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NEUTROPHIL INTERACTIONS WITH FIBROBLASTS IN CULTURE

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ABSTRACT

The fate of senescent neutrophils and their potentially histotoxic contents at inflamed sites remains uncertain. Human neutrophils "aged" in culture for 24h have been reported to undergo apoptosis, or programmed cell death, which leads to recognition and phagocytosis by macrophages. There is also evidence that neutrophil apoptosis occurs at inflamed sites. Although macrophage phagocytosis appears to be the predominant mode of removal of apoptotic cells, other cells may also ingest apoptotic bodies. Haslett observed that fibroblasts, but not a variety of other cell monolayers, also recognised aged neutrophils *in vitro*.

This project investigated the mechanisms responsible for fibroblast interaction with aged neutrophils. This was compared to macrophage interaction since fibroblast phagocytosis of apoptotic neutrophils may represent an important auxiliary neutrophil disposal mechanism, acting before monocytes have matured, or when macrophages are overloaded.

In these studies I have found that:

- (1) The interaction of fibroblasts with aged neutrophils consists of
 - (a) phagocytosis, (5-15% ingest) which is directly related to neutrophil apoptosis as was shown for macrophage phagocytosis of aged neutrophils
 - (b) a larger adhesive interaction, involving non-apoptotic neutrophils aged in tissue culture.
- (2) The phagocytic interaction shows similarities to macrophage recognition of aged neutrophils; with strong dependence upon divalent cations and being inhibited by amino sugars, basic amino acids, trypsinisation of the phagocyte and inclusion of the tetrapeptide arg-gly-asp-ser (RGDS). These characteristics suggest the involvement of an integrin molecule on the fibroblast surface. Preliminary data with the antivitronection receptor antibody, 13C2, implicated the vitronection receptor in fibroblast phagocytosis of aged neutrophils, as in macrophage recognition.
- (3) Phagocytosis by fibroblasts is also inhibitable by mannose, fucose, mannose-6-phosphate and other ligands of the C-type lectins, features not found in macrophage phagocytosis of aged neutrophils. Further, the adhesive interaction shows similar specificity and inhibition of both types of interaction by MEL-14 and DREG-56 antibodies indicated that a molecule of the homing receptor family, on the surface of the aged neutrophil, may be responsible for the lectin specificity exhibited in both the phagocytic and adhesive interactions between fibroblasts and aged neutrophils.

The data presented here indicates a specific interaction between neutrophils and fibroblasts which may occur at the inflamed site. Since the fibroblast is the major cell responsible for the laying down of matrix proteins in scar tissue, further study of fibroblast response to neutrophil phagocytosis may lead us to a greater understanding of the link between inflammation and fibroblast-mediated tissue scarring.

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ABBREVIATIONS

Arg	Arginine
ATP	Adenosine triphosphate
BALF	Bronchoalveolar lavage fluid
Ca ²⁺	Calcium
C5a	Complement fragment 5a
Cl ⁻	Chloride ion
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
ECF-A	Eosinophil chemotactic factor of anaphylaxis
EDTA	Ethylene diamine tetraacetic acid
EGF	Epidermal growth factor
ELAM-1	Endothelial leukocyte adhesion molecule-1
EMEM	Eagle's minimal essential medium
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FIB	Fibroblast
FITC	Fluorescein isothiocyanate
Fn(R)	Fibronectin (receptor)
F-1-P	Fructose-1-phosphate
F-6-P	Fructose-6-phosphate
Fuc	Fucose
Gal	Galctose
Gala	Galactosamine
Glu	Glucose
Glut	Glutamine
Glut acid	Glutamic acid
Glsa	Glucosamine
GPIIb	Platelet glycoprotein IIb
GPIIIa	Platelet glycoprotein IIIa
GRGDSP	Glycine-arginine-glycine-aspartic acid-serine-proline
GRGESP	Glycine-arginine-glycine-glutamic acid-serine-proline
HBSS	Hanks buffered salt solution
H ₂ O ₂	Hydrogen peroxide
HOX	Hypophalous acid
HOCl	Hypophalous hydrochloric acid
ICAM(-1 or -2)	Intercellular adhesion molecule(-1 or -2)
Ig	Immunoglobulin

IGF	Insulin-like growth factor
^{111}In	$^{111}\text{Indium}$
IPF	Idiopathic pulmonary fibrosis
LDG	Lactate dehydrogenase
LFA-1	Lymphocyte function-associated antigen-1
LGL	Large granular lymphocytes
LPS	(Bacterial) lipopolysaccharide
Lys	Lysine
mAb	Monoclonal antibody
Man	Mannose
Mg^{2+}	Magnesium
Mø	Macrophage
M-1-P	Mannose-1-phosphate
M-6-P	Mannose-6-phosphate
MFR	Mannosyl/fucosyl receptor
MPO	Myeloperoxidase
mRNA	messenger ribonucleic acid
NacGala	N-acetyl galactosamine
NacGlsa	N-acetyl glucosamine
NBCS	New born calf serum
$\text{O}_2^{\cdot-}$	Superoxide anion
$\text{OH}^{\cdot}$	Hydroxyl radical
PADGEM	Platelet activation dependent granule-external membrane protein
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PMN	Neutrophil
PN HEV	Peripheral lymph node
PP HEV	Peyer's patch high endothelial venule
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PRPDS	Platelet rich plasma derived serum
RGD	Arginine-glycine-aspartic acid
RGDS	Arginine-glycine-spartic acid-serine
RGES	Arginine-glycine-glutamic acid-serine
RNA	Ribonucleic acid
RNaseB	Ribonuclease B
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	Standard error
TIMP	Tissue inhibitor of metalloproteinases
UV	Ultra violet

VCAM-1	Vascular cell adhesion molecule-1
VLA(-1,2,3,4,5 or 6)	Very late antigen(-1,2,3,4,5 or 6)
Vn(R)	Vitronectin (receptor)

Standard abbreviations also used (eg. mM for millimolar, h for hour, etc.)

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CHAPTER 1:INTRODUCTION

This chapter serves to introduce the background areas of interest and relevant details for this project.

1.1 Inflammation: Stages of the inflammatory response and methods of termination.

It has been known since the earliest times that injury in animals and man is followed by a characteristic series of changes in the damaged area. The tissue response to injury is known as inflammation, the term being derived from the Latin, *inflamare* meaning to burn.

Following injury there is widespread dilatation of arterioles and venules so that blood flow through the injured area may increase as much as ten-fold (Ascheim and Zweifach, 1962). This is accompanied by release of chemical mediators such as C5a (chemotactic for neutrophils; Synderman et al., 1971; Larsen and Henson, 1983), eosinophil chemotactic factor of anaphylaxis (ECF-A; chemotactic for eosinophils; Kay et al., 1971) and polymorph cationic protein (chemotactic for monocytes; Hurley, 1983). 30 to 60 minutes after the onset of inflammation, the blood-borne neutrophils begin to stick to the inner surface of the endothelium and the neutrophil takes on amoeba-like characteristics inserting a pseudopoidal projection in the space between two endothelial cells prior to squeezing into the interstitium (Ross, 1969). By this process of neutrophil exudation, large numbers of neutrophils migrate into an inflamed area. The process is brought about by chemotaxis as chemical mediators generated in the inflamed area diffuse out. Monocytes follow the neutrophil movement into the tissue and, once there, are transformed into macrophages. Maturation of the monocytes into macrophages capable of

phagocytosis takes 24 to 48h (Newman et al., 1982). Thus the phagocytic cells reach the inflamed site ready to perform their function ie. the removal of foreign organisms (or damaged native cells) which are recognised by certain characteristics of their surfaces.

Following this sequence of events, depending on the nature and duration of the injurious stimulus, the type of tissue injured and the extent of tissue destruction caused, subsequent changes result in one of four possible outcomes (Hurley, 1983):

1. Resolution

Resolution results in complete restoration to normal of both the structure and function of the inflamed area and can only occur if the damaging agent does not cause extensive tissue damage. In many tissues, if a single cell or a small group of cells are killed, their remains are removed and the missing cells are replaced by mitotic division of neighbouring cells of the same type. Resolution usually occurs following mild physical or chemical injury of limited duration, or following infections where the causative agent, although widespread throughout the body, does not cause large areas of tissue destruction. However, even massive accumulation of cells, as seen in lobar pneumonia, may be removed completely within a few days and the lung restored to structural and functional normality.

2. Healing by scar, with or without regeneration of lost parenchymal cells

Whenever the causative stimulus destroys a substantial amount of tissue, inflammation results in scar formation. In this case, when the dead tissue has been resorbed, connective tissue is newly synthesised to replace the lost tissue. This process is known as repair, and the newly formed connective tissue is called scar tissue. Newly formed scar tissue is composed of fibroblasts, of their secretion products, collagen and connective tissue ground substance, and of newly formed blood vessels and lymphatics.

3. Suppuration (abscesses)

Suppurative lesions are characterised by the formation of an inflammatory exudate containing large numbers of neutrophils and killed cells which break down to form a thick, creamy fluid called pus. It is thought that inflammation leads to suppuration when the stimulus persists and is of a type that evokes massive emigration of neutrophils. The most common cause of suppuration is invasion of living tissues by pyogenic (pus-forming) microorganisms, such as streptococci, gonococci and *Escherichia coli*.

4. Chronic inflammation

Chronic inflammation may develop by failure of evacuation of an abscess or when the agent initiating inflammation is a foreign body which persists. Chronic inflammation is a feature of many inflammatory diseases of unknown aetiology, where it is often associated with permanent architectural injury and loss of organ function.

Clearly in organs such as the lung and kidney resolution is preferable to development of scarring and chronic inflammation, which may have functional effects on these organs, and abscesses, which may lead to release of bacteria, etc. The rationale for study of the mechanisms of termination of inflammation by resolution is that identification of such mechanisms will lead to new insights into the development of circumstances whereby inflammation may persist and causes excessive tissue injury and scarring.

1.2 The resolution of inflammation and neutrophil disposal at inflamed sites

One important prerequisite for the resolution of inflammation is removal of the neutrophils that have gathered at the site of injury or infection. This would seem particularly important since the human neutrophil has been implicated as

a mediator of tissue injury in inflammatory diseases ranging from rheumatoid arthritis and myocardial reperfusion injury to respiratory distress syndromes, blistering skin disorders and ulcerative colitis (Cochrane, 1968; Malech and Gallin, 1987; Henson and Johnston, 1987). The injury in these, and many other acute inflammatory disorders, has been linked to the neutrophil's ability to release a number of agents that can destroy normal cells and damage connective tissues. Normally, the neutrophil contents are involved in mobilisation of cells to the site of inflammation and bacterial killing, but these contents can also cause extensive host tissue damage in certain inflammatory diseases.

Neutrophil granules contain a large number of enzymes, but three proteolytic enzymes, the serine proteinase, elastase, and the two metalloproteinases, collagenase and gelatinase, have the greatest potential to mediate tissue destruction. Each of these proteinases is able to attack key components of the extracellular matrix, which is composed of a complex mix of collagens, elastin, proteoglycans and glycoproteins that lies under epithelia and surrounds fibroblasts (Weiss, 1989). In addition, each can cause tissue damage by other mechanisms.

Elastase can degrade almost all components of the extracellular matrix and can also cleave a variety of key plasma proteins, such as immunoglobulins, complement proteins and clotting factors, and even attack intact cells (Janoff, 1985; Weiss, 1989). In fact, because of these findings, it has been suggested that the physiological role of elastase is in the phagocytic vacuole where it can break down ingested microorganisms (Janoff, 1985). This hypothesis has been supported by the fact that plasma and interstitial fluids contain a number of antiproteinases, including α_1 -proteinase inhibitor (previously called α_1 -antitrypsin), α_2 -macroglobulin and secretory leukoproteinase inhibitor, that can inactivate neutrophil elastase (Janoff, 1985). Elastase can also directly injure cells, whether via enzymic activity or due to its cationicity is uncertain. Indeed other phlogistic products of the neutrophil may regulate the action of

elastase. The neutrophil is also able to generate reactive oxygen metabolites: the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH\cdot$) (Malech and Gallin, 1987). In combination with H_2O_2 , the neutrophil myeloperoxidase can oxidise halides to their corresponding hypophalous acids (HOX) and, since Cl^- is in higher concentration in plasma than any other halide, the predominant product is HOCl (Weiss, 1989). Relatively small amounts of HOCl, when combined with α_1 -proteinase inhibitor, causes oxidative inactivation thus removing the host-erected barriers designed to protect the host tissues from injury and allowing activation of the elastase (Weiss and Regiani, 1984).

Neutrophil collagenase can cleave the interstitial collagens (types I, II and III collagens) at a single, specific locus, whilst gelatinase degrades native types V, XI (and possibly IV) collagens (Weiss and Peppin, 1986). However, these collagenolytic enzymes are released in latent, inactive forms. Neutrophil collagenase, like the closely related, but distinct, tissue collagenases from other cell types (eg. macrophages, fibroblasts and endothelial cells) is a proenzyme that becomes activated when treated with trypsin *in vitro* (Weiss and Peppin, 1986). Experiments using intact neutrophils have indicated that generation of oxygen metabolites by the neutrophils is an absolute requirement for subsequent activation of collagenase by the intact neutrophils (Weiss et al., 1985). A similar role for oxidative activation of gelatinase has been described. Despite the fact that latent gelatinase is structurally distinct from latent collagenase, endogenously generated HOCl was shown to activate gelatinase in an intact cell system (Peppin and Weiss, 1986).

Once the collagenases have been activated, the ability of antiproteinases to regulate these enzymes is uncertain. α_2 -Macroglobulin and a more specific antiproteinase, the tissue inhibitor of metalloproteinases (TIMP), are powerful regulators of other tissue collagenases, but appear to be less effective against the neutrophil enzymes. Regardless of their ability to regulate neutrophil collagenases, α_2 -macroglobulin can be inactivated by oxidants whereas TIMP

can be destroyed by neutrophil elastase (Weiss and Peppin, 1986; Weiss, 1989). Thus it appears that the body has no defence mechanism to protect it from its own neutrophil enzymes.

In acute inflammation, neutrophils do not continuously degrade host tissues in an uninterrupted manner. Normally the neutrophil influx is finely regulated and ceases when the initiating antigen is destroyed (Clark, 1983; Haslett and Henson, 1988). When the oxidising environment has dissipated, the antiproteinase screen of the inflamed site can be reconstituted. However, if the inflammatory stimuli are chronically directed against host tissues or are not properly down regulated, the results could be irreparable damage to the host tissues, by any of the large number of potentially histotoxic neutrophil products.

It is generally assumed that the neutrophils, after performing their host defence function, remain at the inflamed site and die by necrosis, so disintegrating and liberating their granules and cytoplasmic enzymes, and that the resulting cell debris is phagocytosed by macrophages (Hurley, 1983). This neutrophil fate is likely to result in the release of the potentially histotoxic contents of the neutrophils so resulting in local tissue damage and amplification of the inflammatory response. There is little direct evidence, however, that neutrophils inevitably meet their fate by disintegration. In fact, Metchnikoff (1891a), who assigned engulfment and digestion of microorganisms to two major cell types; the microphages (neutrophils; "little eaters" because they are only capable of phagocytosing small particles eg. bacteria) and macrophages (macrophages; "big eaters" because they are able to ingest large particles eg. erythrocytes, spermatozoa, protozoa) described a very different mechanism. He reported that, in his studies of inflammation induced by puncturing the tail fins of living tadpoles with rose thorns or small glass tubes, "microphages" (neutrophils) were "englobed", apparently intact, by macrophages. Moreover, in later studies of human disease (1891b) he observed that, in erysipelas, the neutrophils englobed the streptococci but

were then, themselves, englobed by the macrophages.

Subsequently, there have been numerous other observations of neutrophils apparently contained within macrophages in both animals (Brewer, 1964; Sanui et al., 1982 and Chapes and Haskill, 1983) and man (Pekin et al., 1967; Kadri et al., 1975; Spriggs et al., 1978; Parmley et al., 1981 and Newman et al., 1982). In the experimental peritonitis induced by thioglycollate broth (Sanui et al., 1982) and *Corynebacterium parvum* (Chapes and Haskill, 1983) it was proposed that macrophage removal of neutrophils is the major neutrophil disposal route.

Newman et al. (1982) first studied neutrophil recognition by macrophages *in vitro* and found that there was a time-related recognition process; although freshly isolated neutrophils were not recognised by monocyte-derived macrophages, populations of neutrophils aging in culture *ex vivo* became recognisable to macrophages in a time-related fashion. It was possible to demonstrate that monocytes, separated from human peripheral blood needed to undergo a maturation process in order to recognise "aged" neutrophils. The mechanisms whereby aging neutrophils became recognised as "non-self" were not, however, identified. Further investigation by Haslett and Savill indicated that macrophage recognition of aged neutrophils was related to a change in neutrophil morphology known as "apoptosis" and which is described in more detail below.

1.3 Mechanisms of cell death

Mechanisms of cell death and removal have been of interest to many scientists especially those concerned with the kinetics of tissue "turnover", tumor growth and embryonic remodelling. It had been previously assumed that cell

death inevitably occurred by necrosis. During necrosis the cell swells prior to rupture of nuclear, organelle and plasma membranes, all basophilia is then lost leaving a faintly stained cell "ghost"; the swollen cytoplasm also loses its basophilia and cell boundaries become indistinct. Necrosis affects large numbers of contiguous cells and, possibly as a result of disgorgement of proinflammatory lysosomal enzymic contents, exudative inflammation develops in the adjoining viable tissue. It appears that one of the critical events leading to necrosis is loss of cellular volume homeostasis. Altered membrane permeability is implicated by potassium loss, entry of sodium and a fall in membrane potential (Prieto et al., 1967; Laiho and Trump, 1974; Jennings et al., 1975) and the cell is no longer able to exclude vital dyes such as trypan blue. Loss of the membrane pump functions may be related to the fall in intracellular ATP levels. Necrosis is usually a response to an obvious insult to the cell such as high toxin concentrations (McDowell, 1972), extremes of temperature (Buckley, 1972), extremes of pH (Kerr et al., 1974) or "attack" by complement components (Hawkins et al., 1972).

However, an alternative type of cell death has also been described; this occurs mainly in situations where death leads to a predictable or programmed removal of unwanted cells such as during embryogenic remodelling or thymic involution and may serve as a balance to mitosis in regulating the size of animal tissues. This mode of cell death has been termed "programmed cell death" or "apoptosis" (Kerr et al., 1972¹). Although apoptosis may occur in response to injury, such as low concentrations of toxins, it tends to be seen where "physiological death" is occurring. It normally affects scattered individual cells which are then phagocytosed and digested by cells in the surrounding tissue but, even when large fields of cells undergo this fate, apoptosis does not appear to initiate an inflammatory response. Apoptosis is a feature of many diverse processes including embryonic remodelling (Ballard

¹ The term "apoptosis" was devised by Prof. James Cormack of the Department of Greek, Aberdeen University at the request of Kerr et al. In Greek "apoptosis" (the 2nd p being silent) is the "dropping off" or "falling off" of petals from flowers or leaves from trees.

and Holt, 1968; Young, 1984), involution of endocrine-dependent organs after withdrawal of trophic hormones (Hopwood and Levison, 1976), cell death in antibody-dependent lymphocytotoxicity (Stacey et al., 1985), cell death and removal in tumors (Wyllie, 1985), T-cell death in infectious mononucleosis (Moss et al., 1985) and, more recently, focal crypt epithelial cell apoptosis of the rectum is reported to be a characteristic pathological feature in AIDS (Kotler et al., 1986).

Immature lymphocytes undergo apoptosis in response to glucocorticoid hormones, calcium ionophores and antibodies to the CD3-T-cell receptor complex (Smith et al., 1989; McConkey et al., 1989; Shi et al., 1989). In contrast to immature T cells (which undergo apoptosis) mature T cells proliferate when treated with anti-CD3 antibodies (Bluestone et al., 1987). Any cells carrying self-reactive receptors need to be eliminated, suppressed or inactivated to avoid destructive autoimmunity, and it has been proposed that anti-CD3 antibody-induced apoptosis of immature T cells may represent the mechanism which produces clonal deletion and consequently self-tolerance (Smith et al., 1989).

There are characteristic morphological features in cells undergoing apoptosis. At the light microscopical level these include; chromatin condensation, cytoplasmic contraction and vacuolation of the cytoplasm (Kerr et al., 1972). The nuclear condensation appears, initially, as deep basophilia, known to some observers as *pyknosis*; later the nuclear buds appear as discrete basophilic masses, giving rise to the histological appearance of *karyorrhexis*. Cells undergoing necrosis can look very similar ie. initially the chromatin often appears fairly uniformly compacted thus giving the appearance of *pyknosis*; later, when the swelling nucleus ruptures its membrane, the marginated chromatin masses may appear as discrete masses as in *karyorrhexis*. However, the differences between necrosis and apoptosis are easily resolved by electron microscopy which demonstrates chromatin aggregation, nucleolar prominence, cytoplasmic vacuolation and that the membrane and organelles, including granules, remain intact in the apoptotic

cell which is in stark contrast to cells undergoing necrosis (Wyllie et al., 1980).

Unlike cells undergoing necrosis, apoptotic cells do not exhibit increased membrane permeability, as shown by the ability to exclude vital dyes such as trypan blue. The integrity of the cell membrane is retained, organelles remain intact and there are no gross changes in ATP levels (Wyllie et al., 1980).

