures which are lost following incubation at 4°C (Kellie *et al.*, 1985). It is likely however that the effect of temperature is not specific for mø phagocytosis of apoptotic neutrophils but that all phagocytic events are inhibited at 4°C. Preliminary experiments in which mø were preincubated with cytochalasin D for 5 minutes resulted in detachment of the mø from the plate during the phagocytosis assay making assessment of whether microfilaments are required for phagocytosis difficult. However, this finding may emphasise the necessity for intact microfilament networks in maintenance of mø adhesion. In parallel experiments, phagocytosis of apoptotic neutrophils was unaffected by pretreatment of mø with the microtubule inhibitor nocodazole. These results suggest that although an intact actin

framework is an important factor for mø phagocytosis of apoptotic neutrophils, microtubule assembly is not required. Furthermore, results presented here suggest that the adhesion state of the macrophage is an important factor in regulating this system. This is investigated further in chapter 5.

# CHAPTER 5.

## 5.1. INTRODUCTION.

Phagocytosis of apoptotic neutrophils was found to be modulated by activating macrophage intracellular protein kinases. Although activation of mø intracellular protein kinases alters the phenotype of the mø with respect to the β3 integrin subunit distribution, evidence presented in this thesis questions the direct involvement of the molecules implicated in mø phagocytosis of apoptotic neutrophils (Savill *et al.*, 1990 and 1992). An alternative interpretation of data presented in this thesis and that previously published by Savill and colleagues is that αvβ3, CD36 and thrombospondin are involved indirectly in the process of mø recognition of apoptotic neutrophils. In support of this, inhibition of mø phagocytosis of apoptotic cells following dbcAMP treatment was also found to disrupt the actin cytoskeleton and cause the loss of focal contacts. Indeed, the cytoskeleton has a major role in controlling cell adhesion (Chong *et al.*, 1987) and adhesion of macrophages to ECM components has previously been shown to modulate complement- or immunoglobulin-receptor mediated phagocytosis (Wright *et al.*, 1983; Pommier *et al.*, 1983; Brown, 1986; Newman and Tucci, 1990).

Historically, the ECM was thought merely to provide an inert scaffold surrounding cells. More recently, however, it has been realised that the ECM actively orchestrates key steps in the process of wound healing and regeneration (Gailit and Clark, 1994; Raghow, 1994). Interactions of cells with individual components of the ECM can initiate signal transduction cascades and, as a reservoir of cytokines and growth factors, the ECM can further modulate a host of cellular responses. It is therefore possible that

the ECM composition of inflamed sites and thus the adhesive state of the mø may exert regulatory effects on the process of removal of apoptotic cells.

In this chapter, I have examined the effects of adhesion of monocyte-derived macrophages to various ECM proteins upon subsequent phagocytosis of apoptotic neutrophils. In addition, I have developed a method for assessment of phagocytosis in suspension to directly address whether substrate adhesion represents a requirement for mø phagocytosis of apoptotic neutrophils.

## 5.2. EFFECT ON PHAGOCYTOSIS OF APOPTOTIC NEUTROPHILS BY MACROPHAGES ADHERED TO EXTRACELLULAR MATRIX PROTEINS.

Vitronectin, fibronectin and collagen are components of the extracellular matrix at different phases of tissue repair (Gailit and Clark (1994). Mø were therefore adhered to tissue culture wells coated with 10$\mu$g/ml vitronectin, fibronectin, collagen I or collagen VI to assess the effect of different substrates on mø phagocytosis of apoptotic neutrophils. Surprisingly, in all cases, the percent of mø phagocytosing apoptotic neutrophils was found to increase when compared to the level of phagocytosis in uncoated control wells (Fig. 5.1). The greatest observed increase (196% of control levels) was for mø plated onto Fn. One possibility is that some mø which phagocytose apoptotic neutrophils do not adhere to un-coated plastic and that coating with matrix proteins allows adhesion of these cells. However, no difference was observed between the substrates with regard to the total number of cells counted

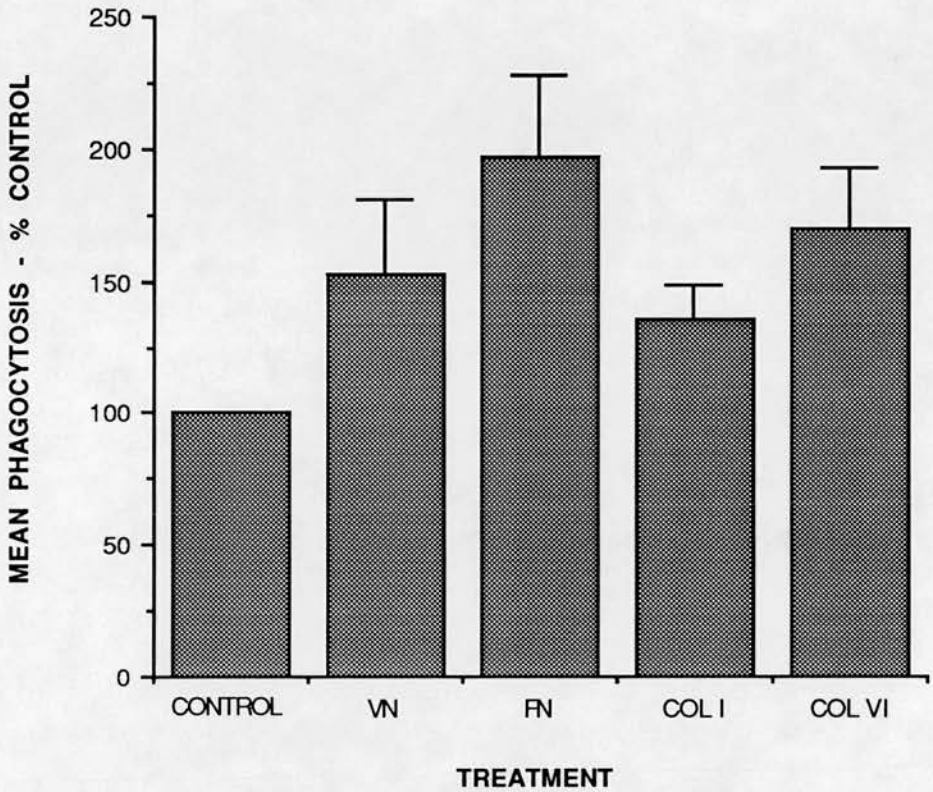



**Figure 5.1. Modulation of macrophage phagocytosis of apoptotic cells as a result of adhesion of macrophages to different substrates.**

Wells of chamber slides were coated for 18 hours at 4°C with 10μg/ml vitronectin (VN), fibronectin (FN), collagen I (Col I) and collagen VI (Col VI) and mø adhered for 60 minutes prior to interaction with apoptotic neutrophils. Phagocytosis increased by 52.14%, 96.75%, 35.26% and 69.59% respectively (n=3).

per field suggesting that the increase in phagocytosis observed is not due to specific adhesion of phagocytic mø to the ECM proteins. It is also possible that adhesion to Fn specifically recruits a subpopulation of mø which are able to phagocytose apoptotic cells or that specific adhesion to matrix proteins augments mø functional activity in terms of phagocytic capacity. Thus, the proportion of mø that are able to phagocytose apoptotic neutrophils is increased.

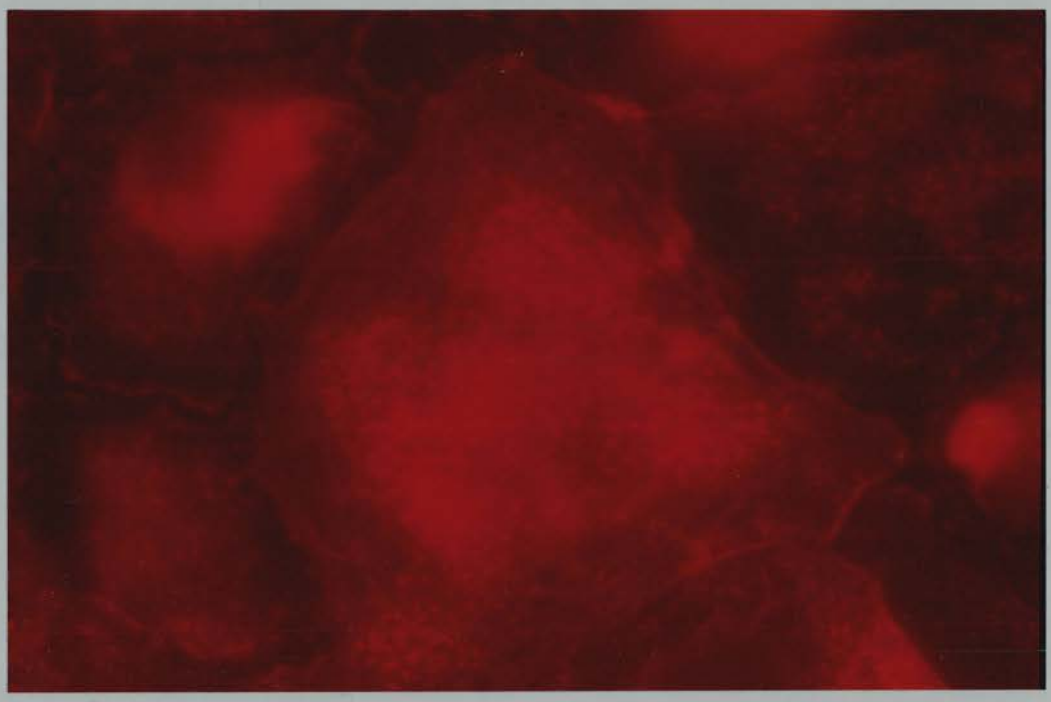
Integrins have no intrinsic enzyme activity and the mechanism of signalling mediated by integrins is believed to dependant on their ability to assemble cytoskeletal frameworks. After binding to ECM components, integrins bind to the cytoskeleton and promote its reorganisation. The frameworks formed can influence adhesiveness, cell shape and motility and may determine how integrin-mediated regulatory signals are propagated throughout the cell. I therefore analysed the effect of adhesion to Fn on the mø cytoskeleton. As can be seen in figure 5.2, the actin framework appears intact with the similar patterns of focal staining as observed in control and PdBu treated cells.