Wyllie has studied the mechanisms of apoptosis in corticosteroid treated thymocytes (Wyllie, 1980; Wyllie et al., 1984) and has reported that there are highly characteristic changes in the nuclear chromatin of such cells. With the onset of morphologically recognisable apoptosis there was a progressive increase in the proportion of extracted chromatin of a molecular weight too low to be sedimented at 25000g. Electrophoresis of DNA extracted from the unsedimented low molecular weight chromatin resulted in a "ladder" pattern indicating internucleosomal cleavage, the amount of DNA in each fragment being a multiple of 180 base pairs. A very similar pattern of chromatin cleavage is induced by bacterial nucleases and no such pattern of chromatin cleavage is seen in necrotic cells (Russell, 1983). Such biochemical changes are reflected by apparently simultaneous ultrastructural changes. The nuclear chromatin marginates in large coarse granular aggregates which join across the whole nucleus to form a "cap"; later, "buds" of membrane-bound, condensed chromatin may separate from the nucleus. Wyllie (1980) concluded that "within glucocorticoid-treated thymocytes there is an endonuclease activity which excises well organised nucleosome chains from chromatin". He, therefore, hypothesised the existence of a class of endonuclease whose activation is associated with the programmed destruction of the genome resulting in controlled cell death and making removal of the intact cell possible.

Unlike necrosis, apoptosis does not itself appear to cause tissue injury or to evoke an inflammatory response even where massive apoptosis occurs during

embryological tissue remodelling. This analogy suggested that its occurrence in aging neutrophils may represent a neutrophil removal mechanism which occurs without inciting further inflammation or tissue scarring.

1.4 Programmed cell death (apoptosis) in neutrophils leads to recognition by macrophages

Following the observations of Newman et al. (1982), Haslett investigated the changes leading to aged neutrophil recognition by macrophages. Working in Henson's laboratory, he developed a method of neutrophil isolation from peripheral blood that resulted in minimal activation of the neutrophils (Haslett et al., 1985) (as assessed by a number of *in vitro* and *in vivo* criteria) and minimal cell loss by clumping, adherence to the incubation vessel or necrosis (as assessed by trypan blue exclusion; Haslett et al., 1987; Savill et al., 1989a), problems which had been experienced with former cell isolation techniques. He observed that the numbers of cells exhibiting features of apoptotic morphology by light microscopy increased with time over 24h of culture. During this time <2% of the neutrophils failed to exclude the vital dye, trypan blue. Associated with the increased proportion of cells with features of apoptosis, there was minimal spontaneous release of lactate dehydrogenase (LDH), a cytosolic marker enzyme, or myeloperoxidase (MPO), a granule marker enzyme (Haslett et al., 1987; Savill et al., 1989a) and electron micrographs of the aging neutrophils showed intact nuclear, organelle and plasma membranes. The demonstration of time-related increases in low molecular weight chromatin which on gel electrophoresis gave rise to a 180 base pair "ladder" indicative of endogenous endonuclease digestion (Haslett et al., 1987; Savill et al., 1989a) confirmed apoptosis. These observations demonstrated that neutrophils undergo apoptosis *in vitro* and that light microscopy can be used to reliably recognise apoptosis in neutrophil

cultures.

Haslett et al., (1987) noted that macrophage recognition of aged neutrophils increased *pari passu* with the proportion of neutrophils undergoing apoptosis. He, therefore, hypothesised that the apoptotic subpopulation of aged neutrophils was that recognised by macrophages.

Histological studies of tissues undergoing apoptosis suggest that apoptotic cells are swiftly recognised and phagocytosed by mechanisms which do not appear to initiate an inflammatory response (Wyllie et al., 1980). This implies that phagocytosis and degradation of apoptotic neutrophils by macrophages may represent an injury-limiting mechanism for the disposal of *senescent* or *effete* neutrophils, which would tend to favour termination of inflammation by resolution. The *in vitro* observations of the rapidity of macrophage recognition and ingestion of apoptotic neutrophils (Savill et al., 1989a) and the observation by Slater (1989) that ingested neutrophils are rapidly degraded, would support this concept. *In vivo*, however, it is likely that monocytes that have migrated to inflamed sites require 24-48h to mature into inflammatory macrophages with the capacity to ingest aged neutrophils (Newman et al., 1982). Furthermore, in embryological remodelling, although apoptotic cells are most often observed within macrophages, other cells such as endothelial cells and epithelial cells also appear to engulf apoptotic bodies (Wyllie et al., 1980). These observations prompted Haslett to explore the possibility that other local cells could provide a macrophage "back-up" system. This might allow inflammation to be kept "under control" during the period of monocyte immaturity or at times when the macrophage "disposal system" has been overwhelmed. Under identical *in vitro* conditions, Haslett examined cell interactions between monolayers of human epithelial cells, endothelial cells and fibroblasts and fresh and "aged" neutrophils. The results indicated that endothelial and epidermal cells were unable to recognise neutrophils, but that fibroblasts (like the macrophages) were able to recognise aged, but not freshly isolated, neutrophils. Since most tissues have a large complement of fibroblasts (Crapo et al., 1982), it is reasonable to suppose

that they could provide an alternative, or ancillary neutrophil removal route.

1.5 The characteristics of fibroblasts

Fibroblasts (*fibra*, fibre and *blastos*, germ) are present in almost every organ of the body (Crapo et al., 1982). The cell was so named because original observers believed that this cell was responsible for the manufacture of the fibres of the intercellular matrix. Nowadays it is generally accepted that fibroblasts are, indeed, responsible for production of fibres and also for most of the amorphous ground substance. Fibroblasts, like many other cells, are derived from mesenchymal cells. Some undifferentiated mesenchymal cells persist in connective tissue and morphologically resemble young fibroblasts. Fibroblasts exist in tissues in different stages of differentiation and may be referred to as "young" and "old" fibroblasts. The term "fibrocyte" is often applied to the latter type of cell.

In conventional histological sections of connective tissue the fibroblast is a spindle-shaped cell with tapering eosinophilic cytoplasmic extensions. Its nucleus is, generally, elliptical, containing two or four nucleoli, and has sparse and scattered chromatin. In very dense connective tissue, fibroblasts are compressed between thick bundles of collagen, their nuclei are more elongated and darkly stained and their cell borders are difficult to see by light microscopy.

The fibroblast shape can vary from the simple elongated spindle depending on the nature of the substrate to which the cell attaches, the space available for movement over the substrate and by stimulation for division. fibroblasts are capable of serial replication *in vivo* and *in vitro*; when a cell enters the mitotic phase, it loosens its attachment to a surface and becomes spherical.

After telophase, the daughter cells flatten onto available surfaces and once more assume extended forms.

The typical fibroblast has a well-developed granular endoplasmic reticulum on which the precursor polypeptides of collagen, elastin, proteoglycans and glycoproteins are synthesised. Fibroblasts contain the usual complement of organelles and inclusions. The mitochondria tend to be long and slender, and fat droplets and primary and secondary lysosomes are occasionally present. Microtubules are present and appear to be required for translocation of secretory vesicular elements (Goldberg and Rabinovitch, 1988). Bundles of actin microfilaments are also prominent; and are implicated in general cellular motility. The cisternae of the rough endoplasmic reticulum contain granular material, which is presumably the soluble precursor molecules which have been precipitated during fixation and staining. The molecules in the cisternae are transported to the well-defined Golgi for chemical modification. Molecules to be secreted pass via smooth-surfaced secretory vesicles to the plasma membrane. The secretory vesicles fuse with the plasma membrane to release their contents into the extracellular space. Further biochemical modifications of the secreted molecules may follow and insoluble collagen and elastic fibres are deposited in the extracellular space close to the cell surface (Goldberg and Green, 1964; Goldberg and Rabinovitch, 1988).

The main role of the fibroblast in tissue homeostasis is the synthesis and degradation (turnover) of connective tissue. Collagen is a protein that forms extracellular fibres or networks of basement membrane. Depending on the structural requirements of a particular tissue, these fibres may be abundant (dense connective tissue) or sparse (loose connective tissue) and may be arranged in distinctive patterns. For example, in tendons and ligaments, the collagen fibres are thick and long and are grouped into large parallel bundles to provide sufficient tensile strength while, in contrast, in such tubular expansile structures as blood vessels, intestine and glandular ducts, collagen fibres tend to be wrapped helically around the long axes (Epstein and

Munderloh, 1975). Inflammatory diseases are often associated with proliferation of fibroblasts and changes in collagen structure as has been reported in interstitial pulmonary fibrosis (Seyer et al., 1976), scleroderma (Fleischmejer et al., 1978) and in fibroblast cultures from gingiva affected by periodontitis (Narayanan and Page, 1976).

1.6. The fibroblast as a "facultative" phagocyte

Fibroblasts have often been referred to as "facultative" phagocytes (meaning that they do not always perform phagocytosis), and they have been reported to phagocytose a variety of particles both *in vitro* and *in vivo* (Rabinovitch, 1970). Generally, however, fibroblasts are considered to be less phagocytic than the "obligatory" or "professional" phagocytes; neutrophils and macrophages (Van Furth, 1970). However, although the ability of fibroblasts to phagocytose particulate material is considered to be less than the phagocytic capability of cells of the monocyte/macrophage lines (Van Furth, 1970), studies performed by Grinnell (1984) suggest that this is not always the case. He observed that baby hamster kidney fibroblasts are able to phagocytose large numbers of 5.7µm fibronectin-coated latex beads, whereas previous studies by Bevilacqua et al. (1981) indicated that monocytes are unable to phagocytose fibronectin-coated particles. Grinnell (1984), therefore, suggested that fibroblasts may require particles to be coated with different opsonins than particles recognised by macrophages, for full expression of their phagocytic potential. Phagocytosis by fibroblasts has been reported in a number of other circumstances, including carmine phagocytosis (Vasiliev et al., 1975) and horseradish peroxidase clearance by synovial fibroblasts (Mitnik et al., 1978). In patients with macular amyloidosis (characterised by hyperpigmented areas on the skin of varying sizes which are associated with small amyloid deposits in the dermal papillae) the digesting action of fibroblasts is well developed and many melanosomes are taken into the cells

so that the cells resemble melanophages. Many cells that appeared to be macrophages by light microscopy were subsequently found to be fibroblasts when assessed by electron microscopy (Ishii et al., 1984). Phagocytosis of inert substances such as poly-l-lysine coated or UV irradiated latex beads, dextran sulphate, carbon particles and mycostatin by rabbit synovial fibroblasts (Werb and Reynolds, 1974) and latex bead phagocytosis by corneal fibroblasts (Berman et al., 1979) have also been described. Microscopic examination of rat skin after the degranulation of mast cells, or the injection of isolated mast cell granules (Tannenbaum et al., 1980) supports the possibility that fibroblasts have phagocytic roles *in vivo*.

Ultrastructural studies of early carrageenin-induced granulomas in guinea pigs and rabbits reveal a heterogeneous cell population consisting mostly of neutrophils, macrophages and fibroblasts (Perez-Tamayo, 1970a; Perez-Tamayo, 1970b). The granulomas were induced by injection of carrageenin solution into the subcutaneous tissues of the lower abdominal wall of the guinea pigs and rabbits. An electron-dense material (which they assumed to be carrageenin) present in the extracellular and intracellular compartments disappeared progressively, first from extracellular spaces and later from cells. In the early stages the electron-dense material was present in neutrophils, and after four days the electron-dense material was seen in macrophages. Parallel with increased collagen breakdown and dissolution, neutrophils and macrophages decreased in number while fibroblasts became predominant. Intracellular collagen fibrils were only observed within fibroblasts and collagen breakdown was observed in extracellular bundles with no obvious relationship to cells, although breakdown only occurred to any great extent when the cellular population consisted virtually solely of fibroblasts. This led to the assumption, by Perez-Tamayo, that the fibroblast was the major cell responsible for phagocytosis and breakdown of collagen. Thus there is clear evidence for a role of fibroblasts in phagocytosis and clearance of particles in the later stages of complex inflammatory responses, but the phagocytic recognition mechanisms concerned remain obscure.

1.6.1. The possible role of fibroblasts in neutrophil disposal

As considered above, a "back up" mechanism may be required for disposal of aged neutrophils at times when the monocytes are still immature or when the macrophages have become overwhelmed. From Haslett's preliminary data we inferred that fibroblasts may be able to perform this role. A possible analogous "back up" mechanism has been described by Mitnik et al. (1978) who observed the entry of horseradish peroxidase into synovium. At low concentrations of antigen, antigen was taken up predominantly by macrophages, whereas at higher concentrations both macrophages and fibroblast-like lining cells accumulated antigen.

1.7 Fibrosis in disease states

Normal fibroblasts synthesise collagen at a rate that is precisely regulated to meet the demands of the extracellular matrix, particularly during periods of development, differentiation and repair. This is a dynamic process: the net result depending on the balance between synthesis and degradation of several matrix components, collagen in particular.

In the normal lung there is extremely rapid turnover of both collagen and non-collagen proteins (9.9%/day compared to 3.3%/day in skeletal muscle, 4.0%/day in skin and 4.7%/day in the heart; Laurent, 1982). Laurent and McAnulty (1983) speculate such rapid turnover reflects the vital function of the lung in gas exchange and the consequent need for rapid adaptation in response to injury. Many lung diseases have been described where inflammation is associated with the deposition of excessive amounts of fibrous tissue. Recovery of increased numbers of neutrophils from lavage fluid has been associated with worse prognosis of the disease as has increased

hyaluronan and type III procollagen peptide since they reflect increased fibrosis (Bjerner et al., 1989). Fibrosis may result in lung malfunction by at least two mechanisms: firstly, in the interstitium it may interfere with gas exchange since gases have a further distance across which to perfuse and they pass more slowly through the denser fibrotic tissue; and secondly, the mechanical properties of the lung are affected since fibrosis results in a loss in elasticity from the lung which, in turn, restricts the expansion and contraction of the lung during inspiration and expiration. The result is breathlessness of the patients as they struggle to maintain adequate oxygenation of the tissues. The destruction of the lung parenchyma in idiopathic pulmonary fibrosis (IPF) appears to be initiated by inflammatory cells infiltrating the alveolar-capillary membrane and small airways (Palmer and Reed, 1974). The injury to parenchymal cells is associated with proliferation of fibroblasts and increased deposition of collagen in the interstitial space. In fibrosing lung diseases the fibrosis is not always limited to the interstitium as fibroblasts may migrate through breaks in the basement membrane of the alveolar epithelium and promote intraalveolar fibrosis (Raghu et al., 1985).

Experimental models of pulmonary fibrosis have been produced by intratracheal instillation of the antineoplastic agent, bleomycin, in a variety of laboratory animals including hamsters (Snider et al., 1978), rats (Thrall et al., 1979) and rabbits (Laurent et al., 1981). The histological changes in this model have been extensively reported and involve the appearance of inflammatory cells followed later by the appearance of fibrous tissue. Laurent and McAnulty (1983) studied collagen turnover in the rabbit model and observed rapid changes in the rates of both synthesis and degradation of collagen during bleomycin-induced pulmonary fibrosis. In particular, they noted an increased rate of synthesis and a decreased rate of newly synthesised collagen degradation, *in vivo*, which contribute to rapid accumulation of collagen in the early stages of pulmonary fibrosis. Shen et al. (1988) also found close correlation in bleomycin injury between initial injury and inflammation and the final fibrotic result.

In the healthy heart, fibroblasts synthesise fibrillar collagens which support and tether myocytes to maintain their alignment (Borg and Caulfield, 1981), while their respective tensile strength and resilience resist deformation, maintain shape and thickness, prevent rupture and contribute to the passive and active stiffness of the myocardium (Weber et al., 1987). An acquired or congenital defect in this collagen network can lead to abnormalities in cardiac structure, mechanics or valve function. The early inflammatory response and alterations in collagen metabolism to myocardial cell loss were monitored by Judd and Wexler (1975) in the dog. Cellular infiltration, including fibroblasts, was at its peak on day 3. Increased collagen synthesis, fibroblast proliferation and structural and biochemical remodelling of the matrix are seen subsequently. An early increase in collagen synthesis prior to fibroblast proliferation suggests that collagen synthesis in existing fibroblasts may increase prior to additional fibroblast recruitment. This fibrillar collagen matrix of the heart is largely involved in the hypertrophy that accompanies chronic pressure overload (ie. aortic stenosis and renovascular and essential hypertension).

In normal human liver, hepatocytes, adipocytes and endothelial cells produce components of the extracellular matrix. In fibrosis, hepatocytes which normally do not synthesise collagens may produce types III and IV collagen (Clement et al., 1986). Fibrosis has been described early in liver disease, be it of toxic, viral or parasitic origin, and can be spontaneously reversible (Sherlock, 1989). Pathological fibrosis in the liver represents excessive or disordered production of hepatic extracellular matrix which contains collagen and a variety of large glycoproteins and proteoglycans (Arenson and Bissell, 1987).

Current concepts of the pathophysiologic mechanisms leading to alterations of connective tissue in fibrotic disease states, including IPF, suggest that fibroblast proliferation and changes in the rates of collagen production and degradation play an important role.

Factors controlling fibroblast proliferation and changes in collagen synthesis and degradation

Initial studies of fibroblast growth were performed by Pledger et al. (1977) on rodent fibroblasts and immortalised human fibroblast cell lines, which had been growth arrested by high cell density. They described a fibroblast cell cycle involving two discrete stages controlled by separate serum components. The first stage, termed **competence**, was found to be a prerequisite for entry into the growth cycle. Competence was conferred by platelet-derived growth factor (PDGF) and later Stiles et al. (1979) found that fibroblast growth factor (FGF) also conferred competence. The time required for a population to attain competence was reported to be dependent on PDGF concentration, such that at high PDGF concentration the entire population rapidly becomes competent. The induction of competence was also found to be temperature dependent, occurring more quickly at 37°C than at 25°C or 4°C (Pledger et al., 1977). Later, competence was characterised by the accumulation of a large number of mRNA species including the protooncogene, c-myc (Cochran et al., 1983; Lau and Nathan, 1985) and the competent state has since been directly related to the presence of the c-myc gene product (Robertson, 1984). However, competent cells appeared unable to **progress** efficiently into S phase unless they were incubated throughout Go/G1 with medium containing plasma (Pledger et al., 1977) or a progression factor such as epidermal growth factor (EGF), insulin-like growth factors (IGFs), stomatomedin A or C or a number of other mitogens (Stiles et al., 1979).

It is unclear which cells release the molecules which account for the enhanced fibroblast growth-promoting activity in IPF bronchoalveolar lavage fluid (BALF); however, the most likely candidate is the macrophage. Firstly, alveolar macrophages comprise 6% of the normal lung and their number are markedly increased in IPF (Crystal et al., 1984). Secondly, alveolar macrophages are able to release molecules that cause fibroblast proliferation (Leibovich and Ross, 1976; Glen and Ross, 1981; Shimokada et al., 1985). Both competence-factors and growth inhibitory factors are released in large

amounts by alveolar macrophages in IPF (see table 1.1). Among the competence- type growth factors are fibronectin (Rennard et al., 1981) and PDGF (Martinet et al., 1987). Finally, alveolar macrophage release of a progression-type fibroblast growth factor is markedly increased in IPF. This growth factor, alveolar macrophage-derived growth factor, in the presence of a competence factor can stimulate fibroblast replication in the absence of serum (Bitterman et al., 1983). In addition to alveolar macrophages, other possible sources of BALF fibroblast growth factors include endothelial cells, lymphocytes, epithelial cells and plasma-derived molecules (Collins et al., 1985; Cantin et al., 1988).

Collagen synthesis involves a number of co-translational and post-translational events, many of which are unique to collagens and a few other proteins with collagen-like amino acid sequences. The post-translational processing takes place in two stages. Intracellular modifications, together with ribosomal synthesis of polypeptide chains, to form triple-helical procollagen molecules. Extracellular processing then converts these molecules into collagens and assembles collagen molecules into stable, cross-linked fibrils (Kivirikko and Majamaa, 1985).

Collagen degradation can occur both intracellularly and extracellularly. Intracellular degradation has been proposed to occur in two sites: within the lysosome or within the cisternae of the endoplasmic reticulum or Golgi apparatus (Berg et al., 1980). Fibroblasts release collagenase to degrade extracellular collagens, but other cells may also play a role in extracellular degradation of collagen, particularly in disease states. A variety of cells, including macrophages and neutrophils, are capable of phagocytosing collagens and releasing proteases which degrade collagens extracellularly or agents capable of stimulating fibroblast-mediated degradation (Nimni, 1983). Modulation of collagen and collagenase production by various products of the immune system and inflammatory cells is also presented in table 1.1.

TABLE 1.1 Fibroblast growth and matrix production modulation by products of the immune system and inflammatory cells.

	Collagen production	Hyaluronic acid production	Collagenase production	Proliferation
100,000-170,000MW lymphokine (CPF)	stimulates			
60,000MW lymphokine		stimulates		stimulates
Transforming growth factor β	stimulates	stimulates		stimulates
Interleukin-1 α and β	stimulates	stimulates	stimulates	stimulates
Gamma-interferon	inhibits			inhibits and stimulates
Platelet-derived growth factor				stimulates
Prostaglandin E_2				inhibits

CPF=Collagen production factor

Modified from Postlethwaite and Kang, 1988.

Thus the fibroblast can contribute to the scarring process by proliferation and laying down matrix, but can also contribute to degradation of matrix proteins and even removal of particles at the inflamed site. The links between inflammatory cells and fibroblasts and their responses, whether direct or indirect (via cytokines), are likely to represent an important area of research in inflammatory disease.

1.8 Aims of the project

Fibroblast recognition of aged neutrophils could represent a neutrophil disposal mechanism during normal resolution of inflammation which is an alternative to neutrophil phagocytosis by the macrophage. Investigation of the mechanisms involved in this recognition may help in the understanding of the links between inflammatory cells and fibroblasts in tissue scarring associated with chronic inflammatory disease. The purpose of this project was to answer two main questions about aged neutrophil recognition by fibroblasts: (1) Is recognition of aged neutrophils by fibroblasts related to neutrophil apoptosis as is the case for macrophage recognition of aged neutrophils? (2) What is the mechanism of recognition of aged neutrophils by fibroblasts and does it resemble aged neutrophil recognition by macrophages?

1.8.1. Is recognition of neutrophils by fibroblasts related to apoptosis?

Macrophage recognition of aged neutrophils is determined by neutrophil apoptosis (Savill et al., 1989a). It was found that macrophage recognition of

aged neutrophils increased in parallel with the percentage of neutrophils showing light microscopical features of apoptosis (**Apoptotic index**). The direct link between neutrophil apoptosis and macrophage recognition was demonstrated by separating 24h aged neutrophils by counterflow centrifugation (chapter 2 section 2.4) into apoptotic and non-apoptotic populations and confirming that macrophages recognised the apoptotic neutrophils but were unable to recognise the non-apoptotic aged neutrophils (Savill et al., 1989a). I was, therefore, interested in studying in fibroblasts the relationship between neutrophil apoptosis and fibroblast recognition.

This question is addressed in chapters 3 and 4 and the methods used are described in chapter 2.

1.8.2. What are the cell-surface mechanisms involved in fibroblast recognition of aged neutrophils?

This question is addressed in chapters 3 to 6 and the methods used are described in chapter 2.

The actual surface changes on the neutrophil that accompany apoptosis have not been fully elucidated. It was first thought, by Duvall et al. (1985), that exposure of increased numbers of N-acetyl glucosamine residues on the apoptotic cell surface may lead to its recognition, by a lectin-sugar mechanism, as senescent-self. This conclusion was based on a study in which Duvall et al. demonstrated that recognition of apoptotic rodent thymocytes by rodent macrophages at 4°C could be inhibited by the presence of (D+) N-acetyl glucosamine. Preincubation studies localised this effect to the macrophage surface (since preincubation of macrophages, but not apoptotic thymocytes, with the sugar inhibited recognition). Parallel studies with gold-conjugated lectins specific for such carbohydrate moieties indicated that

exposure of a galactose, N-acetylglucosamine and N-acetylgalactosamine-inhibitable lectin on the macrophage surface was, in fact, not the case (Morris et al., 1984). This led to the investigation, by Savill, of the cell-surface mechanisms involved in macrophage recognition of aged neutrophils and subsequently to the studies of fibroblast recognition of aged neutrophils detailed in my thesis.

However, since the fibroblast has not been widely recognised as a "professional" phagocyte, fibroblast surface recognition processes have been poorly documented. In other cell-cell recognition systems a variety of adhesive processes, of possible relevance to the fibroblast/aged neutrophil experiments, have been described. These are now considered below:

1.9 Adhesive molecules in cell-cell recognition

Numerous types of adhesive molecule have been described, of which lectins, the fibroblast mannose-6-phosphate receptor, selectins and integrins are described below as they are relevant to my studies.

1.9.1. Lectins

Cell surface-expressed carbohydrates or carbohydrate moieties are present on both prokaryotic and eukaryotic cells and are involved in recognition phenomena in a whole variety of plant and animal species. Carbohydrate molecules are recognised and complementary binding interactions brought about by protein or glycoprotein molecules known as lectins. The term was

originally used to describe carbohydrate-recognising structures from plants such as phytohaemagglutinin from red kidney beans and concanavalin A from jack beans (Goldstein et al., 1980).

The main characteristics of lectins are their ability to bind sugars, agglutinate cells and stimulate lymphocytes. The cell-membrane carbohydrates recognised by lectins are made up of nine basic subunits:- four hexoses (glucose, galactose, mannose and fucose), two pentoses (arabinose and xylose), two amino sugars (N-acetylglucosamine and N-acetylgalactosamine) and sialic acid. Most lectins interact with one sugar specifically, but for some the specificity is broader involving a number of related sugars. Lectins are usually made up of subunits and occur as a group of closely related glycoproteins or isolectins with similar chemical and biological characteristics (Weir, 1980)

Recognition based on carbohydrates has been described for a number of primitive species including the slime mould *Dictyostelium discoideum* (Rosen et al., 1973). At the start of its life cycle, the slime mould is an amoeboid vegetative form but, when its food is exhausted, it becomes a cohesive multicellular sessile slug. The cohesiveness is due to lectins (called discoidins) with a specificity for N-acetylgalactosamine. Lectin-like activity has been described in invertebrates such as lobsters, snails, horseshoe crabs and bivalve clams and it is likely that both the opsonins which promote phagocytosis and the red-cell agglutinating activity in invertebrate haemolymph depend on recognition of N-acetylglucosamine, N-acetylgalactosamine and N-acetylneuraminic acid (Weir, 1980).

In more highly developed species carbohydrate-dependent cell interaction has been observed in embryo neural retinal cells (Rutishauser et al., 1976) but in this case the recognition molecules involved are cell-type specific not species specific. However, it has been shown that surface carbohydrate recognition is species specific in sperm-egg interactions in the ascidian *Ciona intestinalis*, with fucose playing a key role in binding and fertilisation (Rosati and De

Santis, 1980). Lectins appear to be components of intracellular as well as extracellular membranes and a role in regulation and segregation has been proposed for lysosomal enzymes which are glycosylated (Lloyd, 1977; Bowles, 1979).

The lectins have been divided into a number of categories; the C-type (Ca^{2+} -dependent) animal lectins which are structurally related to the asialoglycoprotein receptor; the S-type (thiol-dependent) animal lectins which are widely distributed; fibronectin and laminin, which mediate the interactions of cells with the extracellular matrix (Yamada, 1983); serum immunoglobulins which have specificity for carbohydrates (Feizi and Childs, 1987); the mannose-phosphate receptor, which directs transport of proteins to lysosomes (Kornfeld, 1986); viral haemagglutinins (Wiley and Skehel, 1987); and serum amyloid protein, a member of the pentraxin family (Pepys and Baltz, 1983).

The involvement of a lectin interaction in apoptotic thymocyte recognition by macrophages has been postulated by Duvall et al. (1985) when they observed that apoptotic thymocyte recognition by macrophages (at 4°C) was inhibitable by 10mM galactose, N-acetyl galactosamine and N-acetylglucosamine. This inhibitory activity has been "localised" to the macrophage surface by sugar preincubation studies (Duvall et al., 1985).

Another type of lectin which is of relevance to this project has been isolated from the serum of rats as well as humans (Drickamer et al., 1986). This protein is a member of the mannose-binding proteins which are C-type lectins. The role of this protein has not yet been elucidated, but its synthesis is upregulated in response to heat shock and γ inteferon which suggests a role in natural immunity (Ezekowitz and Stahl, 1988). Binding of this mannose-binding protein is divalent cation dependent and it is thought to bind to mannose residues on cell surfaces (Ezekowitz and Stahl, 1988).

1.9.2 The fibroblast mannose-6-phosphate receptor

Cultured fibroblasts internalise lysosomal enzymes by receptor-mediated endocytosis (Kaplan et al., 1977). Cell surface receptors on fibroblasts recognise a phosphorylated carbohydrate on lysosomal enzymes, most likely a mannose-6-phosphate (M-6-P) residue (Kaplan et al., 1977). Hepatocytes possess receptors with the same ligand specificity (Ullrich et al., 1979). Hepatocytes also interact with terminal galactose (Ullrich et al., 1979), N-acetyl glucosamine (Steer et al., 1978) and mannose (Ullrich et al., 1979) residues on lysosomal enzymes. Macrophages possess a receptor system that interacts with both N-acetyl glucosamine and mannose residues on lysosomal enzymes (Steer et al., 1978) and it is also thought that they possess a M-6-P receptor similar to that of the fibroblast (Jessup and Dean, 1982). A M-6-P receptor has been purified from a variety of tissues and appears to be a single glycoprotein with an apparent molecular weight of 215,000 (Sahagian et al., 1981; Steiner and Rome, 1982), although there is one report indicating that the receptor may consist of smaller subunits (Mitchell et al., 1984). This receptor binds acid hydrolases independent of divalent cations and it has been detected in the Golgi complex, coated vesicles, endosomes and the plasma membrane (Sahagian, 1984). The various pools of the receptor appear to be in rapid equilibrium, since exogenously added antireceptor antibodies quickly interact with all of the intracellular receptor (90% of the total) (Sahagian, 1984; Gartung et al., 1985). A cation independent M-6-P receptor, with a molecular weight of 46,000, has also been reported (Hoflack and Kornfeld, 1985) which is widely distributed among cell types, and consequently, most cells contain both M-6-P receptors. Both receptors bind ligands tightly at neutral pH and release them at pH5 or lower, which is a property of many receptors which deliver or discharge ligands in acidified compartments eg. endosomes and lysosomes.

1.9.3. Selectins

This new family of adhesive proteins is characterised by a structure consisting of a lectin domain, an EGF-like domain, a series of consensus repeats homologous to complement regulatory proteins, a transmembrane region and a small cytoplasmic domain (Johnston et al., 1989; Bevilacqua et al., 1989; Stoolman, 1989). The members of this family are platelet activation dependent granule-external membrane protein (PADGEM; also known as GMP-140), endothelial leucocyte adhesion molecule-1 (ELAM-1), MEL-14 antigen (the murine lymphocyte homing receptor) and DREG-56 antigen (the human equivalent of MEL-14 antigen).

PADGEM is an integral membrane protein of the alpha granules of platelets and Weibel-Palade bodies of endothelial cells that is expressed on the plasma membrane following cell activation and granule secretion. PADGEM is thought to function as a heterotypic intercellular adhesion molecule mediating the binding of activated platelets to certain classes of leucocytes that bear a PADGEM recognition site (Larsen et al., 1989). The physiological role of PADGEM in activated platelets has not yet been elucidated, but it has been speculated that PADGEM may be responsible for the localisation of certain classes of leucocytes to an inflammatory site by binding of activated platelets to injured endothelial or subendothelial surfaces followed by subsequent binding of monocytes and neutrophils at these sites (Larsen et al., 1989).

The membrane glycoprotein ELAM-1 appears on the surface of human endothelial cells exposed to cytokines such as interleukin-1 and tumor necrosis factor (Bevilacqua et al., 1987). Upregulation reaches its maximum 4-6h after exposure to cytokines and requires new synthesis of RNA and protein. ELAM-1 mediates adhesion of neutrophils to cytokine-treated endothelium (Bevilacqua et al., 1985) and can be detected in endothelium *in vivo* at sites of inflammation (Cotran et al., 1986). However, the ELAM-1 recognition site on leucocytes has not yet been identified definitively.

MEL-14 antigen, on mouse lymphocytes was the first described by Gallatin et al. (1983). Normal lymphocytes circulate repeatedly through the blood stream, tissues and lymphatic system. This phenomenon has been termed "recirculation" and is thought to be critical to the normal function of the immune system. The lymphocytes enter the tissues from the bloodstream via the postcapillary venules. In rodents and humans, the postcapillary venules of peripheral lymph nodes (PN) and Peyer's patches (PP) have an unusual histological appearance characterised by a single layer of cuboidal endothelial cells resting on a basement membrane (termed high endothelial venules, HEV). Both human and rodent lymphocyte binding to rat HEV was found to be calcium dependent and trypsin sensitive at the lymphocyte surface (Stoolman et al., 1987). The experiments of Stoolman and Rosen (1983) indicated that a carbohydrate-binding molecule, with fucose/mannose specificity, might be involved in the adhesive interaction between lymphocytes and HEV. Another series of experiments showed that the *in vitro* attachments of both murine and human lymphocytes to PN HEV is selectively inhibited by millimolar levels of mannose-6-phosphate (M6P) or by structurally related fructose-1-phosphate, as well as by nanomolar levels of a M6P-rich yeast mannan core from *Hansenula nositii* phosphomannan, the polyphosphomonoester known as PPME (Slodki, et al., 1973; Stoolman et al., 1987).

A protein from the surface of murine lymphocytes (gp90^{MEL-14}) defined by the monoclonal antibody MEL-14 has been proposed by Gallatin et al. (1983) to be the homing receptor mediating the interaction of lymphocytes with PN HEV. They also reported that the antigenic determinant detected by the antibody MEL-14 is present on those normal lymphocytes and lymphoma cells which can bind to PN HEV, and is absent from lymphoid cells which either do not recognise PN HEV or bind only to PP HEV. While certain types of homotypic cell-cell recognition may be due to like-like interactions at the molecular level, this is unlikely in the lymphocyte-PN HEV model as PN HEV cells do not appear to express significant amounts of the MEL-14 antigen. The MEL-14 antigen has been independently cloned and sequenced

by Weissman (Siegelman et al., 1989) and Rosen (Lasky et al., 1989). Both groups arrived at identical amino acid sequences for this molecule and a schematic representation of its structure is shown in Fig. 1.1. The N-terminal motif of the MEL-14 antigen (residues 39 to 155) has a high degree of homology with a number of calcium-dependent animal C-type lectins (Drickamer and McCreary, 1987; Drickamer, 1988).

The strong homology in this domain between the MEL-14 antigen and the animal C-type lectins, and the fact that the isolated domain has been shown to have carbohydrate-binding activity in several instances (Halberg et al., 1988), strongly suggests that this region functions as a lectin domain in the MEL-14 antigen. Thus it is predicted that this motif accounts for the ability of lymphocytes to bind to PN HEV in a sugar- and calcium-dependent manner.

A similar antigen, gp100^{MEL-14}, on the neutrophil surface was subsequently described and has been implicated in neutrophil extravasation (Lewinsohn et al., 1987). Circulating and bone marrow neutrophils present in the body may be rapidly mobilised, migrate with speed to inflamed sites and rapidly kill bacteria. The first step in extravasation involves adhesive interactions of neutrophils with the vascular endothelium and this must be regulated to allow localisation of neutrophils only to inflammatory sites. The leucocyte integrins, LFA-1 (CD11a/18), Mac-1 (CD11b/18) and p150,95 (CD11c/18) (collectively referred to as the CD11/18 complex) are essential in the neutrophil extravasation process (Arfors et al., 1987; Kishimoto et al., 1989) and it appears that gp100^{MEL-14} is also involved. The neutrophil gp100^{MEL-14} and other molecules related to or identical to lymphocyte homing receptors are expressed at high levels by neutrophils, monocytes and other leucocytes. *Ex-vivo* and *in vivo* studies have shown that mAb MEL-14, raised against MEL-14 antigen, blocks both neutrophil and monocyte interactions with physiologically inflamed venules and inhibits leucocyte accumulation in the inflamed peritoneum and blocks binding of neutrophils to vascular endothelium within tissue sections of lymph nodes *in vitro* (Lewinsohn et

Fig. 1.1 Schematic representation of MEL-14 antigen

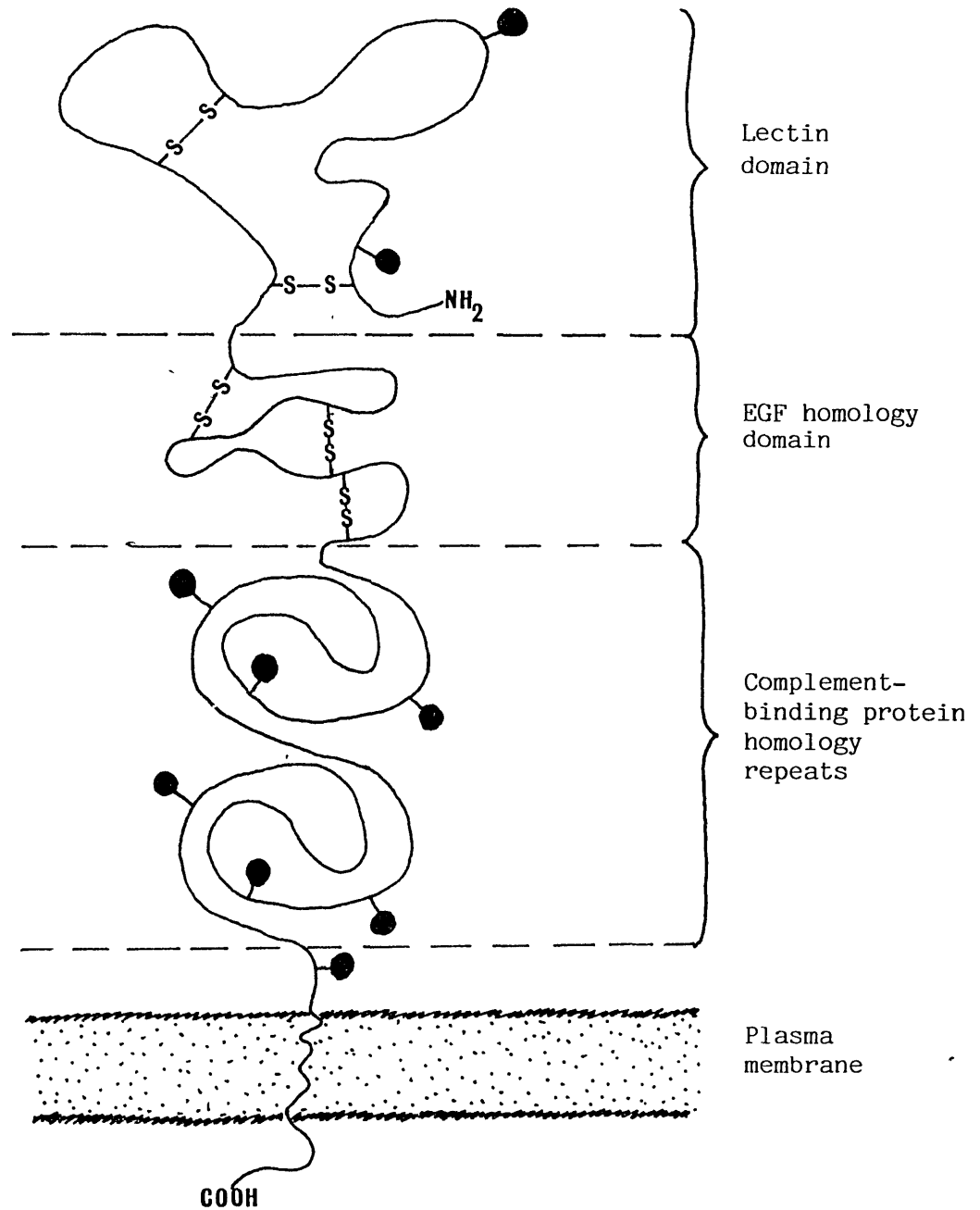


Fig. 1.1 Schematic representation of MEL-14 antigen. Putative N-glycosylation site, —●—. Disulphide bonding of cysteines may also occur in the complement-binding protein homology repeat domains.
Taken from Coombe and Rider, 1989.

al., 1987; Kishimoto et al., 1989; Hogg, 1989). It therefore seems likely that, *in vivo*, homing receptors and leucocyte integrins cooperate to mediate the extravasation of leucocytes. In contrast to Mac-1 activity, gp100^{MEL-14}-mediated binding does not require neutrophil activation. In fact, activation appears to decrease the gp100^{MEL-14} expression of circulating neutrophils to one-tenth (Kishimoto et al., 1989). Kishimoto et al. (1989) hypothesise that gp100^{MEL-14} maybe, in conjunction with as yet unidentified adhesion systems, mediates the initial adhesive event between circulating neutrophils and the vascular endothelium adjacent to inflammatory sites. Chemotactic factors released from the inflamed site may initiate activation in these adherent cells so down regulating gp100^{MEL-14} and engaging Mac-1 activity to allow extravasation to occur.

However, although both rodent and human lymphocytes have been studied, the mAb MEL-14 is essentially a murine antibody since it has been raised against the murine MEL-14 antigen. Activation of rodent neutrophils results in cleavage of gp100^{MEL-14} from the neutrophil surface (Kishimoto et al., 1989), so Kishimoto et al. (personal communication) activated human neutrophils and raised a monoclonal antibody against the "dregs" remaining in the culture medium. They named the resulting monoclonal antibody mAb DREG-56 and it is the human equivalent of mAb MEL-14. As such, mAb DREG-56 is of more relevance than mAb MEL-14 to the human fibroblast/aged neutrophil recognition mechanism studied in this project. The antigen and mAb were termed **DREG** primarily because the antigen is **down regulated** on the neutrophil surface following stimulation but also because the mAb was raised against the "**dregs**" remaining in the culture medium following stimulation of the human neutrophils.

Recently it has been claimed by Camerini et al. (1989) that Leu8/TQ1 is the human equivalent of the MEL-14 lymph node homing receptor, since it bears strong homology with the MEL-14 homing receptor described on rodent lymphocytes and has been shown to be shed upon activation of human

lymphocytes. If this is, indeed, the case then the DREG-56 antigen, against which the mAb DREG-56 is directed, bears the same relationship to Leu8/TQ1 as gp100^{MEL-14} on rodent neutrophils does to gp90^{MEL-14} on rodent lymphocytes.