## 5.3. EFFECT OF MONOCLONAL ANTIBODIES AGAINST THE FIBRONECTIN INTEGRIN RECEPTOR ON MACROPHAGE PHAGOCYTOSIS OF APOPTOTIC NEUTROPHILS.

Since mAb directed against the vitronectin receptor repeatedly failed to inhibit mø phagocytosis of apoptotic neutrophils and in view of the observed increase in phagocytic levels for mø adherent to fibronectin, the effect of mAb against the fibronectin integrin receptor, $\alpha 5\beta 1$, on mø phagocytosis of apoptotic neutrophils was investigated. For these

**Figure 5.2. Actin distribution of macrophages adhered to fibronectin.**
**Top panel:** Mø, adhered for 60 minutes on glass slides, fixed and stained with rhodamine-conjugated phalloidin. (Magnification x1000).

**Lower panel:** Mø adhered for 60 minutes on Fn-coated glass slides (10μg/ml), fixed and stained with rhodamine-conjugated phalloidin. The same point structures observed in untreated cells are apparent in mø adhered to Fn. (Magnification x1000).



experiments I used mAb previously shown to inhibit β1 integrin function (mAb13) and α5 function (BIIG2 and SAM-1) and an antibody which has been demonstrated to increase β1 integrin functional activity (TS2/16). Mø were incubated for 20 minutes at room temperature with mAb against the α5- and β1-subunits prior to assessment of ability to phagocytose apoptotic neutrophils. As can be seen from figure 5.3, preincubation of the mø with mAb against the fibronectin receptor had no significant effect on phagocytosis suggesting that ligation of β1 integrins is not involved directly in mø recognition of apoptotic neutrophils.

Mø were incubated for 30 minutes on ice with 13C2 (1:100; [αv]), PM6/13 (1:50; [β3]), BIIG2 (5μg/ml; [α5]) and both 13C2 and PM6/13 prior to adherence to tissue culture plastic or Fn-coated tissue culture plastic as described in section 2.7.2ii. Incubation with the mAb had no affect on adherence of mø to substrate: after 60 minutes at 37°C, mø were observed to have formed uniform monolayers on both the plastic and Fn-coated wells. The standard apoptotic neutrophil assay was then performed and assessed as previously described (section 2.5.2). In this series of experiments, the neutrophils adhered extensively to the mø in the PM6/13 treatments making assessment of phagocytosis impossible. However, incubation of mø with mAb against αv or α5 had no effect on phagocytosis of apoptotic neutrophils under control conditions or on mø adhered to fibronectin (Fig. 5.4).

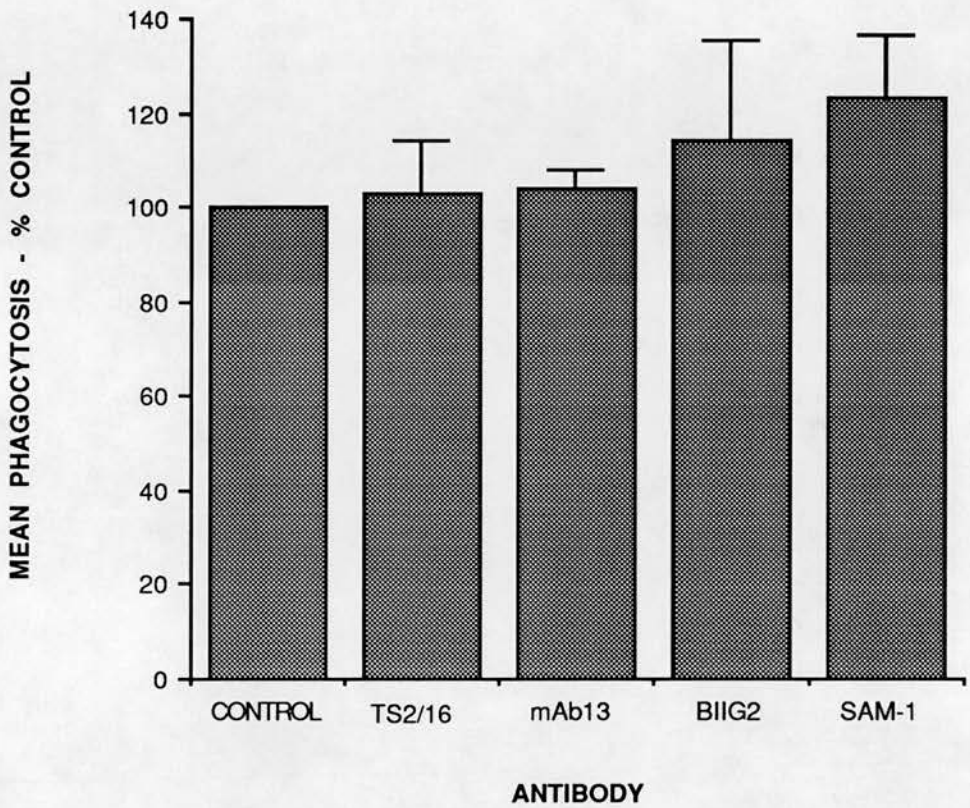



**Figure 5.3. Effect of monoclonal antibodies against the α5β1 fibronectin receptor on macrophage phagocytosis of apoptotic neutrophils.**

Mø were incubated for 20 minutes at room temperature with mAb against the α5- or β1- integrin subunits prior to assessment of their ability to phagocytose apoptotic neutrophils. Preincubation of mø with these mAb caused no significant change in phagocytosis levels compared to control conditions. In this series of experiments, 21.1± 1.9% (mean ± S.E.) of mø phagocytosed apoptotic neutrophils (n = 3). TS2/16 - β1 stimulatory, mAb13 - β1 inhibitory, BIIG2 - α5, SAM-1 - α5.

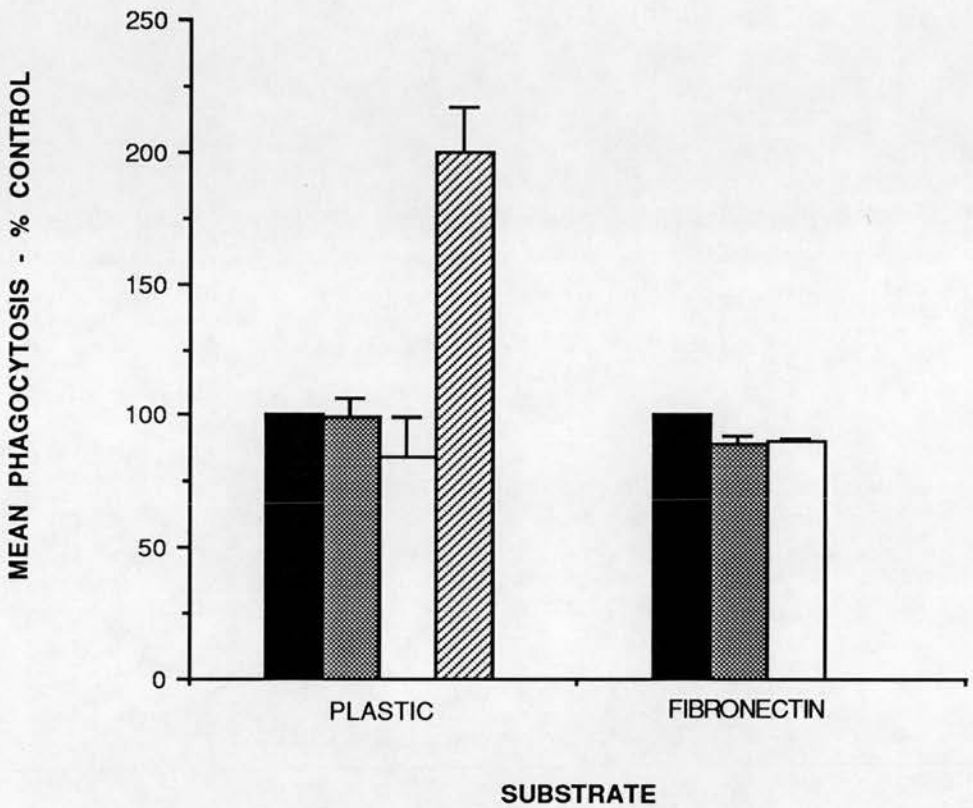



**Figure 5.4. Effect of incubation of macrophages with monoclonal antibodies prior to adhesion to substrate on macrophage phagocytosis of apoptotic neutrophils.**

No antibody (black bars), 13C2 (1:100; [αv]; grey bars), BIIG2 (5μg/ml; [α5]; white bars). The hatched bar shows the level of phagocytosis of mø adhered to Fn as a percent of the plastic control. n = 2.