A receptor has also been described that is involved in lymphocyte adhesion to PP HEV. The lymphocyte surface antigen involved in this adhesion process is known as the Hermes/CDw44/Pgp-1 antigen and an antibody known as the Hermes-1 antibody has been raised against it (Jalkanen et al., 1986a). Hermes-1 antigen is not, essentially, a selectin, but it bears some homology to the MEL-14 antigen so it is relevant to mention the Hermes-1 antigen in this section. The lymphocyte gp90^{Hermes} has been found to be present on some very diverse cell types including fibroblasts, Kupffer cells, epidermis, mesangial cells and alveolar lining epithelium (Picker et al., 1989). The monoclonal antibodies Hermes-1 and MEL-14 are thought to be crossreactive, apparently precipitating the same human lymphocyte 85-95kDa glycoprotein (Jalkanen et al., 1986b; Jalkanen et al., 1987). However, unlike the MEL-14 antigen, the broad distribution of the Hermes-1 antigen means that it is less specific. The Hermes-1 antigen, like the MEL-14 antigen, was cloned independently by two groups using different cloning strategies (Stamenkovic et al., 1989; Goldstein et al., 1989). Seed's and Butcher's groups arrived at essentially the same amino acid sequence and a schematic representation of the Hermes-1 antigen is shown in Fig. 1.2.

Preliminary studies suggest that the lymphocyte Hermes antigen can also interact with fibronectin and collagen (Picker et al., 1989) and these observations indicate that gp 90^{Hermes} may have several binding specificities, perhaps mediated by distinct binding domains. Hermes-defined cell adhesion molecules appear functionally heterogenous, and this heterogeneity could also contribute to or reflect specificity of function in diverse cell-cell or cell-matrix interactions.

Fig. 1.2 Schematic representation of Hermes-1 antigen

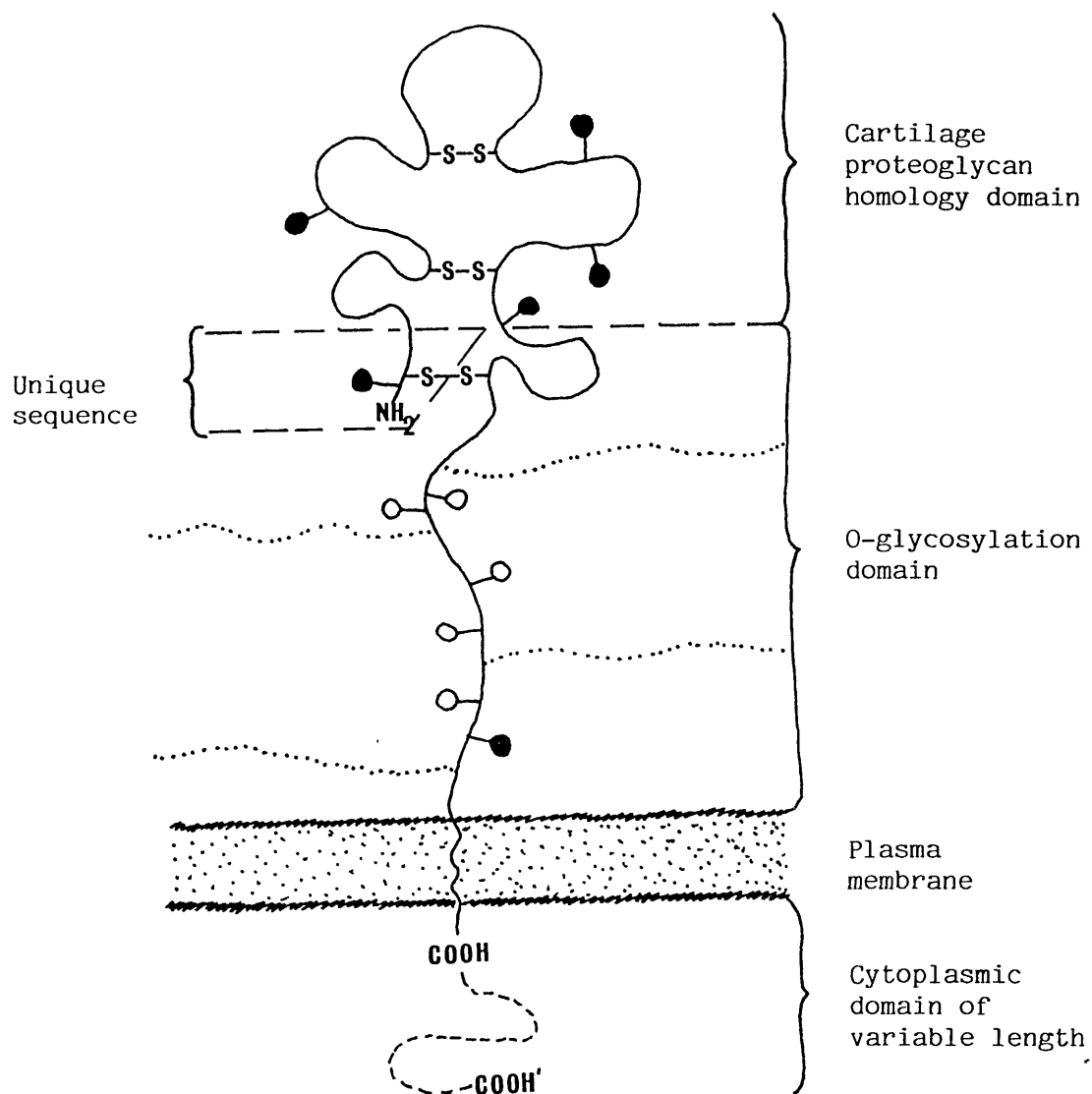


Fig. 1.2 Schematic representation of Hermes-1 antigen (CDw44). Putative N-glycosylation site, —●—; putative O-glycosylation site, —○—; chondroitin sulphate glycosaminoglycan chain in proteoglycan variant, ... Taken from Coombe and Rider, 1989

1.9.4. Integrins: the arg-gly-asp recognition signal and vitronectin receptor

Fibroblasts have been observed to adhere to fibronectin (Fn)-coated tissue culture plastic and to phagocytose Fn-coated latex beads (Grinnell, 1980) and morphological evidence has been presented indicating that fibroblasts either phagocytose or spread around Fn-coated beads depending on bead diameter (Grinnell, 1984). Biochemical studies with cytochalasin D, azide and iodoacetate, as well as temperature-dependence studies, showed similar responses of cell spreading and phagocytosis to these treatments and provided evidence for the hypothesis put forward by Grinnell (1984) that "cell spreading and phagocytosis are similar cell responses to different sized substrata". In fact, the fibroblasts when spreading on Fn-coated tissue culture plastic are thought to be trying to "phagocytose the tissue culture plate".

Fibronectin accumulates at sites of inflammation and wound healing and is known to be one of the fibroblast chemoattractants responsible for fibroblast recruitment to the site of inflammation (Postlethwaite et al., 1981). The Fn molecule is a 2500 amino acid polypeptide which is involved in regulating a number of important biological phenomena, including cell motility and adhesion, and Fn is synthesised by many cells, including macrophages, fibroblasts, endothelial cells and hepatocytes. Both of the plasma Fn chains (secreted by hepatocytes) lack the "extra domain" which is present in the "cellular" Fn (secreted by cells other than hepatocytes) and one chain is slightly smaller than the other because it has 64 fewer amino acids in the variable domain. Despite these differences, the function of the Fns are very similar and most workers have used plasma Fn in cell adhesion studies.

It has been found that a proteolytic fragment of the whole Fn molecule retained the ability to promote adhesion of fibroblasts and that a mouse monoclonal antibody raised against the fragment could inhibit adhesion of cells to surfaces coated with the fragment or whole molecule (Pierschbacher et

al., 1981). The amino acid sequence of the proteolytic fragment of the molecule bearing the cell binding domain was first determined by Pierschbacher et al. (1983). Further analysis of progressively smaller synthetic peptides narrowed the active site down to a tetrapeptide arginine-glycine-aspartic acid-serine (RGDS) (Pierschbacher and Ruoslahti, 1984). The recognition of this sequence is so specific that replacement of aspartic acid with glutamic acid (which bears the same charge as aspartic acid) in the peptide RGES is unable to inhibit binding.

Many adhesive proteins in extracellular matrices and in blood utilise the peptide RGD signal in their cell recognition site (Ruoslahti and Pierschbacher, 1987). These proteins include fibronectin (Fn), vitronectin (Vn), osteopontin, collagens, thrombospondin, fibrinogen and von Willebrand factor. The RGD sequences of each of these proteins are recognised by at least one member of a family of structurally related receptors termed integrins (the name being first applied to the chick fibroblast Fn receptor and considered suitable for the superfamily since the function of these proteins is integrating cells with matrix and with one another) which are heterodimeric proteins with two membrane-spanning subunits. Some receptors recognise the RGD-sequence of only one adhesion protein whereas others recognise whole groups of them. There are a number of receptor molecules that recognise RGD sequences in different proteins (Table 1.2). The RGD receptors may be of a common evolutionary origin and structurally related. This is suggested by their related specificities and their common features in SDS-PAGE (Pytela et al., 1986).

The integrins are a superfamily of adhesive transmembrane heterodimer molecules consisting of non-covalently linked α and β chains. At present this superfamily has been divided into three main families, each family being defined by a common β chain ($\beta_1, \beta_2, \beta_3$) although other members exist (Table 1.2). There are homologies within the various β chains and also within the various α chains, but there is no homology between α and β chains.

TABLE 1.2 The integrin receptor groups and their ligands

Family	Receptor	Ligand	RGD role	Distribution
<u>VLA proteins</u> (β_1)	VLA-1	Collagen, Laminin		
	VLA-2	Collagen, Laminin (endothelium only)	-/?	Widespread
	VLA-3	Fibronectin, Collagen, Laminin	- ?	Widespread
	VLA-4	Fibronectin (CS-1), VCAM-1	-	Lymphocytes, Monocytes?
	VLA-5	Fibronectin (RGD site)	+	Widespread
	VLA-6	Laminin (E8 fragment)	-	Widespread
<u>Leukocyte integrins</u> (β_2)				
<u>CD11/CD18 family</u>	LFA-1	ICAM-1 (CD54), ICAM-2	-	Leukocytes
	CR3/Mac-1	iC3b (Fibrinogen, factor X, LPS?)	+?	PMN, monocytes, LGL
	p150,95	iC3b	?	Mø, monocyte (PMN, some T cells)
<u>Cytoadhesins</u> (β_3)	GPIIb/IIIa	Fibrinogen, Fibronectin, von Willebrand's factor, Vitronectin, Thrombospondin	+	Megakaryocytes, platelets
	Vitronectin receptor (VnR)	Vitronectin, Fibrinogen, Fibronectin, von Willebrand's factor, Thrombospondin osteopontin, bone sialoprotein	+	Widespread
<u>Other</u>				
β_{3b}				Monocytes
β_5 or X		Vitronectin, (Fibronectin minimal binding)	+	Epithelial-type cells
β_s		GRGDSP peptide column	+	Phosphorylated osteosarcoma fibroblasts
β_4		?		Epithelium
β_3 related	Leukocyte Response Integrin	Fibronectin, Fibrinogen, Vitronectin von Willebrand's factor	+	PMN

(Hogg and Dransfield, Personal communication)

(1) The "very late antigens": VLA-1 (present on fibroblasts) and VLA-2 (a collagen receptor) were originally defined as heterodimers appearing 2-4 weeks after *in vitro* activation of T-cells (Hemler et al., 1983). Later VLA-3, VLA-4 and VLA-5 (the fibronectin receptor) from the same protein family were discovered (Hemler et al., 1987) and more recently a sixth member of the family (VLA-6) has also been isolated (Hemler et al., 1989). Expression of these structures is not limited to leucocytes, nearly all cell types, except granulocytes and red blood cells, express one or more of the VLA complexes.

(2) The leucocyte adhesion proteins: Lymphocyte function associated antigen (LFA-1), Mac-1 and p150,95. While LFA-1 is known to be involved in cell-cell interactions, Mac-1 and p150,95 are involved in binding opsonised particles and various solid substrates in addition to being implicated in intercellular adhesion. The molecule LFA-1 contributes to the binding of killer T cells to target cells and is important in T-lymphocyte adhesion to fibroblasts. This binding can be inhibited by anti-intercellular adhesion molecule-1 (ICAM-1) treatment of the fibroblasts or anti-LFA-1 treatment of the lymphocytes, suggesting a possible receptor-ligand interaction between the two molecules (Marlin and Springer, 1987). This interaction between LFA-1 and ICAM-1 was the first known intersection of the integrin and immunoglobulin superfamilies. More recently, LFA-1 has also been shown to interact with intercellular adhesion molecule-2 (ICAM-2) and this indicates that interaction between these superfamilies is more common than was originally thought (Staunton et al., 1989). The combination of integrin and immunoglobulin supergene family members in intercellular adhesion, but not in cell-matrix adhesion systems identified to date, is consistent with the involvement of many immunoglobulin family members in cell-cell recognition (Williams and Barclay, 1988) ; Mac-1, the macrophage receptor for complement iC3b, and p150,95, along with other receptors, appears to be important for the binding of leucocytes to endothelial cells as the leucocytes pass into tissues from the circulation (Springer et al., 1987; Arfors et al., 1987).

(3) Cytoadhesin subfamily: the vitronectin receptor and GPIIb/IIIa. These receptors are known as RGD-directed because they recognise the tripeptide sequence arginine-glycine-aspartic acid (RGD) on their respective ligands. The vitronectin receptor has been described on fibroblasts and is thought to be an integral membrane protein because it can be incorporated into liposomes (Pytela et al., 1985b). GPIIb/IIIa, a platelet integrin, is involved in the platelet adhesive response through its interaction with fibrinogen, fibronectin and von Willebrand factor (Pytela et al., 1985a).

Another characteristic of the integrins, which is apparent from the fact that the amino acid sequence of some sites on each integrin α chain bears some homology to the Ca^{2+} -binding sites of other proteins, is that ligand binding is divalent cation-dependent (Ruoslahti, 1988).

Macrophage recognition of apoptotic cells (both neutrophils and lymphocytes) has been shown to be mediated by the Vn receptor (VnR) (Savill et al., 1990). Previously the functions of the VnR were thought to be purely for cell anchorage (Charo et al., 1987; Cheresch and Harper, 1987), but the findings of Savill et al. indicate a novel role for the VnR in "self-senescent-self" intercellular recognition resulting in macrophage phagocytosis of apoptotic cells.

Vn is the active component of serum spreading factors and is the S-protein of the complement membrane attack complex (Jenne and Stanley, 1985). The VnR is present in various types of cells, including fibroblasts, and can be isolated by affinity chromatography (Pytela et al., 1985b; Pytela et al., 1986). It consists of two major subunits of 150kDa and 115kDa referred to as the α and β subunit, respectively. It is thought to be an integral membrane protein because it can be incorporated into liposomes (Pytela et al., 1985b). The structural arrangement of the α subunit appears to be that the 25kDa chain anchors the α subunit of the VnR to the cell membrane and the 125kDa chain is attached to the 25kDa chain through a disulphide bond (Suzuki et al.,

1986).

Since involvement of the VnR has proved to be of importance in macrophage phagocytosis of aged neutrophils, it seemed highly likely that it would also be involved in fibroblast recognition of aged neutrophils. An investigation of the role of the fibroblast VnR was, therefore, an important part of this project.

1.10 Characteristics of macrophage phagocytosis of apoptotic neutrophils.

The initial approach to the determination of the mechanisms involved in aged neutrophil/fibroblast interaction was by comparison to apoptotic, aged neutrophil/macrophage interaction. The characteristics of apoptotic neutrophil phagocytosis by macrophages are presented in table 1.3.

That the VnR is present on macrophages and not on aged neutrophils was indicated because none of the inhibitory mAbs (7G2, 13C2, 13C6 or 3F12) bound to aged neutrophils as assessed by flow cytometry (Savill et al., 1990). Also, a number of α_{VnR} - and β_3 -specific mAbs (including 7G2 and 3F12) immunoprecipitated no peptides from surface-labelled neutrophils (Gresham et al., 1989). Furthermore, a heterodimer with electrophoretic characteristics similar to the VnR was immunoprecipitable from the macrophage surface.

This chapter has, therefore, served to outline the background areas of relevance to this project. The questions which this project has approached are: Is neutrophil-fibroblast interaction related to apoptosis and, if so, what is the mechanism of interaction between these two cell types?

TABLE 1.3 Characteristics of apoptotic aged neutrophil phagocytosis by macrophages

Inhibits	Stimulates
Low apoptotic index	High apoptotic index
Amino sugars (at aged PMN surface)	
Basic amino acids (at aged PMN surface)	
Low Ca^{2+} , Mg^{2+} conc.	
Trypsin (at Mø surface)	
RGDS	
RGD-bearing proteins eg. Vn and Fn (at Mø surface)	
β_3 mAb 7G2	
α_{VnR} mAbs 13C2, 23C6 and 3F12	

I approached the question of this mechanism by first looking at the similarities between fibroblast and macrophage interaction with aged neutrophils and secondly by looking at the differences. It is hoped that understanding more about the interaction process between fibroblasts and aged neutrophils would then elucidate the mechanisms underlying the resolution of inflammation, but also between inflammatory cells and fibroblasts in the pathogenesis of excessive tissue scarring in inflammatory diseases.

CHAPTER 2: METHODS

2.1 Fibroblast culture

Fibroblasts (FIB) from a number of different sources were used in the experiments described below and included both cell lines and primary cultures. The cell lines used were: F2002 a human foetal lung fibroblast cell line and F4000 a human embryonic kidney fibroblast cell line both from Flow Laboratories Ltd. (Ayrshire, Scotland U.K.); CCL-153, a human foetal lung fibroblast cell line from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, Wilts, England) and the Butcher cell line a human embryonic female fibroblast cell line (Sue Povey) from Goldston labs, University of London, U.K. Primary cultures included fibroblasts derived from neonatal foreskin and foetal skin and lung obtained directly from surgical procedures at the Hammersmith Hospital with the kind cooperation of Mr Singh and the operating theatre staff.

2.1.1. Fibroblast culture from human neonatal skin

Foreskins were obtained immediately after circumcision and washed in Eagle's minimal essential medium (EMEM) or Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, Scotland, U.K.) containing 500 Units/ml Penicillin and Streptomycin (Gibco), 20mM L-Glutamine (Flow Laboratories Ltd.) and 1.5% Sodium Bicarbonate (Gibco). Fibroblasts were extracted using the method described by Ham (1980) with the modifications described below.

The epidermis was scored using a five-bladed scalpel (designed by Dr Bill Otto) and incubated in 0.25% trypsin in Gibco solution A (Gibco) overnight at 4°C.

The epidermis was then scraped away from the dermis, using a pair of curved forceps, and the dermis was cut into small pieces to be placed in a 200 Units/ml solution of collagenase (Type IA, Sigma; Poole, Dorset) at 37°C for 1 hour. After this time the mixture was filtered through a sterile autoclavable 0.5 mm² wire mesh and the filtrate spun at 275g for 6 min to obtain a cell pellet. The pellet was a mixture of fibroblasts, blood cells (mostly erythrocytes) and keratinocytes. The pellet was resuspended in EMEM (or DMEM) plus 20% newborn calf serum (NBCS, Gibco) or foetal calf serum (FCS, Flow Laboratories Ltd.) in a 25 cm² flask (Falcon; Becton Dickinson labware, New Jersey, U.S.A.). During incubation overnight at 37°C, fibroblasts adhered to the flask and contaminating cells were removed with the culture supernatant, which was then replaced with fresh medium (DMEM with 20% FCS).

2.1.2. Preparation of fibroblasts from foetal skin and lung

Skin and lung of 12-15 week fetuses was obtained from Chelsea Women's Hospital by the kind cooperation of Prof. Rodeck's unit as a by-product of routine terminations. The skin and lungs were distinct organs at this stage. The skin or lung was chopped finely using a scalpel blade before being spread across the base of a 25 cm² flask containing 8mls of EMEM (or DMEM) plus 20% NBCS (or FCS). The flask was inverted and incubated overnight at 37°C to facilitate tissue adherence to the flask. Flasks were incubated right side up at 37°C until the fibroblasts originating from the skin formed a confluent monolayer.

2.1.3. Trypsinisation of fibroblasts

Confluent fibroblasts were removed from culture vessels for passage or experi-

mental purposes by trypsinisation. Culture medium was removed, replaced firstly with PBS to remove serum and then with a trypsin-EDTA solution (0.05% w/v trypsin (1:250) and 0.02% w/v EDTA in normal saline, Gibco) and incubated for 20 min at 37°C to detach fibroblasts from the flask and from each other. One half of the cell suspension was then placed in a 75 cm² flask (Falcon) with 15ml EMEM (or DMEM) containing 10% NBCS (or FCS) and the other half diluted to 24ml medium and the suspension plated at 1ml/well on a 24 well tissue culture plate (Falcon) in EMEM (or DMEM) plus 10% NBCS (or FCS).

2.2 Neutrophil isolation

Neutrophils (polymorphonuclear leukocytes; PMN; >98% pure as assessed by May-Grunwald Giemsa stained cytopins and 99.5% viable by Trypan blue exclusion) were isolated from the peripheral blood of healthy human donors using a method proven to maintain neutrophils in an inactivated state according to a number of criteria (Haslett et al., 1985).

Peripheral blood was collected following venepuncture with a wide bore (19 gauge) needle and combined with one part in ten of 3.8% sodium citrate in 50ml polyethelyene tubes (Falcon). Tubes were centrifuged at 300g for 20 min and supernatant platelet-rich plasma (PRP) aspirated. Two thirds of the PRP was centrifuged at 2500g for 10 min to pellet the platelets and the supernatant platelet poor plasma (PPP) was decanted into fresh 50ml polyethylene tubes. The remaining one third volume of PRP was combined with calcium chloride (final concentration 20mM) in a sterile, glass bottle and incubated at 37°C for 1h to aggregate platelets and clotting components. The resultant solution of platelet-rich plasma-derived serum (PRPDS) was used as autologous serum. Following aspiration of the PRP, from above the whole blood pellet, 6ml of 6% dextran (500,000 molecular weight, Sigma Chemicals Co., Poole, Dorset in 0.9% saline) was added to the remaining red and white cells in each tube and the contents of

each tube was made up to 50ml with 0.9% saline at 37°C, mixed by inversion and then allowed to stand at room temperature for 30 minutes for erythrocyte sedimentation. The leukocyte-rich upper layer was aspirated into 50ml polyethylene tubes and centrifuged at 275g for 6 minutes, each pellet was resuspended in 2ml PPP and transferred to a 15ml polystyrene tube (Falcon). The leukocyte suspension was underlayered with 2ml of 42% Percoll solution (Pharmacia, Milton Keynes, Bucks, England; 90% v/v in 0.9% saline) in PPP and then underlayered with 2ml of a 51% Percoll/PPP solution. The discontinuous gradients were then centrifuged at 275g for 10 min. Mononuclear cells form a distinct band at the interface of the saline and 42% Percoll/PPP step, neutrophils band at the interface of the 42% and 51% steps. These bands were aspirated into separate 50ml tubes and sequentially washed first in PPP followed by centrifugation at 320g for 6 minutes (to remove traces of Percoll in the cell suspension) and then with Hank's buffered salt solution (HBSS, Gibco) without calcium and magnesium and finally with HBSS (which contains 1.2mM Ca²⁺ and 0.8mM Mg²⁺) by centrifugation at 275g for 6 min. Cells thus prepared were used for further study or culture. The cells were counted on a haemocytometer (Weber Scientific International Ltd., Lancing, Sussex, England), at the stage of resuspension in HBSS without Ca²⁺ or Mg²⁺, by filling a haemocytometer well by capillary action and counting the number of cells in 4 squares that fill the microscope field of view at a magnification of x300, the number of cells present in the sample is calculated as $4 \times 4 \div 100$ cells/ml.

The neutrophil band was >95% pure with 1-2% erythrocytes, 3-5% eosinophils (in most donors), <0.5% mononuclear cells; basophils were rarely detected. Neutrophil yield varied between donors and from time to time for each donor, but the average yield was $1-2 \times 10^6$ neutrophils/ml of blood (normal range $1-8 \times 10^6$ /ml of blood).

2.3 "Aging" of neutrophils in culture: morphology and viability

Neutrophils were "aged" in culture by maintaining them in suspension cultures for a 24h period as described by Savill et al., 1989a). The method described was devised by Haslett after exhaustive testing of media and culture vessels and results in apoptosis of neutrophils with minimal cell loss due to aggregation or cell necrosis. Neutrophils were suspended at 5×10^6 /ml (unless otherwise stated) in Iscove's modified Dulbecco's medium (Gibco) with penicillin, streptomycin and 10% autologous PRPDS (section 2.2). 5ml of the suspension was added to autoclaved bags of teflon fluorinated ethylene propylene (teflon FEP), from Fothergill Tygaflor Ltd., Littleborough, Lancs.) made by heat-sealing a folded oblong of material to make a bag approximately 6cm wide and 12cm high. One or two bags were taped open side uppermost within a beaker covered by aluminium foil and sterilised by autoclaving at 121°C at 15 PSI for 20 min. The neutrophils were cultured at 37°C and 5% CO₂ for up to 24h.

The concentration of cells in culture was determined by microscopy. Cells were resuspended in their bags and a sample removed and combined with 1 part in 5 of trypan blue (0.4% solution in normal saline; Flow Laboratories Ltd.) for counting on a haemocytometer and assessment of viability. Viable cells exclude the trypan blue dye and dead cells appeared blue.

A similarly prepared sample (not containing trypan blue) was used for haematological staining examination to assess the percentage of neutrophils with morphological features of apoptosis ie. the **apoptotic index** (Savill et al., 1989a). For this assessment the cells were diluted to $1-2 \times 10^6$ cells/ml. An aliquot (200µl) of this cell suspension was added to a chamber of a Shandon cytospin II (Shandon Scientific Products, Cheshire, U.K.), and centrifuged onto glass slides at 400rpm for 3 min. These slides were air dried before fixing in methanol (2min) and staining with May-Grunwald Giemsa (BDH; Dagenham, Essex) or Diff-Quik stains (Merz and Dade, Geneva, Switzerland). Slides were washed in running water and dried before application of a coverslip, the slides were examined by light microscopy under oil (1000x magnification). The proportion of apoptotic

neutrophils in the sample was assessed by counting 1000 cells per slide. Apoptotic cells were scored as those with chromatin condensed into one or more roughly circular masses densely stained with blue dye as described by Haslett et al. (1987) and Savill et al. (1989a); the difference between the dense chromatin of the apoptotic nuclei and the "loose" chromatin in the normal lobed nuclei was easily determined by this method (Plate. 2.1). The appearance of this apoptotic morphology has been shown to correlate well with classical ultrastructural and biochemical features of apoptosis (Savill et al., 1989a).

2.4 Separation of aged neutrophil populations

It was possible to obtain neutrophil populations with varying proportions of apoptotic cells by separation of aged neutrophils into subpopulations using a centrifuge with elutriation rotor (J2-21, Beckman Instruments Inc., Palo Alto, CA, U.S.A.) at 2,500rpm. This counterflow centrifugation method enabled cell separation according to sedimentation velocity (a complex relationship of cell density and the properties of the cell membrane, including shape (Savill et al., 1989a)). In this procedure two opposing forces acted upon the cells housed in the elutriation chamber of the spinning centrifuge. Centrifugal force tended to move the cells away from the centre of rotation into the elutriation chamber and a centripetal force (induced by passing fluid through the elutriator in a direction opposite to the centrifugal force) moved the cells into the centre of the elutriator and in so doing forced the cells out into the outflow pipe from which they could be collected as elution fractions. Thus as the centripetal force exceeded the centrifugal force on a particular cell type within a mixed population, the cells in question were eluted. This method of cell separation was first used by Flanagan (1974) for rat brain cells and was then adapted to purify granulocytes from whole blood (Lionetti et al., 1977). A diagrammatic representation of the system used is shown in Fig. 2.1.

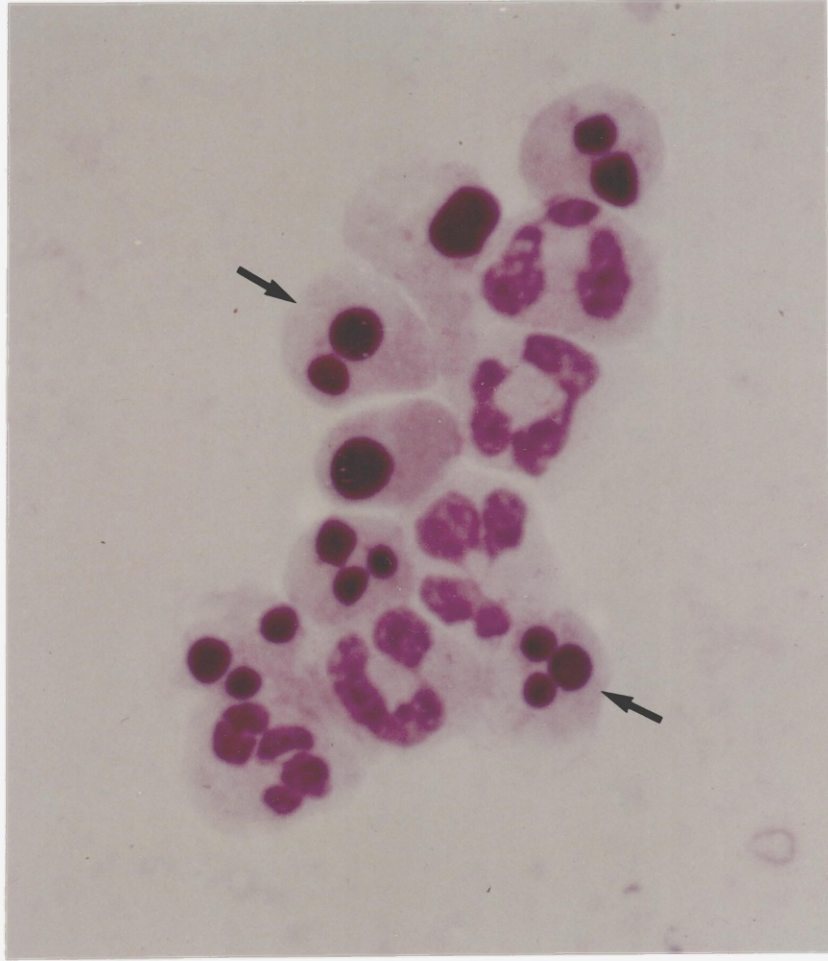


Plate 2.1: Photomicrograph of neutrophils which have been in culture for 13h.

Neutrophils, isolated from peripheral blood (section 2.2), were aged in culture for 13h. After this time, the cell suspension was cytopun and stained with May-Grunwald Giemsa stain (section 2.3). At this time point two distinct neutrophil populations can be seen: neutrophils with "fresh" neutrophil morphology and neutrophils which express apoptotic morphology (arrows). The apoptotic neutrophils have dense chromatin and cytoplasmic vacuolation.

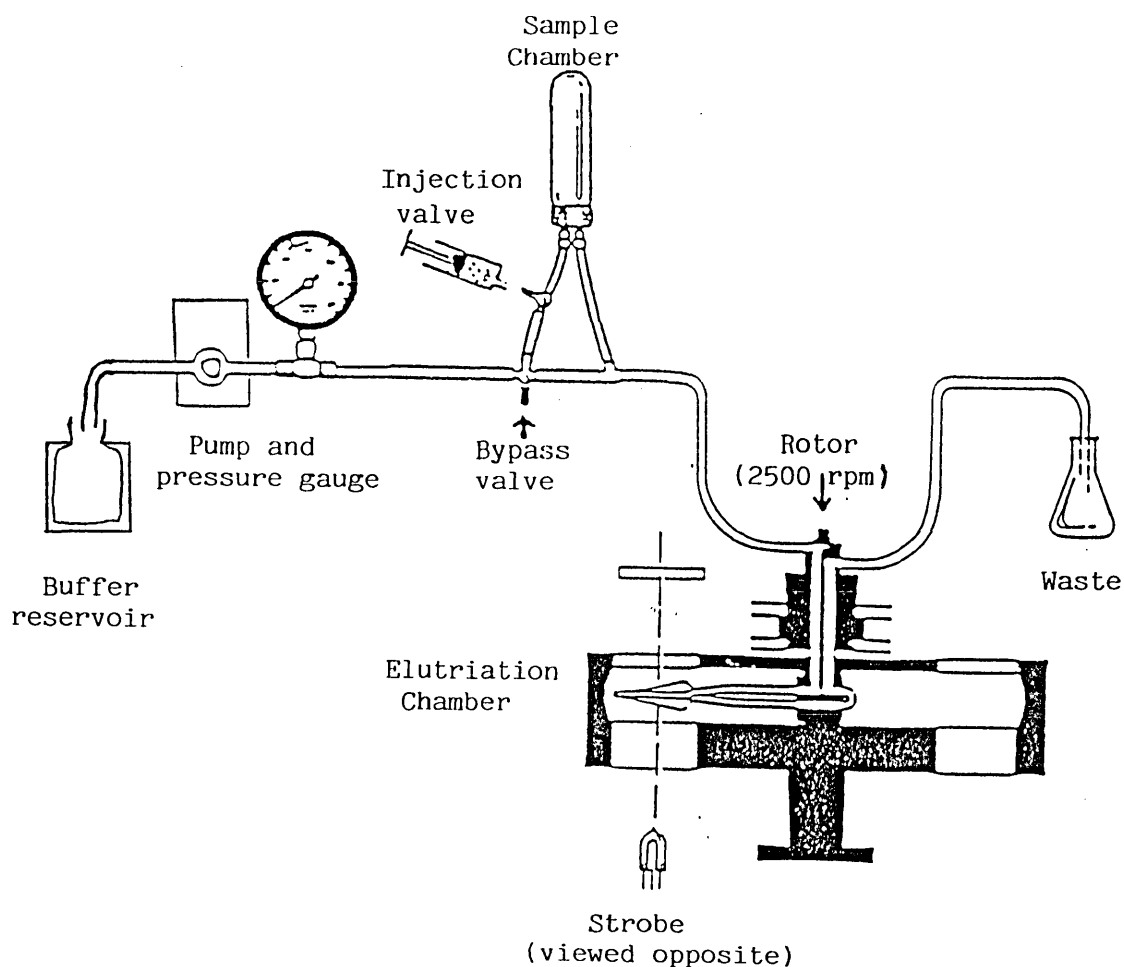


Fig. 2.1. Diagram of the elutriation system. Before use, the pump setting for each flow rate was assessed using a measuring cylinder and stop watch, so that during elutriation the desired flow rate could be obtained by altering the pump setting. Medium usually flows through the sample chamber but, during loading of the cells, the sample chamber was bypassed as shown, after this the medium was again allowed to flow through the sample chamber and fractions were collected into tubes where "waste" is marked on the diagram.

The elutriation system and chamber was first flooded with HBSS without Ca^{2+} and Mg^{2+} which was used throughout as the elution buffer. Aged neutrophils were washed with HBSS without Ca^{2+} and Mg^{2+} (the absence of Ca^{2+} and Mg^{2+} reduced clumping) and resuspended in 5ml of HBSS without Ca^{2+} and Mg^{2+} but with 10% autologous serum at a concentration of $50 \times 10^6/\text{ml}$. The cell suspension was then added to the chamber, via the injection valve, using a syringe with the 3-way tap on the elutriator set so that the elution buffer HBSS by-passed the sample chamber. The elutriation process took about 30 minutes. The 3-way tap was turned so that the elution buffer passed through the sample chamber thus carrying the neutrophils into the elutriation chamber. The cells were loaded into the elutriation chamber at an elutriation flow rate of 14 ml/min which was maintained at that rate using a variable speed peristaltic pump (Masterflex) until all of the cells were in the elutriator chamber. (The pump setting for each flow rate was assessed using a stop clock and measuring cylinder, so that during elutriation the flow rates could be set by adjusting the pump setting). The flow rate was then increased stepwise to 26, 28, 30, 32, 34, 37, 40, 44 and 48 ml/min. At each flow rate 50-100ml of cell suspension was collected and the viability and apoptotic index of each population fraction was assessed by the usual methods. The cell concentration was adjusted to $5 \times 10^6/\text{ml}$ for interaction with fibroblasts. The apoptotic and non-apoptotic populations of the aged neutrophils were eluted as shown in Fig. 3.8a.

Recovery of cells was routinely 70%. The main cause of cell loss was the initiation of cell clumping in the tip of the elutriation chamber. However, such clumping did not appear to distort the proportions of apoptotic and non-apoptotic neutrophils (and eosinophils) recovered when compared to the proportions in the loading sample.

2.5 Assay for neutrophil interaction with fibroblasts

Recognition of neutrophils by fibroblasts was assayed by minor modifications

of the methods described for investigation of macrophage (Mø) recognition of aged neutrophils (Newman et al., 1982; Haslett et al., 1987; Savill et al., 1989a). Confluent fibroblast monolayers in 24 well plates (Falcon) were washed with HBSS and overlaid with suspensions (1ml) of freshly isolated or aged neutrophils at 5×10^6 /ml (previously washed in HBSS).

During incubation at 37°C for 1h, the neutrophils rapidly settled to form a carpet in close association with the fibroblast monolayer. After incubation as many as possible of the excess, non-adherent neutrophils were shaken off the plate by a flick of the wrist. Any non-adherent neutrophils still on the plate after this were washed off by dipping the plate into ice-cold 0.9% saline followed by flicking the plate as before. This dipping and flicking process was repeated until microscopic examination of the plate indicated that none of the non-adherent neutrophils (identified by gentle agitation of the plate under the microscope) had remained on the plate. The interaction was assayed by three methods:

(1) Neutrophils in some wells were stained with a cytochemical stain for the enzyme myeloperoxidase (MPO) which is present in neutrophils but not in fibroblasts (see 2.5.1) facilitating microscopic estimation of the number of neutrophils either adhering to or housed within the fibroblasts.

(2) Phagocytosis of neutrophils was assessed microscopically on stained cytopsin preparations of fibroblast/neutrophil monolayers removed from wells by trypsinisation.

(3) Neutrophils were radiolabelled with $^{111}\text{Indium}$ prior to "aging". After application to fibroblasts and washing the remaining cell material was removed from wells and the association of neutrophils with the fibroblast monolayer assessed by scintigraphy.

Where necessary, the data was assessed statistically using a paired student t-test.

2.5.1. Myeloperoxidase (MPO) stain assay

Cells were fixed in wells by covering with 2% glutaraldehyde (BDH) in PBS. The glutaraldehyde was removed and each well washed with 0.9% saline before staining for MPO using 0.005% hydrogen peroxide and 3mM dimethoxybenzidine (o-diansidine; Sigma) as substrate. This resulted in a brown deposit over the neutrophils (Henson et al.,1978; Newman et al.,1982; Savill et al.,1989b). The extent of interaction was quantitated by microscopic counting of the number of MPO-positive neutrophils interacting with fibroblasts in five randomly selected fields of view. Each field of view was equivalent to the area inside the oblong (marked for camera viewing) at x200 magnification where cells more than half way into the viewing area were counted. Fields for counting were selected with the cells just out of focus so that the eye was not drawn to an area of high or low interaction, but sufficiently in focus that there was no chance of moving into the adjacent wells without knowing.

2.5.2. "Phagocytic" assay

After interaction and washing of the fibroblast plate, the fibroblasts in some of the wells were removed from the plate by trypsinisation as described earlier in section 2.1.3. Trypsinisation was terminated by the addition of serum (which inhibits trypsin action) to the wells. The cell suspensions were transferred to slides by the use of a cytocentrifuge (cytospin II, Shandon; Runcorn, Cheshire) at 400rpm for 3 mins. The concentration of the cells was diluted prior to cytocentrifugation sufficiently to minimise any chance of superimposition of cells ie. neutrophils within the boundary of fibroblasts on the cytospin slide could reasonably be assumed to have been phagocytosed by the fibroblast. Furthermore, it appeared that trypsinisation detached adherent but not ingested neutrophils from the fibroblasts. Cells were fixed to slides by immersion in 2% glutaraldehyde for 4 mins and stained by one of two methods:

(1) 30 min with myeloperoxidase stain which stained the neutrophils dark brown

followed by counterstaining for 10 min with Haemalum (BDH) which facilitated visualisation of the fibroblasts (stained purple). This was the routine staining method used (see Fig. 4.2a).

(2) Staining with May-Grunwald Giemsa as described for assessment of apoptotic index in section 2.3. Following this staining technique, the nuclei of both the fibroblasts and the neutrophils were stained deep purple against the lighter purple cytoplasm. Again, the neutrophil morphology could be assessed (see Fig. 4.2b).

Thus by light microscopy, it was possible to assess the extent of neutrophil phagocytosis by the fibroblasts.

2.5.3. ^{111}In assay

Freshly isolated neutrophils were radiolabelled with ^{111}In at the PPP stage of the neutrophil preparation method (Danpure et al., 1982). Tropolonate (100 μl of 4mM; Sigma) in HEPES:saline (2:10 for pH 7.4) was then added to ^{111}In in 0.04M HCl (approx. 20 μCi in 10 μl). This was allowed to chelate, at room temperature for 2 mins, prior to labelling. The neutrophils were resuspended in PPP to 0.9ml and the tropolonate/ ^{111}In complex added. Integration of the labelled tropolonate complex into the neutrophil membrane was allowed to proceed at room temperature for 5 mins before an excess of HBSS without Ca^{2+} or Mg^{2+} was added. The purpose of this wash was to remove unbound label because labelling is 80% efficient (Danpure et al., 1982). The neutrophil preparation was then completed as normal (ie. HBSS wash and then resuspension in Iscove's plus 10% PRPDS before being placed in teflon bags.

2.5.4. Interaction of aged neutrophils with tissue culture plastic

It was important to prove that any effect of a test solution on aged neutrophil/fibroblast interaction was not actually due to the neutrophils "squeezing" under the fibroblast monolayer and interacting with the tissue culture plate itself. Aged neutrophils, suspended in test solutions, were, therefore, added directly to the tissue culture plates. These experiments confirmed that any changes in aged neutrophil/fibroblast interaction seen were not due to interaction of the neutrophils with the tissue culture plate itself.

2.6 Variations of the fibroblast/aged neutrophil interaction

2.6.1. Effect of molecules in solution

A number of different soluble molecules were used to test their effect on aged neutrophil/fibroblast interactions. The pH of each solution was adjusted as necessary to pH 7.4 using sodium bicarbonate, which did not itself influence the fibroblast/neutrophil interaction. As shown by studies performed comparing control interactions to medium containing the same amount of sodium bicarbonate as used to adjust sugar solution pHs together with hydrochloric acid to maintain the medium at pH 7.4.

The molecules used fell into five main categories and are listed below:-

monosaccharides: D-isomers of glucose, galactose, mannose, fucose, glucosamine, galactosamine, N-acetyl glucosamine, N-acetyl galactosamine, mannose-1-phosphate, mannose-6-phosphate, fructose-1-phosphate and fructose-6-phosphate. (all from Sigma)

amino acids: L-arginine, L-lysine, L- glutamine, and D-isomers of glutamic acid and histidine (Sigma)

polyanions/polysaccharides: mannan and fucoidin (Sigma)

peptides and proteins: Ribonuclease B (Sigma), arg-gly-asp-ser (RGDS)(Peninsular laboratories, St. Helens, Lancs.), gly-arg-gly-asp-ser-pro (GRGDSP) and gly-arg-gly-glu-ser-pro (GRGESp). (Telios pharmaceuticals Inc.,San Diego, CA, U.S.A.)