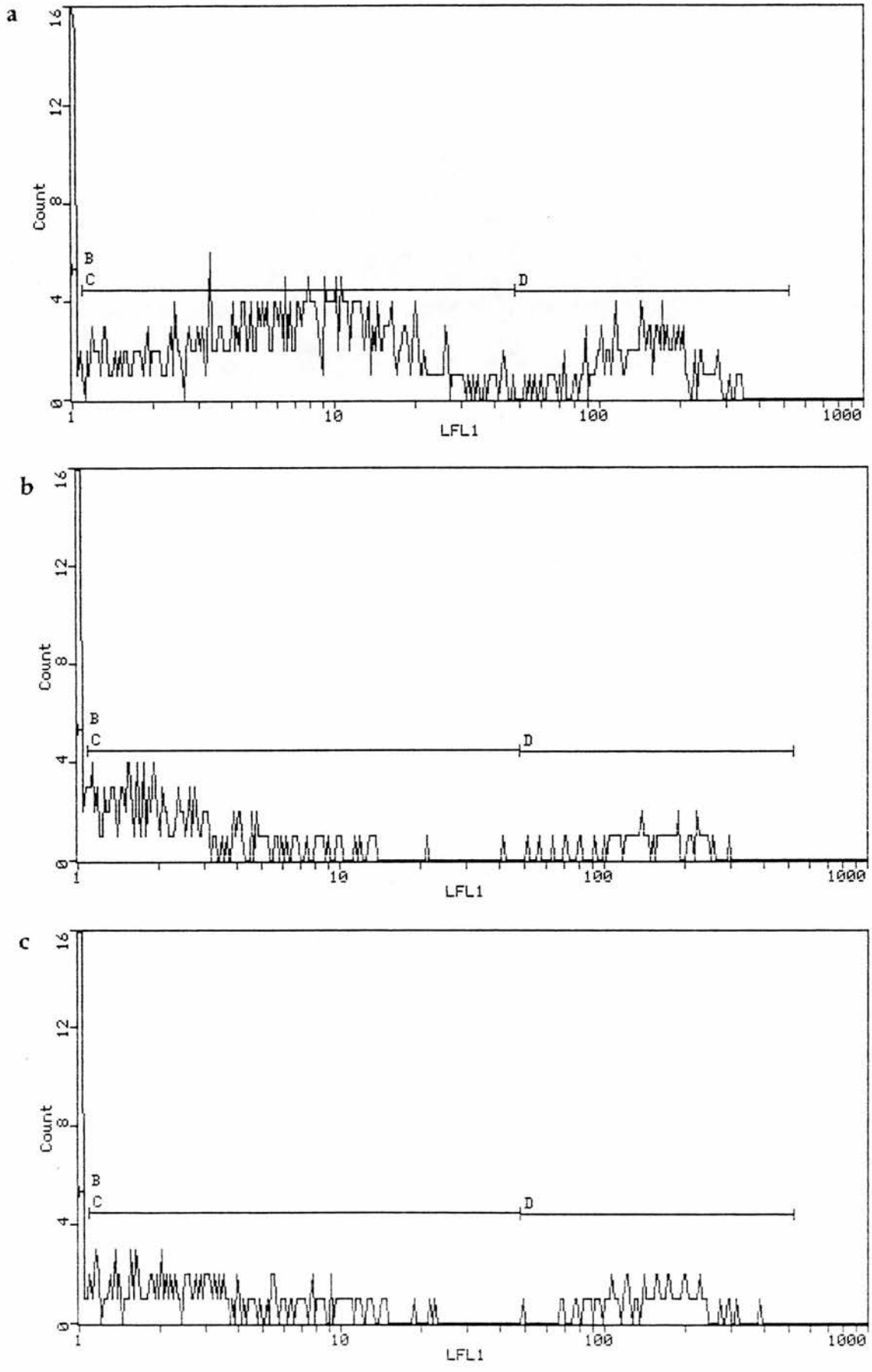
## 5.4. APOPTOTIC NEUTROPHIL PHAGOCYTOSIS ASSAY IN SUSPENSION.

To determine if the adhesion state of the macrophage was important for phagocytosis of apoptotic neutrophils, a modified phagocytosis assay was developed in suspension (as described in section 2.12.1). Mø and neutrophils were differentially labelled with fluorescent dyes and incubated together for 30 minutes at either 4°C or 37°C ± EDTA, a protocol similar to that used to assess cytotoxic T lymphocyte conjugation (Cavarec *et al.*, 1990). The samples were then analysed by flow cytometry to determine if there was any interaction between the two cell types: mø (red positive) were analysed for green fluorescence (apoptotic neutrophils). Characteristic profiles were obtained for each experimental condition (Fig. 5.5). Three distinct peaks could be identified in all samples, a negative peak (–ve), a weak positive peak (+) and a strong positive peak (++) (gates B, C and D respectively in figure 5.5). The experimental condition appeared to alter the proportion of cells in each peak (Table 5.1). A standard adherent apoptotic neutrophil phagocytosis assay was performed in parallel to each suspension assay. Data shown in figure 5.5 and table 5.1 are representative of six separate experiments.

I next considered the interpretation of this data. One possibility was that the green –ve peak represented mø with no associated neutrophils, the green ++ peak represented neutrophils adherent to the surface of the mø while the green + peak represented mø with internalised neutrophils, the green signal therefore being weaker. In keeping with this, the greatest proportion of green + cells occurred at 37°C and there were more green –ve cells at 4°C and in the EDTA treatment (shown to inhibit adherent mø phagocytosis of apoptotic neutrophils).

**Figure 5.5. Flow cytometric profiles of apoptotic neutrophil phagocytosis suspension assay.**

Mø were labelled with phycoerythrin-conjugated CD14 and apoptotic neutrophils with CFDA. The two cell types were mixed and incubated together for 30 minutes at (a) 37°C, (b) 37°C in the presence of 5mM EDTA or (c) at 4°C before being fixed. The flow cytometer, gated on the red positive mø, simultaneously analysed for green fluorescence (apoptotic neutrophils). The profiles are representative of six separate experiments. Gate B = green -ve cells, gate C = green + cells, gate D = green ++ cells. The proportion of cells in each gate is shown in Table 5.1.


a
b
c
Count
LFL1
B
C
D
1
10
100
1000
0
4
8
12
16

| Experimental condition | Proportion of green positive cells (%) | | |
|---|---|---|---|
| | –ve | + | ++ |
| 37°C | 46.0 ± 0.1 | 42.7 ± 0.9 | 11.2 ± 1.8 |
| 4°C | 80.2 ± 1.1 | 14.2 ± 1.5 | 7.1 ± 2.5 |
| EDTA | 81.1 ± 0.2 | 14.7 ± 0.2 | 4.0 ± 0.1 |

**Table 5.1. Flow cytometric analysis of phagocytosis assay performed in suspension.**

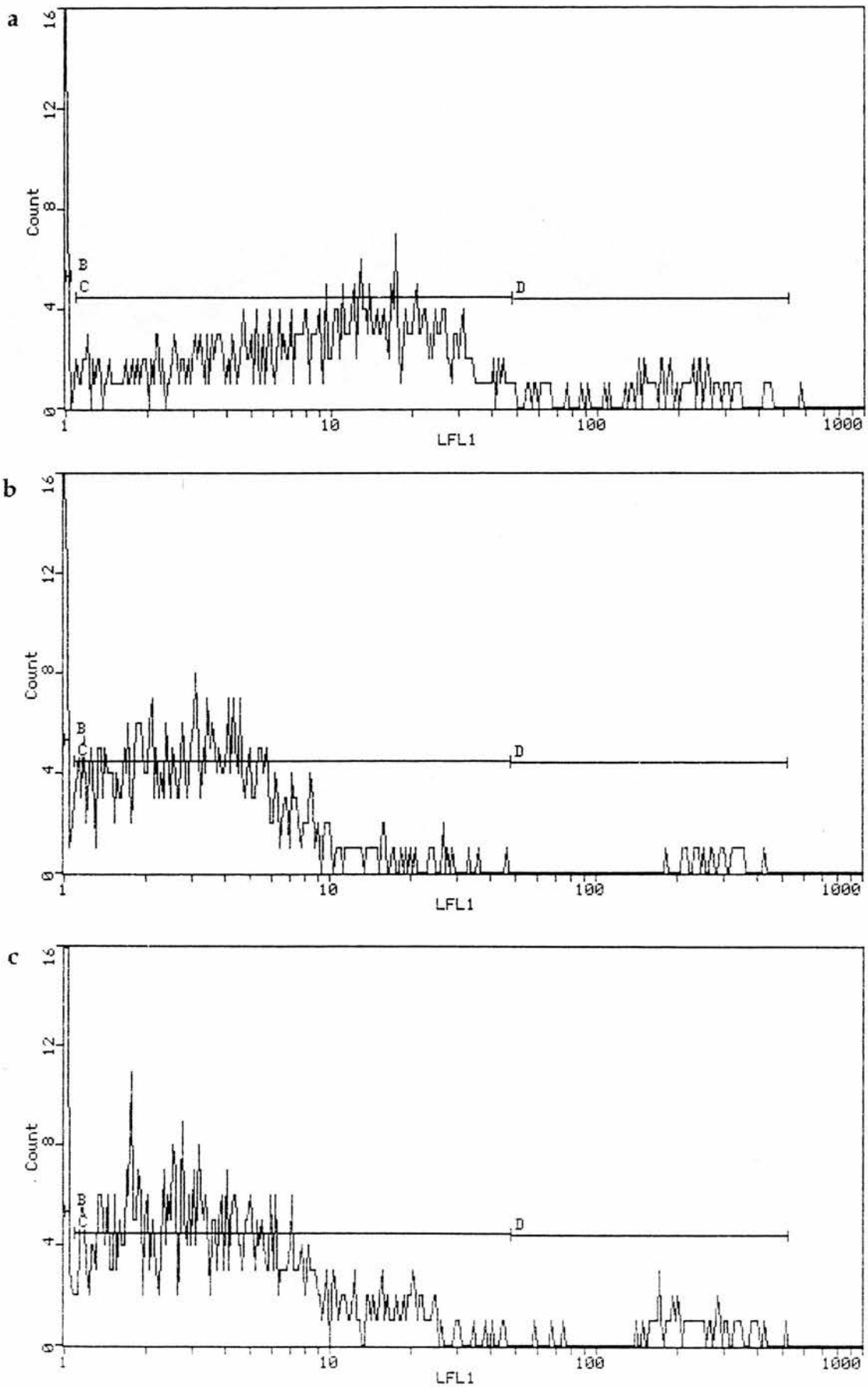
The flow cytometry profiles were gated into three distinct areas (shown in figure 5.4) and the percent of cells in each gate recorded (mean ± S.D. from duplicate samples). In the standard adherent assay performed in parallel with the same cells, 33.1 ± 7.2% (mean ± S.D.) of macrophages phagocytosed apoptotic neutrophils.

There are a proportion of apoptotic and non-apoptotic cells present in a population of aged cultured neutrophils. One possibility was that the green ++ and green + cells represent non-apoptotic and apoptotic cells respectively. To address this question, the aged non-apoptotic cells were removed immunomagnetically and the enriched apoptotic population was used in the suspension assay (as described in section 2.12.2). Similar profiles were obtained using enriched apoptotic cells (Fig. 5.6) however, the proportion of –ve and + cells appeared to differ with the population of neutrophils used, most obviously at 37°C (Table 5.2). The levels of phagocytosis also increased when the enriched apoptotic neutrophil population was used in the adherent assay. Similar results were obtained in a second experiment performed using enriched apoptotic neutrophils.

The increase in the proportion of green + cells at 37°C when an enriched apoptotic neutrophil population is used, along with the increase in phagocytosis in the adherent assay, would be in agreement with the suggested interpretation of the profiles made above. However, when a similar series of experiments were performed in parallel but with instantaneous fixation of mø and neutrophils, it was found that a high proportion of green + cells were present. This raised the possibility that the high neutrophil/mø ratio used in these experiments was giving false co-incident signals and that standard flow cytometry does not permit discrimination of mø and neutrophils at this cell density. However, the 4°C and EDTA data suggest that some resolution of mø and neutrophils may be achieved. Furthermore, the high proportion of green + cells present when the assay is performed in the presence of EDTA or at 4°C suggests that this interpretation is not correct.

**Figure 5.6. Flow cytometric profiles of enriched apoptotic neutrophil phagocytosis suspension assay.**

The same cells were used as in figure 5.5, except the cultured neutrophils were enriched from 47.5% to 73.8% apoptotic cells. a) 37°C, b) 37°C in the presence of 5mM EDTA and c) 4°C. The proportion of cells in each gate is shown in Table 5.2.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| Experimental condition | Proportion of green positive cells (%) | | |
|---|---|---|---|
| | –ve | + | ++ |
| 37°C | 22.2 ± 0.4 | 68.4 ± 0.3 | 9.3 ± 0.1 |
| 4°C | 68.6 ± 8.1 | 29.6 ± 7.3 | 1.9 ± 1.0 |
| EDTA | 60.5 ± 2.2 | 37.0 ± 2.6 | 2.2 ± 0.1 |

**Table 5.2. Flow cytometric analysis of phagocytosis assay performed in suspension using enriched apoptotic neutrophils.**

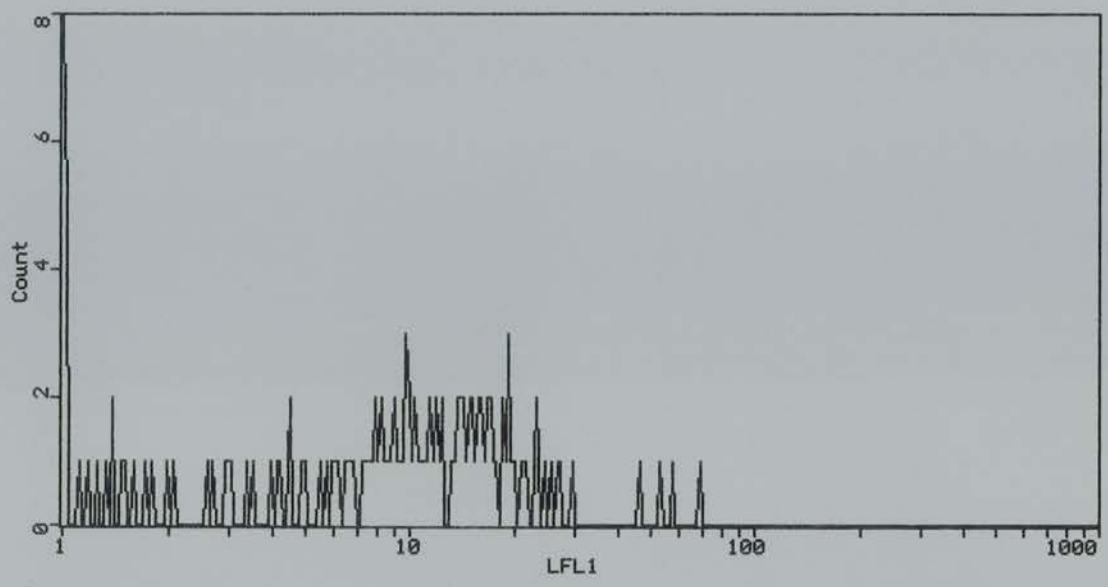
The percent of cells from each gate (shown in figure 5.5) are recorded (mean ± S.D. from duplicate samples). In the standard adherent assay performed in parallel with the same cells, 49.3 ± 8.0% (mean ± S.D.) of macrophages phagocytosed the enriched apoptotic neutrophils.

To resolve these difficulties with the flow cytometric analysis, fluorescent activated cell sorting was used to separate suspension samples and sorted fractions were re-analysed by flow cytometry and examination of cytospin preparations to determine the cell types within each peak. However, because of the small numbers of mø involved initially, insufficient cells were obtained at the end of the sort to make analysis possible. Instead, a magnetic cell sorting method was developed using a MiniMACS separation unit as described in section 2.12.3ii. The sample loaded onto the column has a flow cytometric profile in keeping with previous suspension assays (Fig. 5.7a). Fraction 1 was found to contain a pure population of aged neutrophils (Fig. 5.7b) and the flow cytometric profile was identical to that of the cultured neutrophils taken prior to incubation with mø (not shown). The column was washed twice, fractions 2 and 3 contained aged neutrophils (not shown). The column was then removed from the magnetic separation unit and washed through a final time. The eluent (fraction 4) was shown to contain a relatively pure population of mø. A few of these fraction 4 mø contained apoptotic neutrophils within cytoplasmic vacuoles (Fig. 5.7c). The flow cytometric profile of fraction 4 was very different to the original sample loaded onto the column (Fig. 5.7a) but similar to the profile obtained for mø prior to the phagocytosis assay (data obtained from mø flow cytometry profile is quoted in Fig. 5.7c).

Although phagocytosis was observed in suspension samples, the proportion of mø ingesting neutrophils was reduced when compared to the adherent assay, phagocytosis in suspension is less than one fifth of that observed in adherent cells (table 5.3). This reduced phagocytosis was not a result of manipulation of the cells *in vitro* as phagocytosis levels were still as markedly reduced in experiments where handling of the cells

**Figure 5.7. (a) Flow cytometric profile and photograph of suspension assay sample loaded onto miniMACS separation column.**