Divalent cation chelator: Ethylene diamine tetraacetic acid (EDTA) (Sigma)

Solutions of the above molecules in HBSS were tested for an effect on the fibroblast/neutrophil interaction by inclusion in the usual assay (coincubation) or, in order to determine upon which cells these agents acted, by pre-treatment of one cell type before inclusion in a standard assay (preincubation).

2.6.1(a) Effect of coincubation

Coincubation studies were used to assess the effect on aged neutrophil/fibroblast interactions of the presence of various soluble molecules throughout incubation.

coincubation of a single solution

Fresh or aged neutrophils were washed twice with HBSS prior to resuspension at 5×10^6 /ml in test solutions. The neutrophil suspension in control and test solutions were placed on fibroblast wells previously washed with HBSS. The assay was then performed as described above (section 2.5).

Combinations of monosaccharides and polysaccharides were sometimes used to determine whether such sugars were additive in their effect on the aged neutrophil/fibroblast interaction. Appropriate compensations for pH and concentration were made in the preparation of solutions.

2.6.1.(b) Effect of preincubation

Preincubation studies were performed in an attempt to "localise" an effect of various soluble molecules on aged neutrophil/fibroblast interaction to either the

aged neutrophil or fibroblast surface.

(i) Neutrophil preincubation

Fresh and aged neutrophils were washed twice in HBSS before resuspension in control or test solutions to $5 \times 10^6/\text{ml}$ prior to incubation at 4°C in 15ml polyethylene tubes (Elkay, Basingstoke, U.K.) for 30 min. The preincubated neutrophils were then washed twice with HBSS and resuspended in HBSS to $5 \times 10^6/\text{ml}$ before being placed on prewashed fibroblast wells under standard assay conditions (section 2.5).

(ii) Fibroblast preincubation

Fibroblast wells were washed with HBSS prior to the addition of 1ml of a control or test solution to each well and incubation at 37°C for 1 hour. Wells were then washed twice in HBSS before proceeding with the standard assay (section 2.5).

(iii) EDTA preincubation of neutrophils followed by mannose treatment

This was performed to exclude the possibility of a divalent cation-bound structure on the neutrophil surface participating in the interaction process. The neutrophils were washed twice in HBSS before resuspension at $5 \times 10^6/\text{ml}$ in a range of concentrations of EDTA/HBSS solutions (0-5mM) and being incubation at 37°C for 1 hour. Neutrophils were then washed twice with HBSS and were resuspended to $5 \times 10^6/\text{ml}$ in a mannose solution and included in the standard interaction assay (section 2.5).

2.6.2. Effect of divalent cations

Interaction studies were performed in the absence or presence of varying concentrations of divalent cations.

Fresh and aged neutrophils were washed twice in [HBSS without Ca^{2+} or Mg^{2+}] and then resuspended to $5 \times 10^6/\text{ml}$ in [HBSS without Ca^{2+} or Mg^{2+}] but with Ca^{2+}

(as CaCl_2) or Mg^{2+} (as MgCl_2) added back at known concentrations. HBSS ($1.2\text{mM}\text{Ca}^{2+}$ and $0.8\text{mM}\text{Mg}^{2+}$) was the control solution. The fibroblast wells were washed twice with [HBSS without Ca^{2+} or Mg^{2+}] and 1ml of the resuspended neutrophils added in a standard interaction assay (section 2.5).

2.6.3. Effect of proteolytic enzyme treatment of fibroblasts and neutrophils

Treatment of both aged neutrophils and fibroblasts with trypsin was performed to determine the possible involvement of a trypsin-sensitive protein structure on either cell surface in aged neutrophil/fibroblast interaction.

(i) Fibroblast trypsinisation

Fibroblasts were washed twice in HBSS and covered with 1ml of trypsin solution (type III from bovine pancreas, 10,200Units/mg; Sigma) in HBSS at a range of concentrations (0-0.1mg/ml). After incubation for 10 mins at 37°C serum was added to the wells to inhibit trypsin activity. The wells were washed twice with HBSS before 1ml of neutrophils was placed on each well according to standard assay conditions (section 2.5).

(ii) Neutrophil trypsinisation

Trypsin was thermally denatured by incubating at 50°C for 1 hour. Neutrophils which had been washed in HBSS were resuspended in active or denatured trypsin solutions and incubated for 1 hour at 37°C . After this time serum was added to inhibit trypsin action and the neutrophils were washed twice in HBSS. Cells were resuspended in HBSS at $5 \times 10^6/\text{ml}$ and 1 ml added to fibroblast wells (washed twice with HBSS) in a regular interaction assay (section 2.5).

2.6.4. Effect of monoclonal antibodies on aged neutrophil/fibroblast interaction

The effect of various monoclonal antibodies on aged neutrophil/fibroblast interaction was assessed to determine the involvement of their specific antigens in the interaction mechanism.

Mel-14 (a murine monoclonal IgG_{2a} antibody against the lymphocyte homing receptor MEL-14 antigen; Gallatin et al., 1983),

DREG-56 (a human monoclonal IgG₁ antibody equivalent to the murine MEL-14 antibody)

and Hermes-1 (a murine IgG_{2a} monoclonal anti-lymphocyte antibody of sufficient specificity to justify its use as a control antibody; Jalkanen et al., 1986a) antibodies were kindly donated by Dr. Eugene C. Butcher, M.D., Stanford University School of Medicine, Stanford, California, U.S.A.

13C2 (an anti-vitronectin alpha chain monoclonal IgG₁; Davies et al., 1989) was kindly donated by Dr. Nancy Hogg, (I.C.R.F., Lincolns Inn Fields, London, England).

Due to scarcity of antibodies, the fibroblast/neutrophil interactions were performed in a final volume of 300µl, with all other parameters remaining the same. The neutrophils were washed twice before resuspension in 300µl of HBSS or antibody diluted in HBSS and application to washed fibroblast wells for a regular interaction assay.

In the case of MEL-14 and Hermes-1, the neutrophils were preincubated with the antibody to determine whether or not the effect of MEL-14 localised to the neutrophil surface. For these experiments the neutrophils were washed twice, resuspended in 300µl of antibody (or HBSS as control) and incubated at 37°C for 1 hour in polyethylene tubes. After washing twice with HBSS, the neutrophils were resuspended in HBSS and placed on washed fibroblast wells in the regular interaction assay (section 2.5).

2.7 Latex bead ingestion assay

This assay is a modification of the method of Okada et al. (1981) and was used as a positive control for phagocytosis to prove that the fibroblasts were phagocytically competent and to determine whether the solutions employed in 2.6.1. were specific in their effects upon fibroblast/neutrophil interaction or showed general effects upon the phagocytic process itself. An aqueous suspension of 1µm latex beads (Sigma) was diluted 1:999 in 10ml of phosphate buffered saline (PBS; Oxoid Ltd., Basingstoke, Hants, England) containing 0.1% poly-L-lysine (Sigma) and incubated at room temperature for 15 mins. In this time the beads became coated with poly-L-lysine (uncoated beads are not ingested by fibroblasts). Beads were then washed twice in PBS before resuspension in 25ml of HBSS. The coated latex bead suspension (1ml) was added to fibroblast wells as a neutrophil substitute and a regular interaction assay performed (section 2.5).

2.8 Immunofluorescence flow cytometry

This method was used to indicate the presence or absence of a specific antigen on a cell surface by binding of a primary antibody to the cell by its Fc portion followed by binding of a F(ab')₂ fluorescein conjugate so that the presence of a specific antigen was reflected by the fluorescence observed.

The cells (neutrophils) were maintained at 4°C during labelling to reduce the motility of the cell membrane and inhibit capping of antibody which would affect the results obtained. The washing solution used was PBS containing 2.5% FCS and 0.1% azide (the presence of azide also prevented capping). Neutrophils (usually 2×10^6) were washed twice and pelleted each time by brief (approximately 20 sec) centrifugation at 13000g in a microcentrifuge (MSE, Loughborough, Leicestershire, U.K.). The medium was aspirated and the neutrophils resuspended in 30µl of primary antibody solution and incubated at

4°C for 20 min. The neutrophils were then washed three times and resuspended in 50µl of secondary antibody, an FITC conjugate of a F(ab')₂ fragment of a rabbit anti-mouse immunoglobulin antibody (Dakopatt, High Wycombe, Bucks, U.K.) at a dilution of 1 in 30. After 20 min incubation at 4°C the cells were washed four times and immediately fixed in paraformaldehyde (2% in PBS) for subsequent flow cytometry. The cells were stored in the dark at 4°C for a maximum of 4 days.

Flow cytometry measured the relative intensity of fluorescence and the percentage of cells binding antibody and it was performed using a Coulter Epics II Profile (Hialeah, Florida, U.S.A.). Each experiment included negative controls in which P3 (an IgG₁ monoclonal antibody that has no known specificity for human leucocytes; Morganelli and Guyre, 1988) was used to measure nonspecific fluorescence. These were used to set the fluorescence intensity gates so that 99% of the control population were "negative", cells with a fluorescence intensity greater than this were designated "positive". Analyses were done with fluorescence intensity on a three decade log scale, and mean channel fluorescence was reported as that of the "positive" population. Forward scatter ("size") and side scatter ("granularity") parameters for the population were set by cross correlation of these parameters in cells with a "positive" fluorescence intensity using a "positive control" antibody (Hermes-1; Jalkanen et al., 1986a) which is known to be present on the neutrophil surface. The purity of populations and the characteristic size distributions of neutrophils meant that the size/granularity gate stored by the machine's computer rarely had to be changed from one analysis to the next.

CHAPTER 3

Introduction

It has been shown that neutrophils aged in culture for 24 hours, but not freshly isolated neutrophils are recognised and ingested by monocyte-derived macrophages (Mø), but not by monocytes (Newman et al., 1982). The ability of cells, other than macrophages, to recognise and ingest aged neutrophils was originally investigated by Haslett (personal communication). The cell types investigated included microvascular endothelial cells, human epidermal keratinocytes and fibroblasts. His results showed that of these only fibroblasts interacted with aged neutrophils and that under the conditions of this assay (which involved vigorous washing of the monolayers) none of these cell types recognised freshly isolated neutrophils. Furthermore, he found that fibroblasts from a number of different sources were able to participate in aged neutrophil recognition. (Fig. 3.1).

However, neither the nature of the interaction nor the cell surface recognition mechanisms involved in aged neutrophil interaction with fibroblasts were characterised. Nevertheless, as is true for macrophage recognition of aged neutrophils (Newman et al., 1982) the presence of serum was not a prerequisite of recognition (Haslett, unpublished).

Variation in receptor expression with fibroblast source and passage number has been reported by Lamberigts et al. (1979) who estimated receptor expression by measuring 5 α -reductase activity in fibroblasts from different human skin sources (foreskin, labia majora, scrotum, perineum and forearm). They reported that the amount of 5 α -reductase activity is higher in fibroblast lines established from explants of genital skin than from similar lines derived from non-genital skin regions and also that activity was, in part, dependent on the age of the donor. They also observed that the characteristics of cells may change during serial sub-culture and that 5 α -dihydrotestosterone receptor binding remains constant while

Fig.3.1 Interaction of various human cell monolayers with fresh and aged neutrophils*

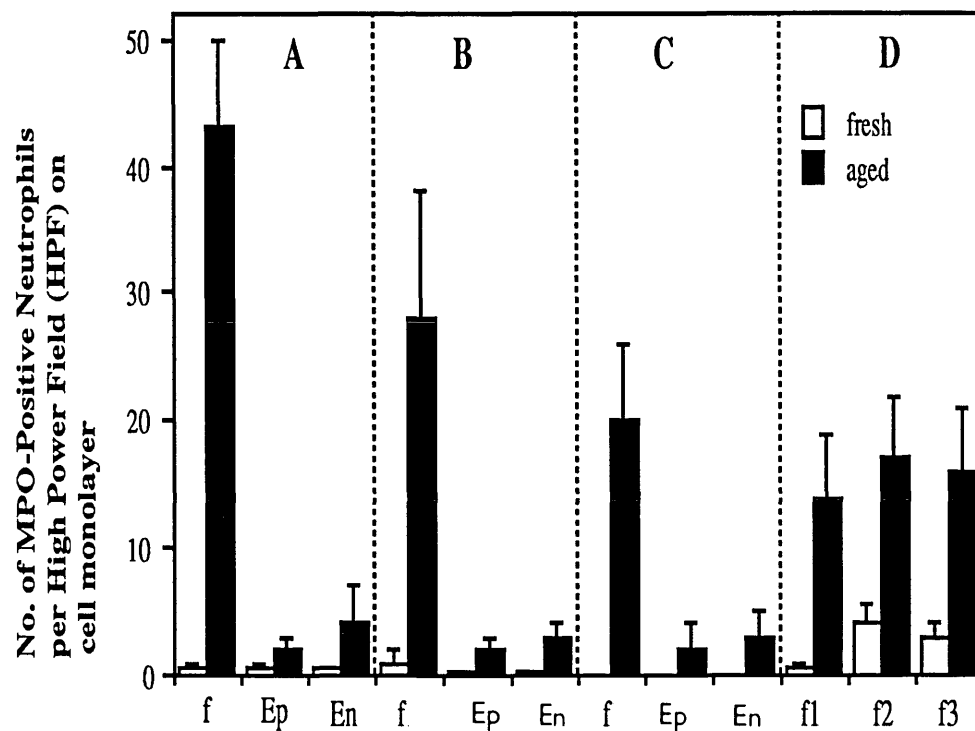


Fig. 3.1

Experiments A,B and C.

Preliminary data showing that fibroblasts, but not endothelial or epithelial cells, recognise aged neutrophils.

f=Fibroblasts

Ep=Human epidermal keratinocytes

En=Microvascular endothelial cells

Experiment D

Preliminary data showing that fibroblasts from a number of different sources are able to recognise aged neutrophils.

f1=Lung fibroblasts

f2=Skin fibroblasts

f3=Kidney fibroblasts

* Haslett, unpublished observations.

5 α -reductase activity increases (up to 8-fold over 40 passages) and 17 β -hydroxysteroid dehydrogenase and 3 α -hydroxysteroid dehydrogenase activities change unpredictably with serial subculture. The observed variability in receptor responses may be due to either selective outgrowth of particular cell types or alterations to the original cell characteristics. Bearing this in mind, changes in the extent of interaction with fibroblast source and passage number was investigated in this project.

3.1 Fibroblast interaction with aged neutrophils

Fibroblast recognition of freshly isolated neutrophils and neutrophils aged for 24h in culture was compared and quantified by microscopic counting of the number of MPO-positive neutrophils remaining on the fibroblast monolayer after 1h coincubation and vigorous washing (chapter 2, section 2.5). The aged neutrophils were interacted with fibroblasts, but there was very little interaction with the freshly isolated neutrophils (plate 3.1, Fig. 3.2), confirming Haslett's original observations. The number of aged neutrophils recognised by fibroblast monolayers was compared in 136 experiments. The number of neutrophils observed per 5 high power fields of the fibroblast monolayers following interaction was counted and these values were grouped into categories of neutrophil numbers (0-49,50-99,etc.). The number of observations (experiments) within each category was then plotted on a histogram (Fig.3.3). The results indicated that the reaction is normally distributed about 100-150 cells/high power field. The extent of aged neutrophil interaction depended on the fibroblast source and passage no. (Figs. 3.4 and 3.5) ie. for the cell line F2002, the greatest interaction was between passage numbers 21 and 25, whilst for the neonatal foreskin fibroblasts, the greatest interaction was at passage numbers 2 and 3 and between passage numbers 6 and 10. For aged neutrophil interaction with fibroblasts, it was noted that the primary skin fibroblasts interacted to a greater extent than all the other fibroblasts used and the extent of interaction

3.1a



3.1b

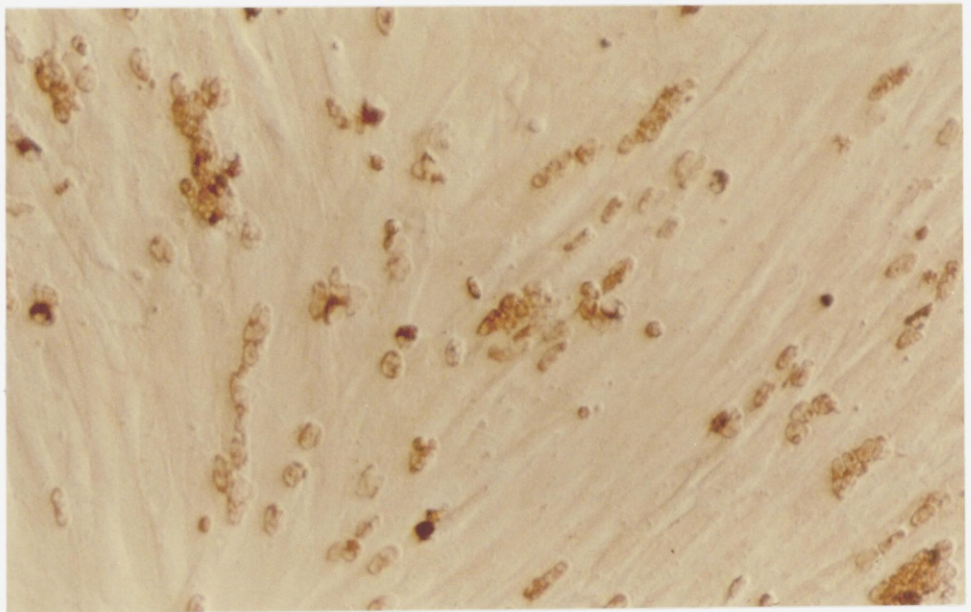


Plate 3.1 Difference between fresh and aged neutrophil interaction with fibroblasts

Fresh and aged neutrophils were washed prior to resuspension in HBSS and being placed on washed, confluent fibroblast monolayers. After 1h at 37°C, any non-adherent neutrophils were removed by vigorous washing and the remaining neutrophils were stained brown using MPOstain.

3.1a Fresh neutrophil/fibroblast interaction. There are virtually no neutrophils left on the fibroblast monolayer following vigorous washing.

3.1b Aged neutrophil/fibroblast interaction. Many neutrophils have interacted with the fibroblast monolayer and remain following vigorous washing.

Fig. 3.2 Interaction of fibroblasts with fresh and aged neutrophils

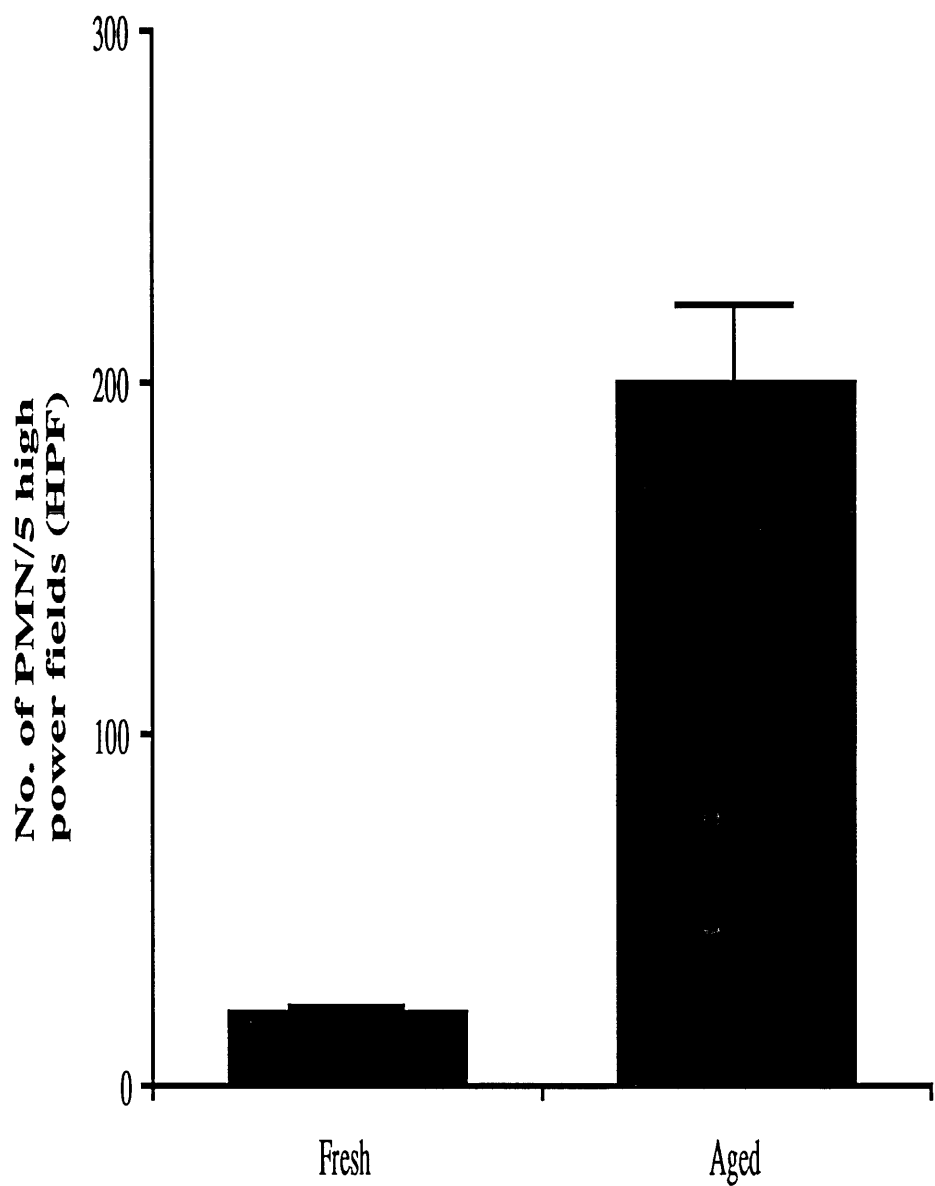


Fig. 3.2 n=60

Fresh neutrophils and neutrophils which had been aged in culture for 24h were placed on confluent fibroblast monolayers and incubated at 37°C for 1h. After vigorous washing and staining of the neutrophils with a myeloperoxidase stain, the number of neutrophils interacting with fibroblasts per 5 high power fields was counted and expressed as mean±SE. It can be seen from the graph that fibroblast interaction with aged neutrophils was at least 10 fold greater than with fresh neutrophils.