Mø and cultured neutrophils, incubated together for 30 minutes in suspension at 37°C, were loaded onto a miniMACS separation column placed in a miniMACS separation unit. The sample has a flow cytometric profile typical of previous experiments. Proportion of cells in each peak: 91.6% green –ve cells; 8.2% green + cells; 0.2% green ++ cells.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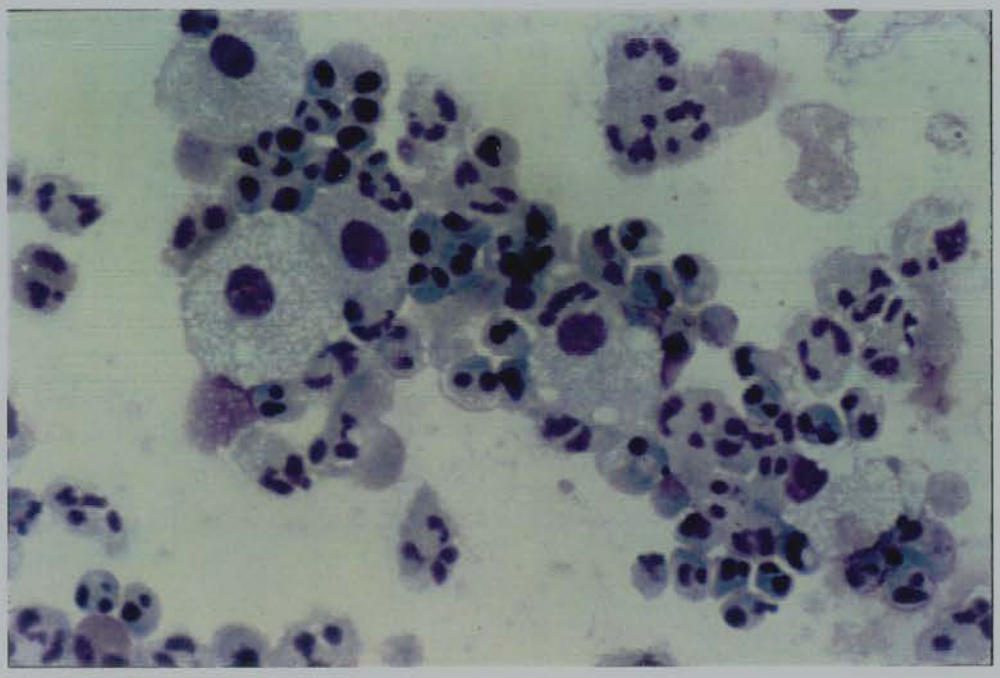
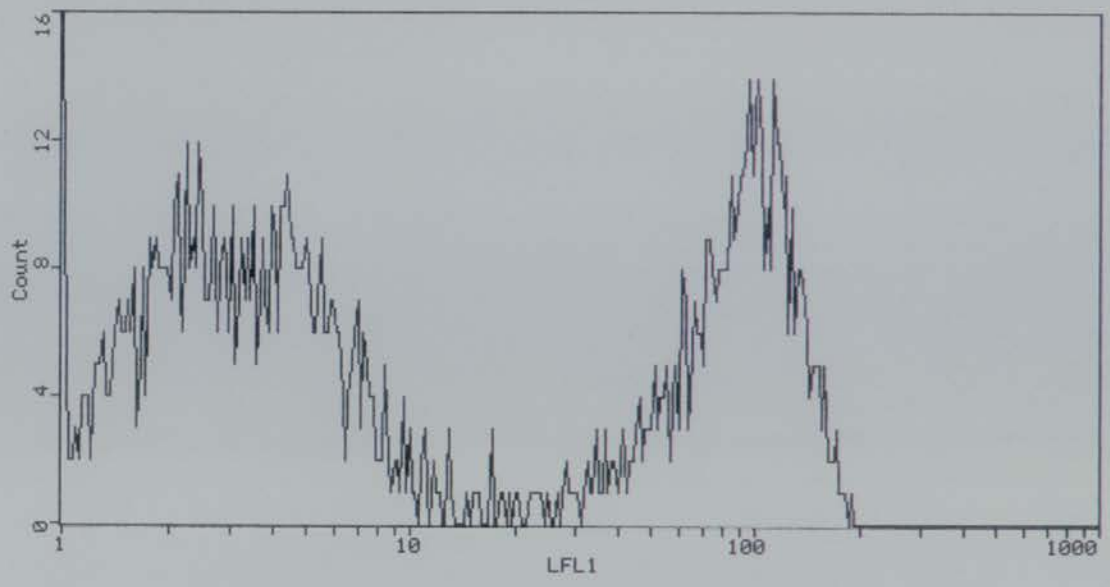


**Figure 5.7. (b) Flow cytometric profile and photograph of fraction 1 collected from miniMACS separation column.**

Fraction 1 is seen to contain a pure population of cultured neutrophils. The flow cytometric profile of fraction 1 matches that of the cultured neutrophils used in the assay.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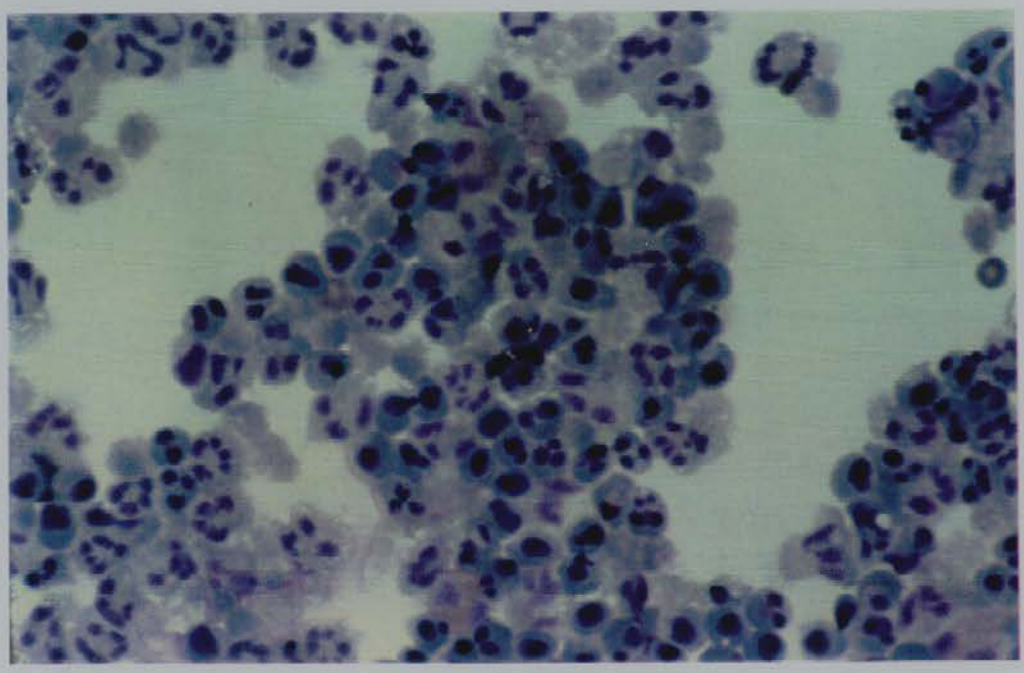
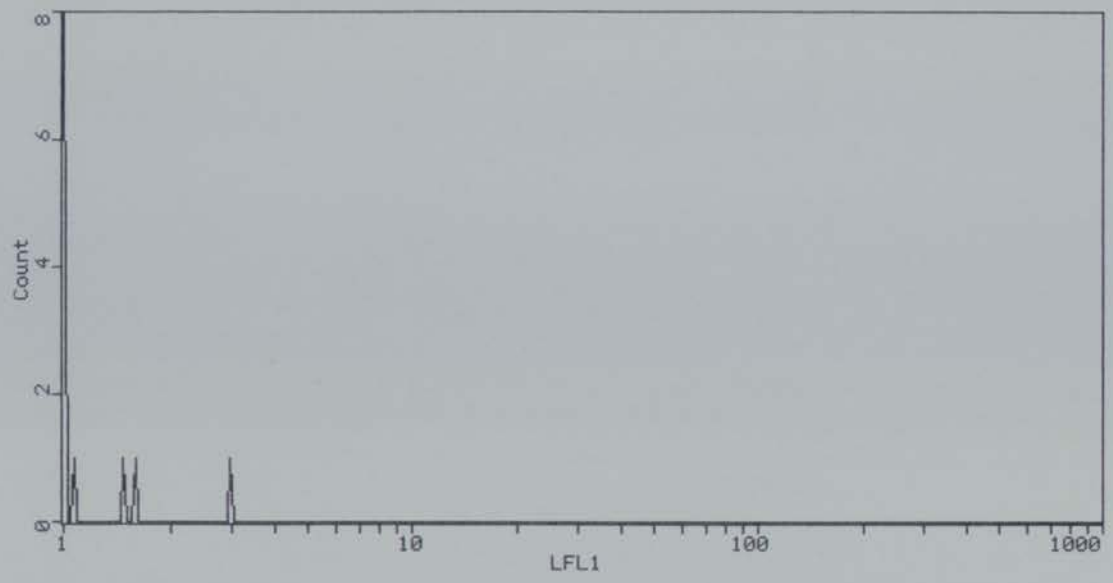


**Figure 5.7. (c) Flow cytometric profile and photograph of fraction 4 collected from miniMACS separation column.**
The sample can be seen to contain a purified population of mø, one of which has a cytoplasmic vacuole containing an apoptotic neutrophil (arrow). Some non-phagocytosed neutrophils are also present. Porportion of cells in each peak: 98.9% green –ve cells; 1.1% green + cells; 0% green ++ cells.
(Proportion of cells in each peak from mø sample prior to phagocytosis assay: 99.6% green –ve cells; 0.4% green + cells; 0% green ++ cells).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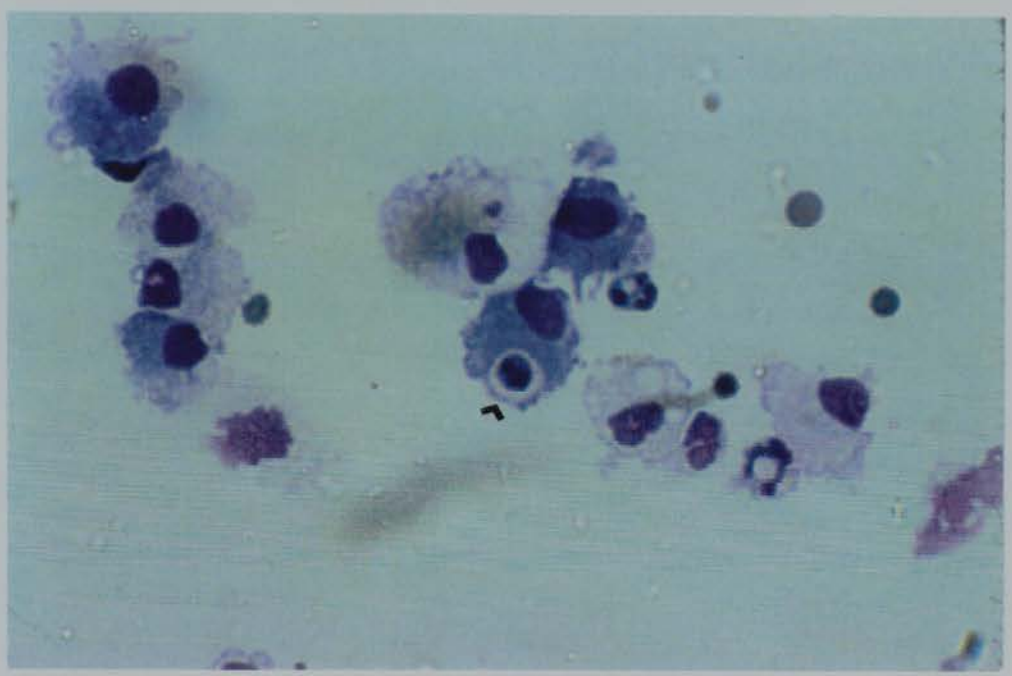