Fig. 3.3 Variation of the number of aged neutrophils interacting with fibroblasts in control wells

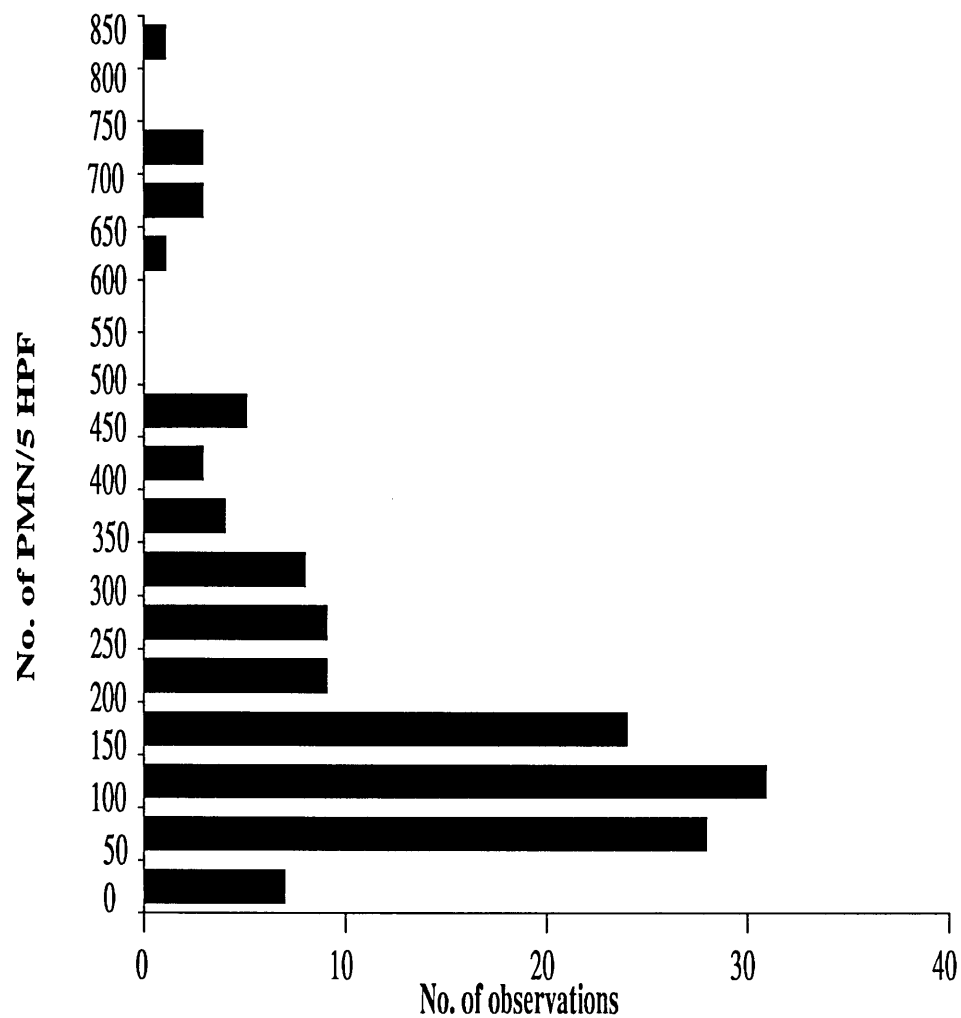


Fig. 3.3 n=136

This graph represents the number of aged neutrophils interacting with fibroblast monolayers in control wells. The aged neutrophils were placed on fibroblast monolayers for 1h after which the fibroblasts were washed vigorously and the remaining neutrophils stained with myeloperoxidase stain. The number of neutrophils interacting with the fibroblast monolayer in 5 high power fields were counted for each experiment and the results expressed as the number of observations within each category (0-49, 50-99, etc.). These data indicate a great degree of variation in the number of aged neutrophils recognised by fibroblasts from one experiment to the next. However, most of the interactions appear to be normally distributed about 100-150 cells/5 HPF.

Fig. 3.4 Variation in F2002 fibroblast/aged neutrophil interaction with passage number.

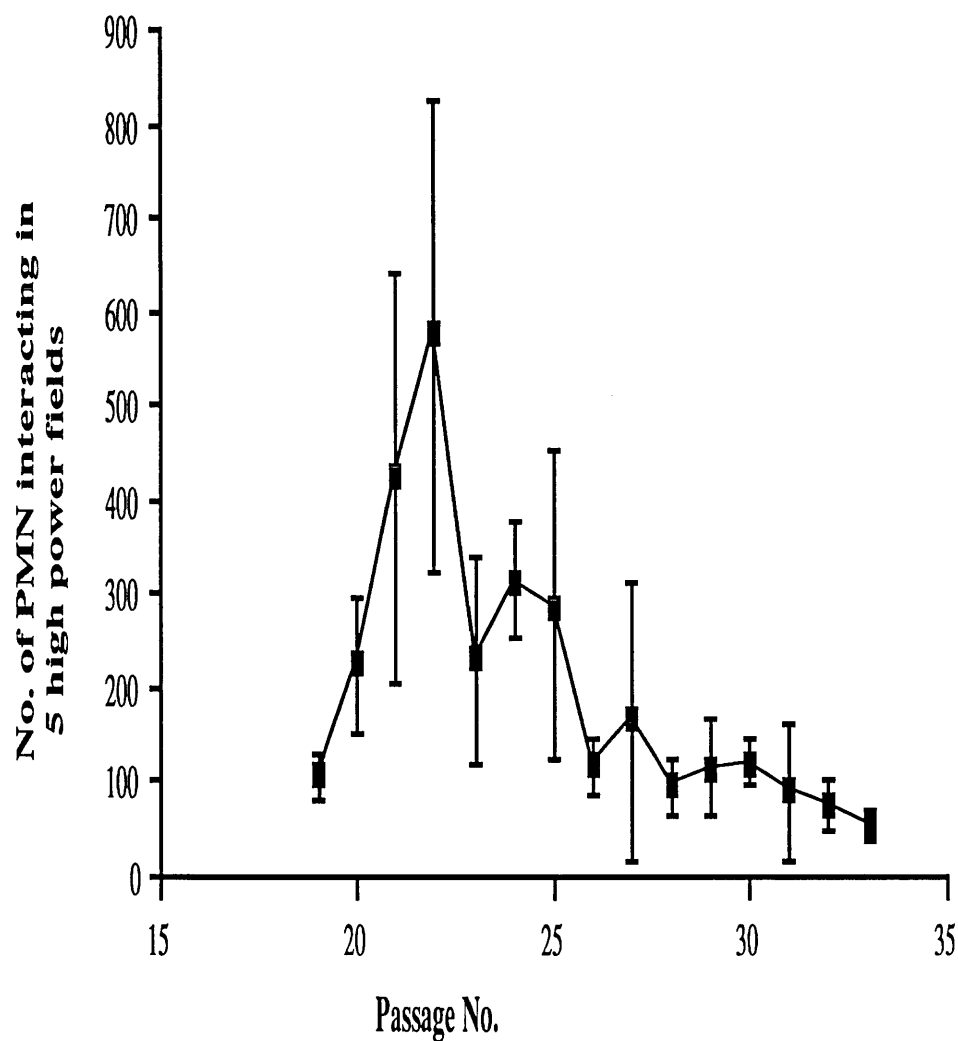


Fig. 3.4 n=20

Neutrophils, aged for 24h at 37°C, were washed twice in HBSS prior to resuspension in HBSS and being placed on washed F2002 fibroblast monolayers at varying passage number. After 1h at 37°C and vigorous washing, the interacting neutrophils were visualised with MPO stain and counted by light microscopy and represented as mean±SE. The results indicated great variability in interaction and passage 21 to 25 appeared to interact to the greatest extent with aged neutrophils, so later experiments were performed using F2002 between passage number 21 and 25.

Fig. 3.5 Variation in foreskin fibroblast/aged neutrophil interaction with passage number.

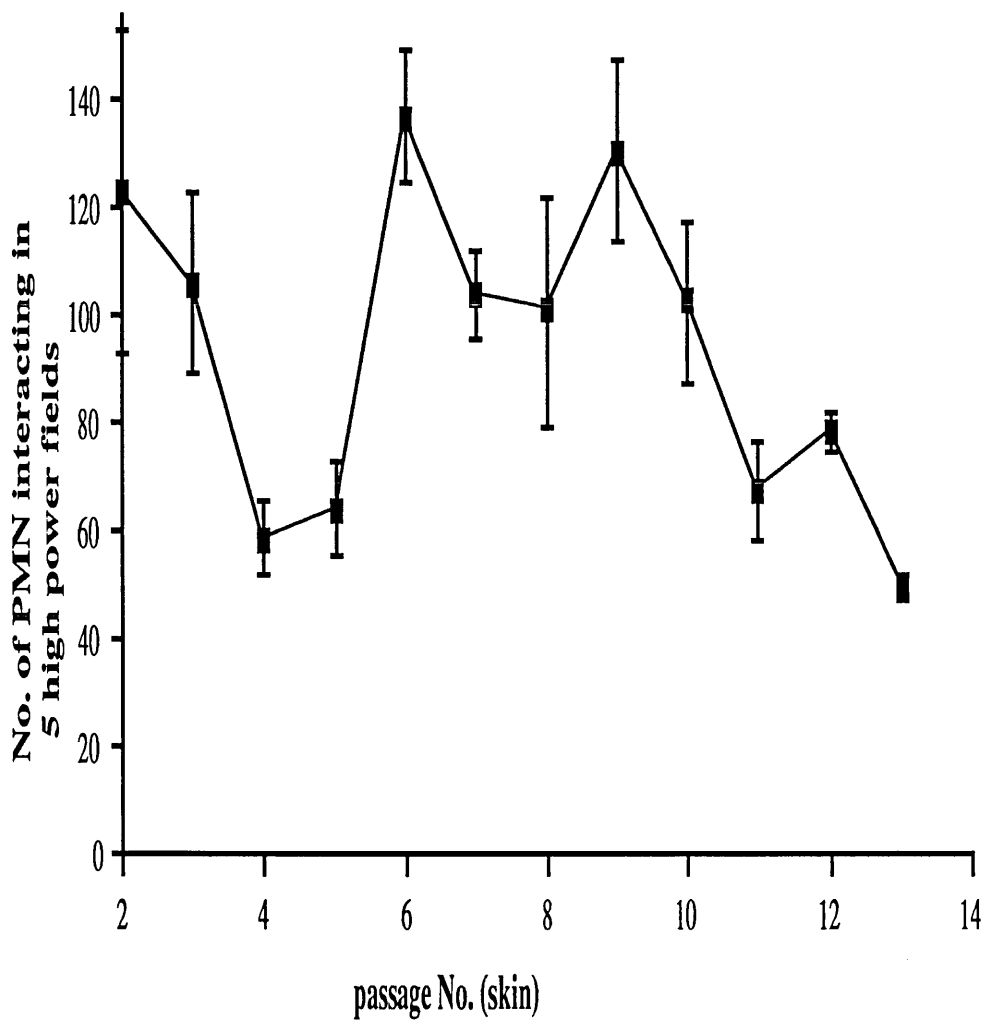


Fig. 3.5 n=20

Neutrophils, aged for 24h in culture, were washed twice in HBSS prior to resuspension in HBSS and being placed on washed neonatal foreskin fibroblast monolayers at varying passage number. After 1h at 37°C and vigorous washing, the interacting neutrophils were stained with MPO stain and counted by light microscopy. The results are represented as mean±SE and indicated that interaction between aged neutrophils and neonatal foreskin fibroblasts was greatest at passage numbers 2 and 3 and between 6 and 10, so in subsequent experiments foreskin fibroblasts were used between those passage numbers.

remained relatively stable throughout the passages used (passages 3 to 10), although there appeared to be a slight peak between passages 6 and 10. However, a definite “peak” interaction was noted for the F2002 fibroblasts between passages 21 to 23. Due to the great variation in interaction, in further experiments the interaction was, therefore, expressed as a percentage of the interaction of 24h aged neutrophils with fibroblasts. Fig. 3.6 shows, the intraexperimental variation from the mean (arbitrarily 100%) expressed as a percentage variation from the mean and it can be seen that 97% of the control duplicates fell within 20.4% of the mean value for that experiment (in an approximately Gaussian distribution).

Fibroblast interaction with aged neutrophils having been confirmed, it was necessary to discover the factors affecting this interaction and the mechanism of recognition involved. Time-course studies have shown that the proportion of a population of neutrophils showing morphological features of apoptosis increases progressively with time in culture and that the recognition of neutrophils by macrophages increases approximately in parallel with increases in neutrophil apoptosis (Savill et al., 1989a)

Time-course studies of fibroblast recognition of neutrophils showed a similar increase in interaction with neutrophils which appeared to plateau after 9h (Fig. 3.7). Neutrophils already aged for 24h were included in the early time points and interacted avidly with the fibroblasts (not shown) indicating that the low level of neutrophil recognition by fibroblasts at early time points related to neutrophil age rather than impaired ability of fibroblasts to recognise neutrophils.

Assessment of the time course of neutrophil/fibroblast interaction showed a similar variation with time when assessed by two methods, staining for MPO-positive neutrophils followed by microscopic examination and radioactive counting of ¹¹¹In-labelled neutrophils (Fig. 3.7).

Fig. 3.6 Within assay variation of control interactions from the mean of the control.

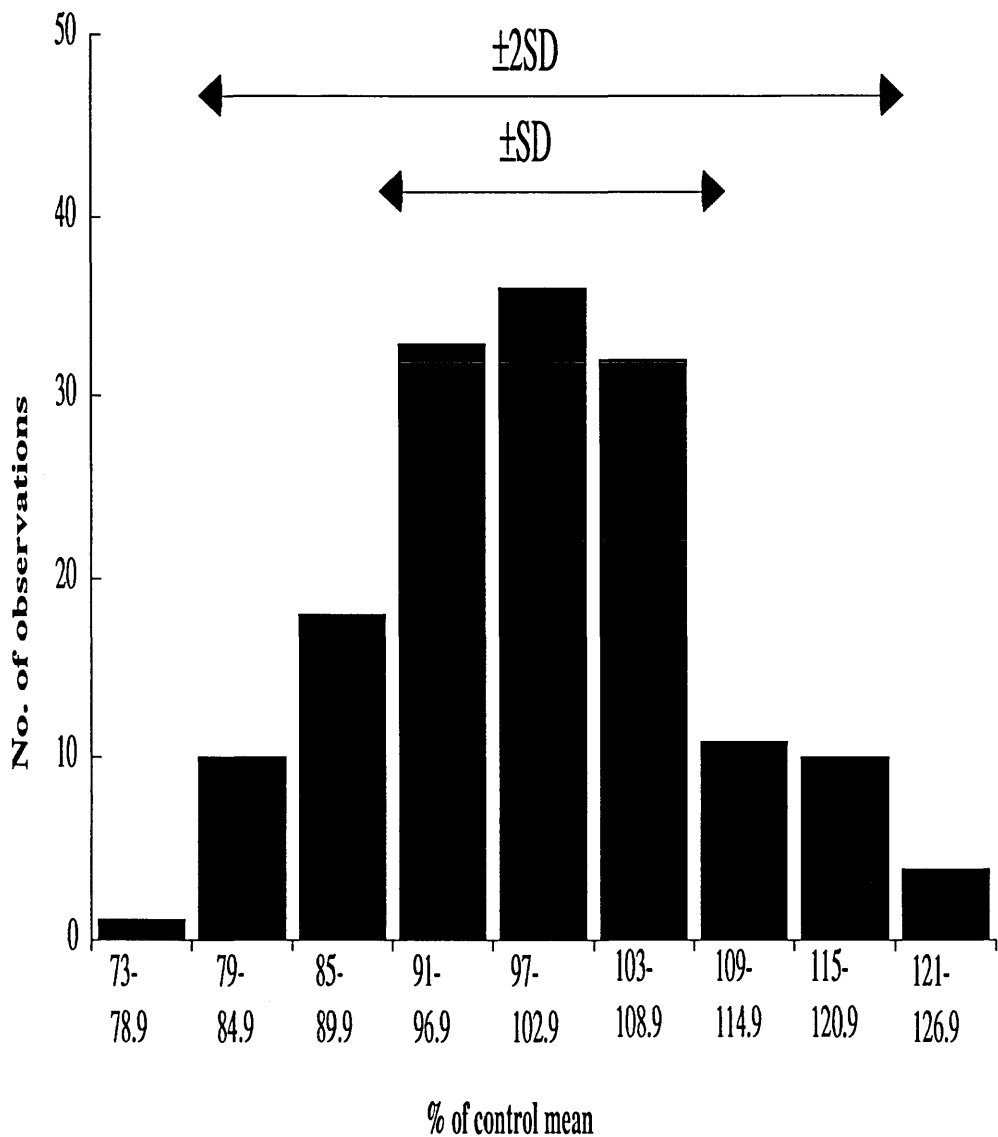


Fig. 3.6 n=155

This experiment was performed to determine the variability of the extent of aged neutrophil recognition by fibroblasts because, if the interaction was found to be extremely variable, inhibition or potentiation of the interaction would prove difficult to observe. The mean of two control interactions in each experiment was regarded as 100% and each individual control observation was expressed as a percentage of the relevant mean value. This graph shows within each assay the individual control well interactions varied normally about the mean. 97% of observations fell within 21.4% of the mean (arbitrarily 100%).

Fig. 3.7 Variation of aged neutrophil/fibroblast interaction with neutrophil time in culture.

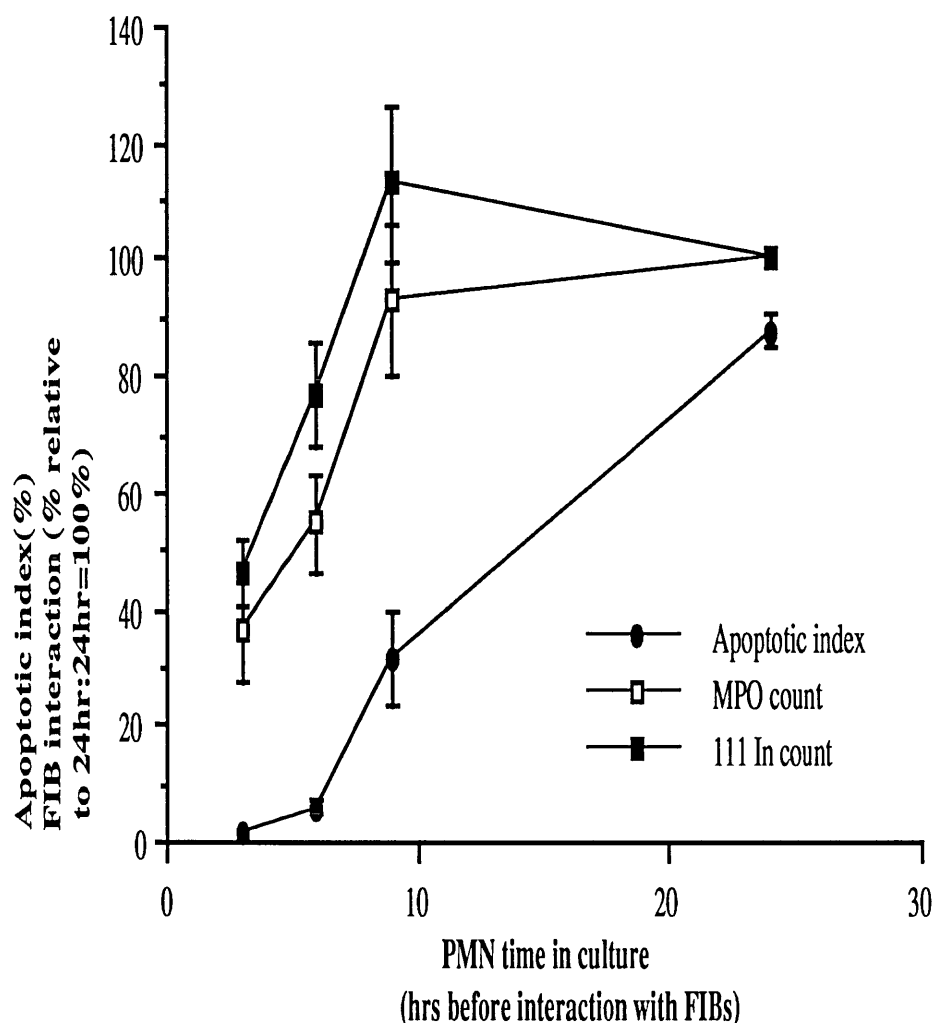


Fig. 3.7 n=16

Neutrophils, labelled with ¹¹¹In and aged in culture for 3,6,9 or 24h, were washed twice in HBSS prior to resuspension in HBSS. The cell suspensions were then placed on washed fibroblast monolayers and incubated at 37°C for 1h. After vigorous washing, the interacting neutrophils on some wells were stained using MPO stain and counted by light microscopy. The number of neutrophils which had interacted on other wells was assessed by radioactive assay of ¹¹¹In. The number of neutrophils exhibiting signs of apoptosis as assessed by light microscopy were also counted. The data is expressed as mean±SE relative to the 24h interaction (227±42 neutrophils/5HPF) and indicates that aged neutrophil interaction with fibroblasts increases up to 9h but not thereafter despite the apoptotic index continuing to increase.