**Table 5.3. Summary of results obtained from the phagocytosis assays performed in suspension.**

§ - counted from cytospin preparations of purified fraction 4 from miniMACS separation column except for experiments 9 and 10 in which results were compared between suspension assay (top values) and fraction 4 from the miniMACS column (bottom value)

¤ - mean ± S.D. of duplicate samples except experiments 9 and 10 (single samples)

* - experiments in which the aged neutrophil populations (top values) were enriched for apoptotic cells (bottom values)

† - minimum handling experiments.

| Experiment number | Percent phagocytosis | | Flow cytometry readings¤ | | |
|---|---|---|---|---|---|
| | Adhesion | Suspension§ | -ve | + | ++ |
| 1 | 21.1 ± 3.0 | | 54.3 ± 0.5 | 28.7 ± 0.1 | 11.2 ± 0.6 |
| 2 | 25.0 ± 4.1 | | 60.4 ± 2.5 | 33.0 ± 2.5 | 6.4 ± 0.1 |
| 3* | 33.1 ± 7.2 | | 46.0 ± 0.1 | 42.8 ± 0.9 | 11.2 ± 1.8 |
| | 49.3 ± 8.0 | | 22.2 ± 0.4 | 68.4 ± 0.3 | 9.3 ± 0.1 |
| 4* | 22.9 ± 0.9 | | 57.1 ± 9.9 | 32.7 ± 6.8 | 9.8 ± 2.7 |
| | 26.94 ± 5.8 | | 10.2 ± 0.4 | 79.7 ± 0.1 | 8.0 ± 0.4 |
| 5 | 16.8 ± 2.5 | 8.2 ± 2.7 | 5.3 ± 0.1 | 90.7 ± 0.2 | 4.1 ± 0.1 |
| 6 | 17.4 ± 2.6 | | 36.4 ± 3.6 | 57.3 ± 2.9 | 6.3 ± 0.8 |
| 7 | | 2.0 ± 2.8 | | | |
| 8 | | 4.9 ± 2.9 | | | |
| 9 | | 0 | 84.6 | 15.4 | 0 |
| | | 0 | 96.9 | 3.1 | 0 |
| 10 | | 0 | 91.6 | 8.2 | 0.2 |
| | | 0 | 98.9 | 1.1 | 0 |
| 11† | 36.8 ± 8.4 | 2.0 ± 2.8 | | | |
| 12† | 71.7 ± 5.8 | 12.3 ± 7.0 | | | |

was kept to a minimum (2.12.3ii). Neither was it a consequence of the labelling protocol as cells used in the parallel adherent assays were also labelled. Table 5.3 shows a summary of all results obtained for this thesis of the phagocytic suspension assays performed at 37°C.

## 5.5. SUMMARY OF RESULTS.

Adhesion of mø to ECM components, notably fibronectin, increases phagocytosis of apoptotic neutrophils. However, mAb studies suggest that the α5β1 fibronectin receptor alone is not involved in either recognition of apoptotic neutrophils or mø adhesion to fibronectin. Execution of the apoptotic neutrophil phagocytosis assay in suspension results in comparatively low levels of phagocytosis in relation to parallel adhesive assays implying that the adhesion state of the mø is an important factor in the modulation of apoptotic neutrophil phagocytosis.

## 5.6. DISCUSSION.

The composition, structure and functional heterogeneity of the extracellular matrix depends on tissue origin and stage of development. However, most ECM contain a backbone of fibrillar proteins (collagen) into which a variety of glycoproteins, carbohydrates and proteoglycans are enmeshed. Vitronectin is a multifunctional glycoprotein found in tissues and plasma. Recognised as an adhesive substrate, cell interaction with Vn may induce spreading and migration and have an effect on cell growth and differentiation in processes such as tumour growth and metastasis, wound healing, bone resorption and viral infection (Preissner, 1991; Felding-Habermann and Cheresh, 1993). Fibronectin is a widespread ECM

and body fluid glycoprotein involved in embryogenesis, wound healing, haemostasis and thrombosis. It is the best studied adhesive protein component of the ECM involved in cell adhesion and chemotaxis (Ruoslahti, 1988; Potts and Campbell, 1994). Collagen is the most abundant protein, accounting for approximately one third of total body proteins. It is the major structural protein, holding cells together and giving organs their characteristic architecture (Gordon and Olsen, 1990).

Results in this chapter suggest that adhesion of monocyte-derived macrophages to ECM components enhances phagocytosis of apoptotic neutrophils in relation to control conditions, most significantly when adhered to Fn. Macrophages themselves are a source of ECM components and matrix deposition by mø *in vitro* might account for the variation of the levels of phagocytosis observed as a consequence of mø donor heterogeneity.

A previous study (Savill *et al.*, 1990) has described inhibition of apoptotic neutrophil phagocytosis when macrophages were adherent to Fn and Vn. One possible explanation for these apparently contradictory results is the higher concentration of ECM protein (80μg/ml) used by Savill *et al.* (1990). It has been shown that the number of ligand-receptor contacts is a important factor in regulating cell adhesion (Massia and Hubbell, 1991). It is therefore possible that the levels of expression of ECM components may exert different effects upon mø phagocytosis of apoptotic neutrophils in a manner similar to the effects of chemotractants which typically inhibit migration at high concentrations. At low levels of ECM components, adhesion and migration may be promoted whereas at higher concentrations, migration may be inhibited. Of interest are the

results from a preliminary experiment carried out for this thesis in which mø were adhered to different concentrations of Fn. Phagocytosis levels fell with increasing concentrations of Fn The percent of mø adhered to 80μg/ml Fn phagocytosing apoptotic neutrophils was found to be the same as mø adhered to tissue culture plastic. While further experiments are necessary to uphold this result, the initial data would tend to support the possibility discussed above.

Vn is an early and transient component of the ECM in wound repair while Fn expression persists throughout the entire process (Clark, 1990; Gailit and Clark, 1994). Intact Vn and Fn can also be detected in acute wound fluid (Grinnell *et al.*, 1992). The observation that phagocytosis of apoptotic neutrophils is enhanced upon adhesion of mø to ECM proteins that are present in wounds at a time when neutrophils would be removed from damaged tissue is of great significance in terms of composition of the ECM and the resolution of inflammation. On the other hand, wound fluid from chronic inflammation contains degraded polypeptides of ECM components which have been shown to have an inhibitory effect on cellular adhesion (Grinnell *et al.*, 1992). In view of this, it would be of value to assess the effects of degraded ECM polypeptides upon mø phagocytosis of apoptotic neutrophils. Perturbation of apoptotic neutrophil phagocytosis by mø in the presence of degraded ECM might be associated with disease pathogenesis and also account for the persistence of chronic inflammation. The composition of the ECM may therefore provide an additional level of control for the rate at which apoptotic neutrophils are removed.

In terms of cytoskeletal organisation, mø adherent to Fn appear to have an intact actin cytoskeleton. It has recently been reported that macrophages adhered to Fn-coated wells for 30 minutes show a decrease in filamentous actin content (Yang *et al.*, 1994). However, results were obtained by flow cytometric analysis and it is therefore not known to what extent the actin framework was disrupted. Also it is possible that further time points, beyond 30 minutes, might have shown a return to resting levels. Interestingly, a preliminary experiment in which mø were adhered to Fn and treated for 15 minutes with dbcAMP prior to the apoptotic neutrophil assay, showed a decrease in phagocytic levels when compared to untreated mø adhered to Fn. Further experiments will be required to verify this initial observation and it will be of interest to investigate the effect of dbcAMP on the actin cytoskeleton of mø adhered to Fn.

Integrins are the major receptors involved in cell-matrix interactions. As adhesion of mø to fibronectin enhanced apoptotic neutrophil phagocytosis, a role for the fibronectin integrin receptor, $\alpha5\beta1$, in mø phagocytosis of apoptotic neutrophils was investigated. TS2/16 is an anti-$\beta1$ mAb, shown to up regulate $\beta1$-integrin mediated cell attachment to different ligands (Arroyo *et al.*, 1992) by inducing an high affinity state of $\beta1$ integrins by directly changing the receptor conformation (Arroyo *et al.*, 1993). In contrast, mAb13 is an anti-$\beta1$ mAb that inhibits early cellular binding to ECM (Berdichevsky *et al.*, 1992). BIIG2 is an adhesion blocking mAb against the $\alpha5$-subunit of the fibronectin receptor that interferes with the initial attachment of many cell types to Fn but not to Col I, Col IV or Vn (Werb *et al.*, 1989). SAM-1, directed against the $\alpha5$-subunit, has been reported to block the uptake of apoptotic B-cells (Flora and Gregory,

1993). No significant effect was observed upon mø phagocytosis of apoptotic neutrophils as a result of preincubating mø with mAb against α5β1 suggesting that the α5β1 fibronectin receptor is not involved mø phagocytosis of apoptotic neutrophils. Nor does it appear that mø are adhering to substrate (tissue culture plastic or Fn-coated plastic) via the αvβ3 or α5β1 receptors as incubation of mø with mAb against the α-subunit of these receptors prior to substrate adhesion did not affect the number of cells viewed per microscope field or the subsequent level of phagocytosis of apoptotic neutrophils. However, mø have multiple receptors that promote adhesion to Fn and it is possible that blocking one receptor merely induces the cell to utilise another adhesion pathway. Studies in which all mø Fn receptors are blocked simultaneously may allow this possibility to be tested.

The relatively low levels of phagocytosis observed in the suspension assay suggest that adhesion to substrate is a major requirement for mø phagocytosis of apoptotic neutrophils. It would be of interest to investigate if pretreatment of suspension mø with PdBu or addition of ECM components to the suspension assay (both methods shown to enhance phagocytosis of apoptotic neutrophils in adherent assays) enhanced the low levels of phagocytosis observed. In addition, the integrity of the actin filament network is required for cell locomotion (Bearer, 1993; Stossel, 1993). Secondary to altered cellular adhesion may be the effect on mø motility. Indeed, the organisation of cytoskeletal components within mø observed for this thesis is consistent with the presence of motile cells. Thus mø phagocytic responses may reflect a combined requirement for cell adhesivity and cell motility.

# CHAPTER 6: SUMMARY.

This thesis has focused upon the model of macrophage recognition and phagocytosis of apoptotic neutrophils proposed by Savill *et al.* (1992) with the aim of defining mechanisms that modulate this phagocytic process.

Apoptosis is the mechanism by which cells are physiologically deleted from a wide variety of tissues, apoptotic cells being taken up by surrounding resident tissue cells (Wyllie *et al.*, 1980) or macrophages (Savill *et al.*, 1989a; Jones *et al.*, 1993). In the multicellular organism *C. elegans*, in which 131 cells die by apoptosis during development, recognition and phagocytosis of apoptotic bodies involves at least 10 gene products. *ced-3* and *ced-4* induce cell death, ced-9 is crucial for prevention of apoptosis while seven genes (*ced-1, ced-2, ced-5, ced-6, ced-7, ced-8* and *ced-10*) contribute to engulfment of apoptotic cells (Driscoll, 1992). Given the homologies between invertebrate and mammalian systems in terms of the machinery that regulates cell death e.g. *ced-3* and ICE (Lazebink *et al.*, 1994) and *ced-9* and *bcl-2* (Hengartner and Horvitz, 1994), it is likely that many of the genes involved in engulfment of apoptotic cells in *C. elegans* will have a similar role in mammalian systems. Thus, the molecular mechanisms by which apoptotic cells are recognised and phagocytosed in mammalian systems may also be complex and involve multiple gene products.

In addition to the characteristic morphological and biochemical changes of apoptosis, specific membrane changes have been described including glycosylation changes (Duvall *et al.*, 1985), altered membrane lipid asymmetry (Fadok *et al.*, 1992a) and reduction in expression of cell surface receptors (Dransfield *et al.*, 1994: Homberg *et al.*, 1995). Expression of

phosphatidylserine on the outer leaflet of the plasma membrane has been suggested to occur during apoptosis and has been reported to be involved in macrophage phagocytosis of apoptotic cells (Fadok *et al.*, 1992a). The surface molecule on apoptotic neutrophils that signals macrophage recognition is as yet unknown. However, apoptotic neutrophil recognition by mø has been found to be charge sensitive at the neutrophil surface, as indicated by inhibition in the presence of amino sugars and basic amino acids. Characterisation of the surface of apoptotic cells may define molecules involved in providing phagocytic signals to mø and perhaps reveal further novel mechanisms of recognition.

To date, three mechanisms have been described for phagocytosis of apoptotic neutrophils by macrophages. Savill and co-workers have defined a human system that involves the macrophage αvβ3 vitronectin receptor and CD36 acting in concert to bind thrombospondin which serves as a molecular bridge between the macrophage and apoptotic cell (Savill *et al.*, 1990 and 1992). In a murine system, Fadok *et al.* (1992b) suggest that the mechanism by which macrophages recognise apoptotic neutrophils is determined by the subpopulation of macrophages being studied. They show that murine peritoneal macrophages utilise a phosphatidylserine dependent mechanism for the phagocytosis of apoptotic neutrophils whereas murine bone marrow macrophages, like human monocyte-derived macrophages, employ the vitronectin receptor (Fadok *et al.*, 1992b). Most recently, Flora and Gregory (1994) describe macrophage phagocytosis of apoptotic cells via a 61D3 antigen-dependent pathway distinguishable from the αvβ3-dependent mechanism. However, the mechanism used for recognition of apoptotic cells was found to be dependent on the macrophage donor: A-type donors cells utilised both the 61D3- and αvβ3-dependent pathways to recognise

apoptotic cells while B-type donors were only able to employ the 61D3-dependent mechanism. However, in all of the mechanisms described above, total inhibition of phagocytosis has never been shown suggesting the potential for further phagocytic pathways, distinct from those described above.

Although "professional" phagocytes such as macrophages are able to efficiently remove apoptotic cells, the role of stromal cells in this process has received relatively little study. Recently, Hall *et al.* (1994) have reported that fibroblasts recognise apoptotic neutrophils. Two distinct mechanisms appear to be involved: one utilising $\alpha v\beta 3$, the other employing a mannose/fructose-specific lectin which plays no part in phagocytosis of the apoptotic cell. Interaction between fibroblasts and apoptotic neutrophils may be of relevance to the relationship between inflammation and scarring associated with disease.

It is well documented that macrophages do not recognise and phagocytose freshly isolated neutrophils (Newman *et al.*, 1982; Savill *et al.*, 1989a) but that macrophage recognition of neutrophils progressively increases with time in culture. Morphological changes characteristic of apoptosis were observed in aged neutrophil populations and a highly significant correlation between recognition and apoptosis was found. Consequently, apoptosis in the aged neutrophil population was directly linked to recognition by mø (Savill *et al.*, 1992a). However, it has recently been reported that phagocytosis of aged neutrophils occurs independently of apoptosis. The oncogene *bcl-2*, has been shown to block the molecular processes of cell death (Vaux *et al.*, 1988). Over expression of Bcl-2, which may be localised to the inner mitochondrial membrane, promotes cell survival without promoting cellular division

(Hockenbery *et al.*, 1990) indicating its critical role in regulation of cell death in some systems. Lagasse and Weissman (1994) found that while induced *bcl-2* expression in mature neutrophils inhibited apoptosis, phagocytosis of the aged cells still took place. They propose that neutrophils exhibit cell surface changes, independent of apoptosis, that enable recognition and engulfment by macrophages. Since this model is based on a murine system, comparison with the removal of human cells is difficult and the mechanism by which macrophages ingested *bcl-2* expressing non-apoptotic neutrophils was not discussed. However, it is likely that in the future, such model systems will provide useful molecular information as to how apoptotic cells are recognised.

Phagocytosis of apoptotic neutrophils by macrophages represents a non-inflammatory mechanism for the removal of senescent cells from inflamed sites. In view of the capacity of many granule contents to cause tissue injury (Weiss, 1989) and cleave tissue matrix proteins into chemotactic fragments (Vartio, 1981), necrotic neutrophil cell death would tend to damage healthy tissues and exacerbate inflammatory processes. However, during apoptosis, membrane integrity is preserved (Savill *et al.*, 1989a) and neutrophil functions, including granule secretion, are markedly down-regulated (Whyte *et al.*, 1993b) thus limiting the potential for neutrophil-mediated tissue damage. Furthermore, phagocytosis of apoptotic neutrophils via the αvβ3/CD36 recognition mechanism does not induce the release of pro-inflammatory macrophage mediators such as thromboxane B2 or N-acetyl-β-D-glucosaminidase (Meagher *et al.*, 1992). Therefore, modulation of this phagocytic event may limit tissue injury and promote the normal resolution process in inflammation.

Integrin function has been shown to be modulated by protein kinase activation (Dustin and Springer, 1989: Shimizu *et al.*, 1990: Stupack *et al.*, 1992). The αvβ3 integrin has been implicated in macrophage recognition of apoptotic neutrophils (Savill *et al.*, 1990) therefore initial work in this thesis involved short pretreatment of monocyte-derived macrophages with activators of protein kinase C and A to assess their effect on phagocytosis of apoptotic neutrophils. I have shown that activation of intracellular protein kinases alters the phagocytic potential of mø for apoptotic neutrophils. Activation of PKC enhanced mø recognition of apoptotic neutrophils while PKA activation caused down regulation of recognition. Inflammatory mediators have multiple effects upon leukocyte function however, those that stimulate PKC activity may increase apoptotic cell removal from inflamed sites. Interestingly, preincubation of day four mø with 'proinflammatory' cytokines e.g. GMCSF or LPS for six hours has been found to enhance apoptotic neutrophil phagocytosis (Ren and Savill, personal communication) although the mechanism by which phagocytosis is modulated is not yet known. It is thought that the capacity of cytokines (which tend to promote inflammation by recruitment of leukocytes to inflamed sites) to promote resolution by increasing the ability of mø to remove apoptotic cells may represent a form of negative feedback controlling inflammation.

Throughout the course of my investigations, I have found no evidence to support the model proposed by Savill *et al.* (1990 and 1992) implicating a direct interaction of αvβ3, CD36 and thrombospondin with the apoptotic neutrophil. However, it is likely that there are indirect associations that modulate macrophage phagocytic responses. Thrombospondin could not be detected from macrophage supernatants and CD36 mAb inhibition studies reveal additional complexities that require further study (Dransfield *et al.*,

1995). Furthermore, in a series of blocking experiments, a panel of mAb against the vitronectin receptor α- and β-subunits did not reduce the proportion of mø able to phagocytose apoptotic neutrophil. In previous mAb studies, reduction of mø phagocytosis of apoptotic was reported using high concentrations (1:25) of mAb ascites fluid (Savill *et al.*, 1990, 1992). In this thesis, purified mAb were used and no significant reduction of mø phagocytosis of apoptotic neutrophils was observed. There are several problems which may be associated with use of high concentrations of mAb. First, there may be specific receptor cross-linking that may result in activation of intracellular signalling pathways that subsequently modulate cellular function. As I have demonstrated in this thesis, elevation of intracellular cAMP in mø exerts an inhibitory effect on mø recognition of apoptotic cells. Alternatively, receptor cross-linking may cause internalisation of receptors which may also modulate cellular behaviour. An additional complication is that the use of whole antibody may co-cross-link specific receptors and Fc receptors present on the mø surface (via the Fc portion of antibody), again possibly activating intracellular pathways. One approach which would bypass these problems is the use of Fab' fragments of inhibitory antibodies thereby avoiding both cross-linking effects and possible engagement of Fc receptors. Furthermore, as noted in chapter four, ascitic fluid may also contain other proteins e.g. matrix molecules that might affect mø recognition of apoptotic neutrophils.

I therefore considered whether the αvβ3 receptor might affect phagocytosis indirectly. Integrins are capable of transducing signals from the exterior of the cell to the interior and also altering cytoskeletal architecture as a result of their association with the cytoskeleton. Altered distribution of αvβ3 following protein kinase activation (described in section 3.8) may exert

indirect control over the process of mø phagocytosis of apoptotic neutrophils, possibly via the macrophage actin cytoskeleton. Although αvβ3 could not be seen to localise with the focal contacts present in mø, it is possible that αvβ3 is associated with less well defined structures within the actin cytoskeleton. While not ruling out a role for αvβ3, I believe the role of the vitronectin receptor in macrophage phagocytosis of apoptotic neutrophils needs to be reviewed. The use of Fab' fragments of antibody and perhaps αvβ3 negative cell lines may allow the role of αvβ3 in phagocytosis of apoptotic neutrophils to be re-assessed. Savill and colleagues have recently demonstrated CD36 transfected melanoma cells phagocytose apoptotic neutrophils. Transfection of αvβ3 negative melanoma cell lines with CD36 may provide an alternative strategy for examining the role of αvβ3.

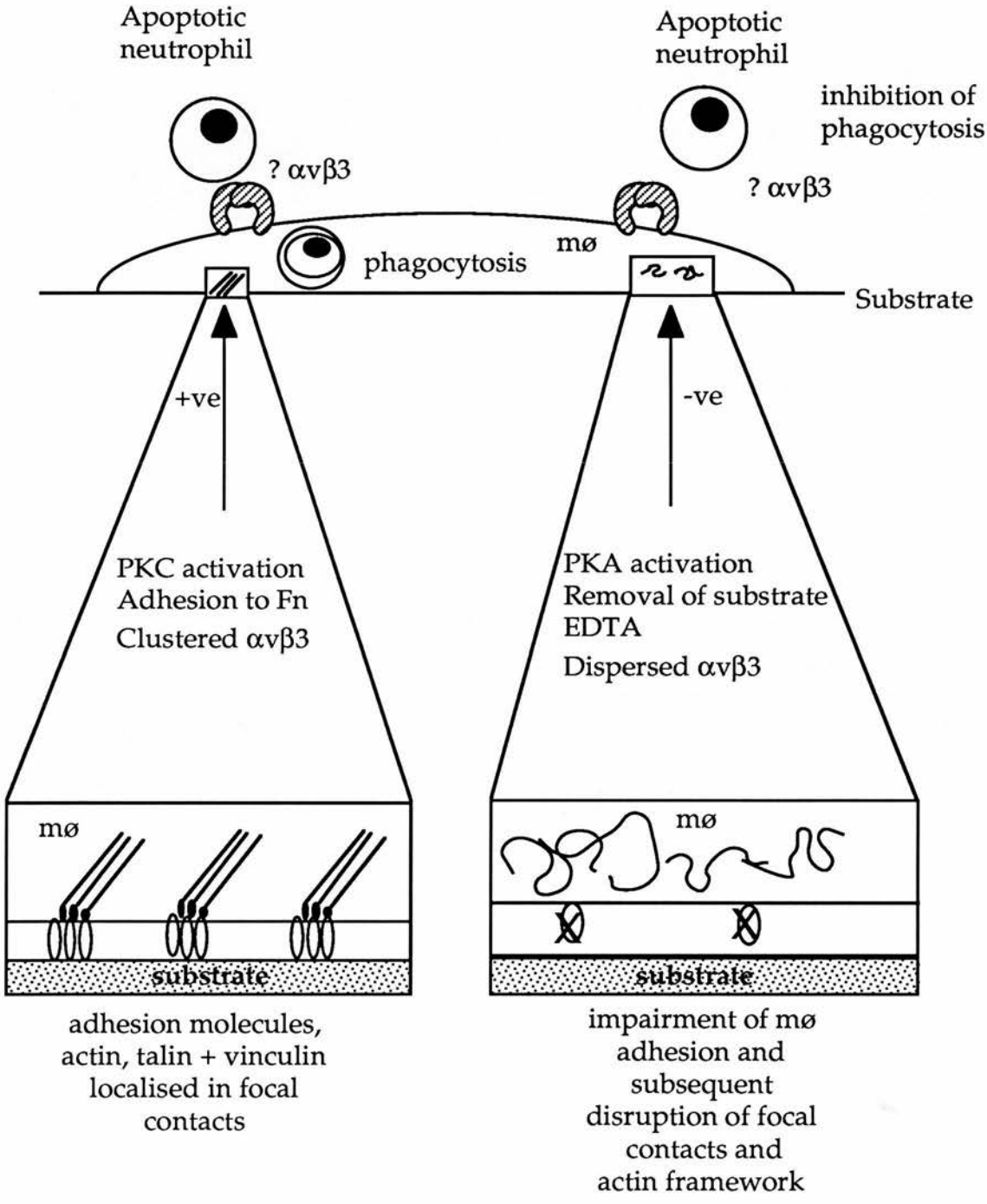
A variety of agents, including dbcAMP, that inhibited apoptotic neutrophil phagocytosis were also found to disrupt the mø actin framework. The actin cytoskeleton is an integral part of the cell adhesion process and in subsequent experiments, mø adherent to ECM proteins were found to be more efficient phagocytes of apoptotic neutrophils suggesting that the adhesion state of the mø is an important regulatory factor in mø phagocytosis of apoptotic cells. This is supported by the results obtained when the phagocytosis assay was performed in suspension - the mean phagocytosis level in suspension is only 13% of that observed in adherent assays.

In conclusion, work presented in this thesis has addressed mechanisms involved in modulating the capacity of macrophages to recognise and phagocytose apoptotic neutrophils. Taken together, these data support a

model for macrophage recognition of apoptotic neutrophils in which the adhesion state of the macrophage is an important regulatory factor (Fig. 6.1). No evidence has been obtained for a direct role of αvβ3 in apoptotic neutrophil recognition which may indicate that this receptor exerts a regulatory effect indirectly. Indeed, αvβ3 mediates adhesion to a variety of substrates, including fibronectin and thrombospondin. Integrin adhesion may modulate cellular behaviour via initiation of intracellular signalling pathways and it is possible that the αvβ3 may act as a regulator of apoptotic neutrophil recognition mechanisms. I have demonstrated that rapid modulation of the macrophage potential for apoptotic cell removal is achieved by activation of intracellular protein kinases. This effect is, in part, achieved by alteration of the architecture of the macrophage actin cytoskeleton and thus the adhesive state of the macrophage itself. Since the extracellular matrix components produced at inflamed sites are altered during the progression of wound healing responses, I would hypothesise that the precise composition of the extracellular environment encountered by macrophages regulates their ability to recognise and phagocytose apoptotic cells. Thus, macrophage responses to mediators of inflammation and peturbation of normal matrix deposition during inflammatory responses may be important factors in determining the process of resolution of inflammation and disease pathogenesis.

**Figure 6.1. Proposed model for modulation of macrophage phagocytosis of apoptotic neutophils.**

Phagocytosis of apoptotic neutrophils by monocyte-derived macrophages was found to be modulated by short term pre-treatment of mø with activators of PKC and PKA. Localised β3 distribution is associated with activation of PKC, however, no evidence has been obtained for a direct role of αvβ3 in apoptotic neutrophil recognition and although not found to localise to focal contacts, αvβ3 may act as a regulator of apoptotic neutrophil recognition mechanisms via initiation of intracellular signalling pathways. Activation PKA was seen to cause disruption of the macrophage actin cytoskeleton. Adherence of macrophages to extracellular matrix proteins was found to increase levels of phagocytosis compared to control conditions. By contrast, very little phagocytosis was observed when the assay was performed in suspension. These data suggest that the adhesion state of the macrophage is important in regulating phagocytosis of apoptotic neutrophils and that the tissue micro-environment of the macrophage at inflammed sites may exert control over removal of apoptotic cells and hence the process by which inflammation resolves.


Apoptotic neutrophil
? αvβ3
phagocytosis
mø
Substrate
+ve
PKC activation
Adhesion to Fn
Clustered αvβ3
mø
substrate
adhesion molecules, actin, talin + vinculin localised in focal contacts
Apoptotic neutrophil
inhibition of phagocytosis
? αvβ3
-ve
PKA activation
Removal of substrate
EDTA
Dispersed αvβ3
mø
substrate
impairment of mø adhesion and subsequent disruption of focal contacts and actin framework

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