3.2 Separation of aged neutrophil populations into fractions of varying apoptotic indices prior to interaction with fibroblasts

The time course studies just described were performed using the total populations of neutrophils which had been aged for varying lengths of time ie. each interaction study was performed on cell populations containing both apoptotic and non-apoptotic neutrophils.

Time course studies inevitably involved comparison between cells aged in culture for varying lengths of time. One could, therefore, not assume that any changes in fibroblast recognition were necessarily directly related to the increase in apoptotic index; increased recognition could have been related to some phenomenon, other than apoptotic index, which is related to neutrophil time in culture.

It is possible to separate a single population of neutrophils aged for 24h into its apoptotic and non-apoptotic fractions by counterflow centrifugation (chapter 2, section 2.4). This yields fractions of neutrophils with apoptotic indices varying from <5% to >90%, but similar viability as assessed by trypan blue exclusion (>98%). The neutrophil population fractions obtained at different elutriation flow rates were then placed on fibroblast monolayers to determine their interaction with the fibroblasts. It was found that fibroblast interaction with aged neutrophils was bimodally distributed among the different elutriation flow rates, peak interaction coinciding with the elution of highly apoptotic and highly non-apoptotic fractions (Fig. 3.8). Fibroblast interaction with aged neutrophils differed from macrophage recognition in that both apoptotic and non-apoptotic aged neutrophils interacted with fibroblasts, whereas macrophages only recognised apoptotic aged neutrophils (reflected by a single peak which corresponded to the apoptotic aged neutrophil fractions; Savill et al., 1989a). Further studies were needed to elucidate the mechanism of aged neutrophil recognition by fibroblasts and to discover whether two mechanisms of interaction (one for apoptotic and one for non-apoptotic aged neutrophils) or one mechanism for both governed the fibroblast recognition.

Fig. 3.8 Interaction between fibroblasts and aged neutrophils separated into fractions containing varying proportions of apoptotic and non-apoptotic neutrophils.

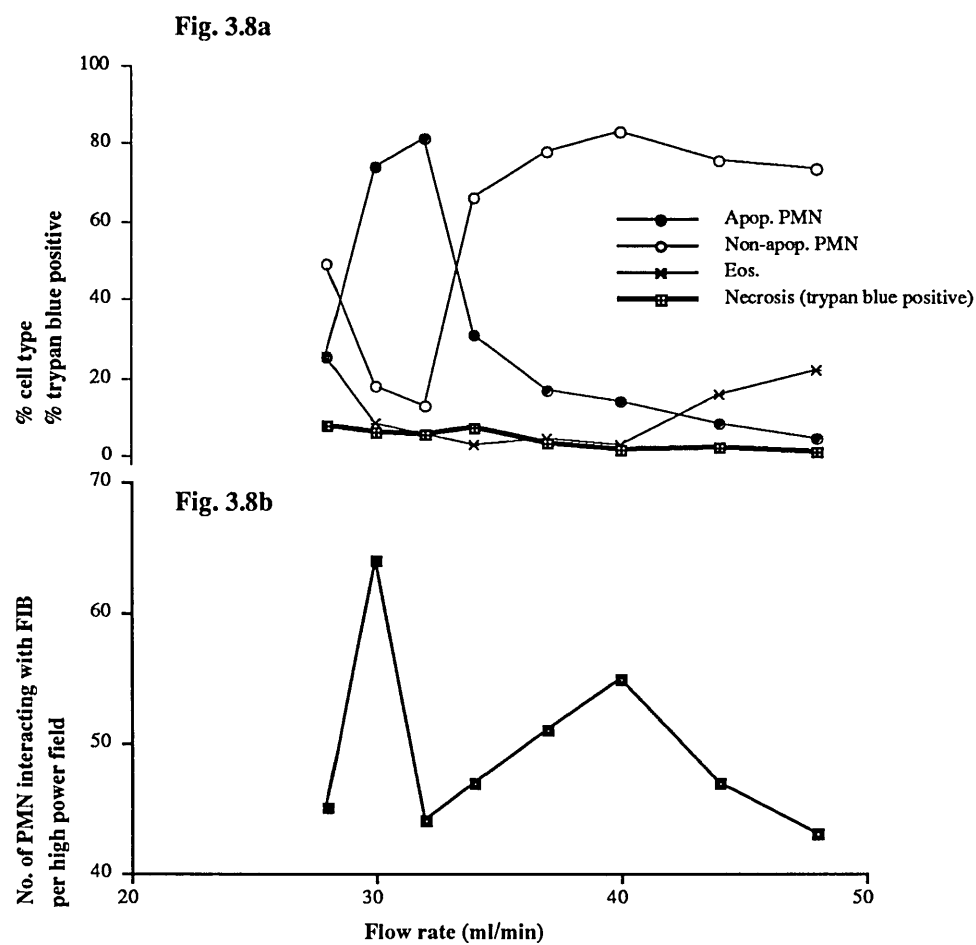


Fig. 3.8

These graphs represent the results from a typical elutriation experiment. Graph 3.8a represents the proportion of each cell type in fractions obtained from elutriation of a mixed population of aged neutrophils. The eluted fractions were placed on fibroblast monolayers for 1h at 37°C prior to vigorous washing and myeloperoxidase staining to visualise the neutrophils.

Fig. 3.8b represents the extent of interaction seen with each fraction and indicates that the interaction was bimodal, peaking at fractions obtained at flow rates of 34ml/min and 40ml/min which represented apoptotic and non-apoptotic aged neutrophils, respectively. It should be noted that the second "peak" was very variable, sometimes appearing as constant interaction, but never as a decline in interaction.

3.3 Inhibition of aged neutrophil recognition by fibroblasts in the presence of certain sugars

Very little study has been made of phagocytosis of senescent cells in general. In particular, no studies of fibroblast recognition of senescent cells has been studied. However, macrophage recognition of both apoptotic thymocytes and apoptotic neutrophils has been subjected to some previous studies.

Binding of apoptotic mouse thymocytes at 4°C by isologous peritoneal macrophages has been shown to occur via a sugar-lectin monosaccharide-inhibitable mechanism (Duvall et al., 1985). Duvall's work suggested that the inhibitory action of such sugars as N-acetyl glucosamine (10mM) occurs through inhibition of macrophage surface receptors for the apoptotic cell, and it was proposed that this indicated that binding was mediated by a macrophage lectin.

Recognition of human apoptotic neutrophils by human monocyte-derived macrophages has also been studied. In this case a charge-sensitive macrophage recognition mechanism, specifically inhibited by amino sugars at varying concentrations (in the range 5-50mM) and 10mM basic amino acids was demonstrated (Savill et al., 1989b).

In order to determine whether the mechanism of fibroblast interaction with aged neutrophils bore any resemblance to either of the previously studied systems, the effects of a range of sugars upon fibroblast interaction with aged neutrophils were tested. Monosaccharides (in the D+ isomeric form) were added to the interaction medium at 10mM, buffered to pH7.4 when necessary. These sugars included those reported to inhibit apoptotic thymocyte recognition by macrophages (N-acetylglucosamine, N-acetylgalactosamine and galactose; Duvall et al., 1985) and those reported to inhibit apoptotic neutrophil recognition by macrophages (glucosamine and galactosamine; Savill et al., 1989b). These results demonstrated that: (1) fibroblast interaction with aged neutrophils did not exhibit a sugar specificity comparable with that for the proposed macrophage lectin involved in binding of apoptotic murine thymocytes (since there was no

inhibition of interaction in the presence of N-acetylglucosamine), (2) there is a similarity to the mechanism of macrophage recognition of apoptotic neutrophils in that glucosamine and galactosamine were also found to be inhibitory in the fibroblast system (Fig. 3.9). However, there was a definite difference between macrophage and fibroblast recognition of aged neutrophils because 10mM mannose and fucose inhibited fibroblast recognition of aged neutrophils but were known not inhibit macrophage/neutrophil recognition (Savill et al., 1989b).

Exclusion of the following possible artefacts was necessary to validate the above results:

(1) That the inhibition of recognition was due to a non-specific toxic effect of the sugars. This was excluded firstly in fibroblasts by demonstration that none of the sugars affected the uptake of a control particle (poly-l-lysine-coated latex beads; data not shown here, see Fig. 4.6 for phagocytosis); secondly, neutrophils retained their ability to exclude trypan blue after incubation with the sugar solutions under conditions identical to those resulting in recognition inhibition (Fig. 3.9).

(2) That the inhibition was due to a change in ionic strength following adjustment of pH of these sugars in HBSS to pH7.4 with sodium bicarbonate (chapter 2, section 2.6) was excluded, since the resulting concentration of sodium bicarbonate in HBSS (with the addition of hydrochloric acid to maintain the HBSS at pH7.4) had no effect.

(3) That the difference between control and "inhibitory" sugars was not infact due to inhibition of interaction with fibroblasts but was due to inhibition of a component of the interaction representing direct adhesion of aged neutrophils to putatively exposed areas of the tissue culture plate. Although it was considered highly unlikely (since confluent fibroblast monolayers were used for experiments, see chapter 2, section 2.5.4) this possibility was excluded since neutrophil adhesion to the tissue culture plate was not inhibited in the presence of the sugars used (Fig. 3.10).

Fig. 3.9 The effect of various 10mM sugar solutions on aged neutrophil/fibroblast interaction.

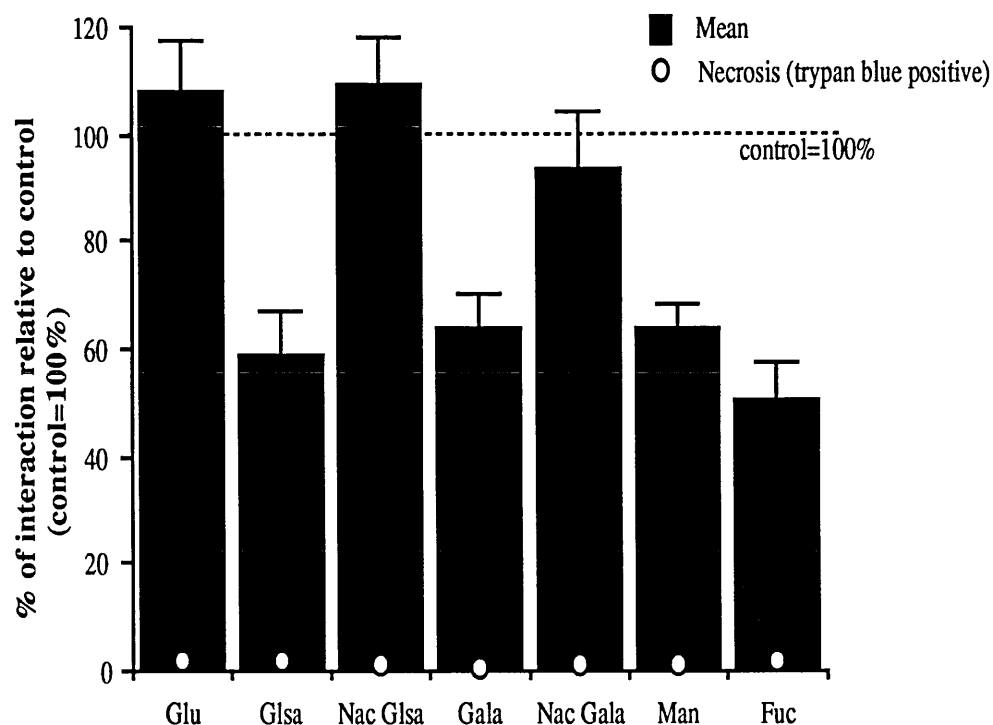


Fig. 3.9 n=30

Neutrophils, aged in culture for 24h, were washed twice in HBSS before resuspension in HBSS containing 10mM sugar solutions. The aged neutrophil resuspensions were then placed on washed fibroblast monolayers and incubated at 37°C for 1h. After vigorous washing, the neutrophils interacting with fibroblasts were stained with MPO stain and counted by light microscopy. The results are represented as mean±SE relative to the control interaction and indicate that glucosamine, galactosamine, mannose and fucose all inhibited aged neutrophil recognition by fibroblasts. Under control conditions 238±28 neutrophils/5HPF interacted with the fibroblast monolayer.

Glu=Glucose

GlSa=Glucosamine

NacGlSa=N-acetyl Glucosamine

Gala=Galactosamine

NacGal=N-acetyl Galactosamine

Man=Mannose

Fuc=Fucose

*** p < 0.001

Fig. 3.10 The effect of various 10mM sugar solutions on aged neutrophil adhesion to tissue culture plastic.

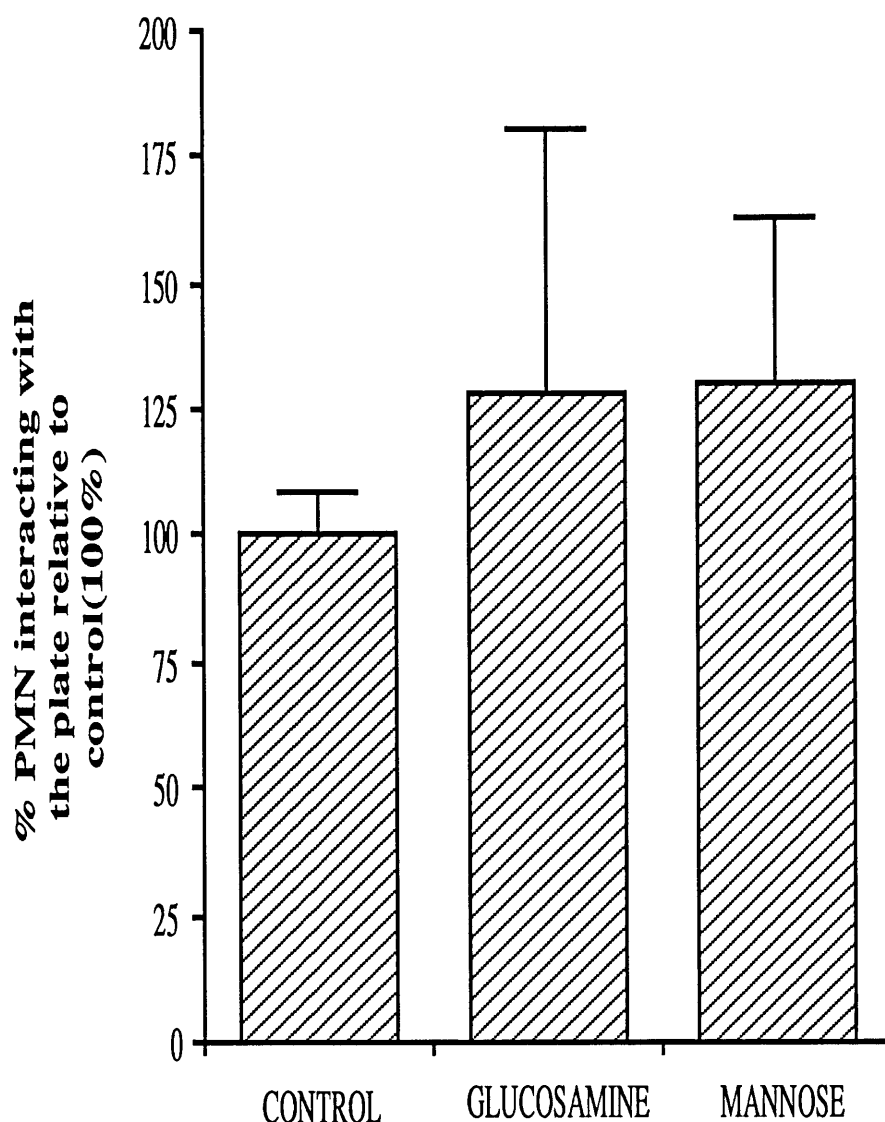


Fig. 3.10 n=4

Aged neutrophils were washed twice in HBSS prior to resuspension in HBSS or a 10mM sugar solution in HBSS. The cell suspensions were then placed directly onto tissue culture plastic and incubated at 37°C for 1h. After vigorous washing, adherent neutrophils were stained with MPO stain and counted by light microscopy. The results are expressed as mean±SE relative to the adhesion to the plate under control conditions (229±14 neutrophils/5HPF) and indicate that glucosamine and mannose did not inhibit neutrophil adhesion to the plate.

3.4 Inhibition of aged neutrophil interaction with fibroblasts in the presence of basic amino acids

As shown by Savill et al. (1989b) for macrophage recognition, neither the parent sugars nor the N-acetyl derivatives of the amino sugars caused inhibition of recognition of aged neutrophils by fibroblasts. A major difference between these sugars and their amino sugar is that the free amino group allows the amino sugars to act as weak bases. At pH7.4, the majority of glucosamine and galactosamine molecules are cationically charged (eg. the pK value for glucosamine is 7.8) (Lehninger, 1981; Dawson et al., 1988). In the case of macrophage recognition of apoptotic neutrophils, Savill (1989b) found that basic, but not neutral or acidic amino acids were also able to inhibit recognition. Similarly, I found that 10mM L-lysine and L-arginine (cationic amino acids) inhibited aged neutrophil recognition by fibroblasts, whilst glutamine (neutral) and glutamic acid (anionic) did not (Fig. 3.11).

Fig. 3.11 Inhibition of aged neutrophil interaction with fibroblasts by basic amino acids.

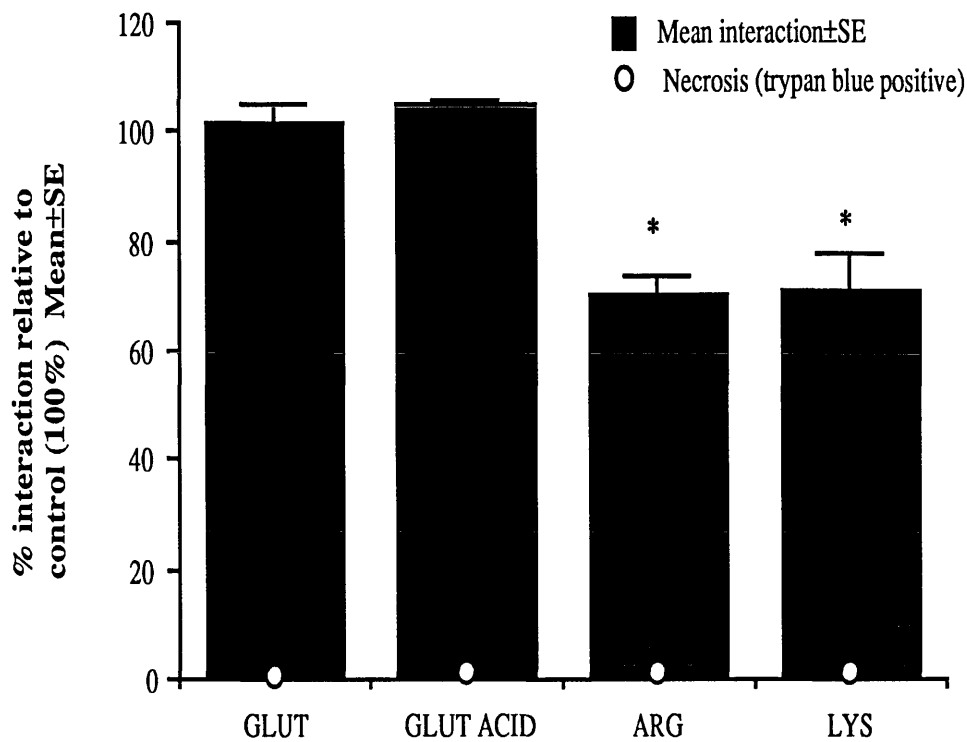


Fig. 3.11 n=10

Aged neutrophils were washed twice in HBSS prior to resuspension in HBSS or 10mM amino acid solutions in HBSS. The cell suspensions were then placed on washed fibroblast monolayers and incubated at 37°C for 1h. After vigorous washing the interacting neutrophils were stained using MPO stain and counted by light microscopy. The results indicate that the basic amino acids, L-arginine and L-lysine, but not neutral or acidic amino acids inhibited the interaction.

It should be noted that the ability of the neutrophils to exclude trypan blue was assessed following coincubation with the amino acid solutions and was found not to increase in their presence. This verified that any inhibition of the interaction was not due to neutrophil death. The results are expressed as mean $\pm$ SE relative to the control interaction. Under control conditions 268 $\pm$ 55 neutrophils/5HPF interacted with the fibroblast monolayer.

GLUT=L-glutamine

GLUT ACID=L-glutamic acid

ARG=L-arginine

LYS=L-lysine

*p < 0.01

SUMMARY OF CHAPTER 3

(1) Fibroblasts interacted with aged, but not freshly isolated neutrophils. This interaction varied with source and passage number of the fibroblasts.

(2) Comparison of the relationship between recognition and apoptosis indicated that fibroblasts interacted with both apoptotic and non-apoptotic aged neutrophils. In contrast, macrophages interact only with the apoptotic population of aged neutrophils.

(3) The interaction of aged neutrophils with fibroblasts was similar to macrophage recognition of apoptotic neutrophils in that amino sugars and basic amino acids inhibited recognition of aged neutrophils by fibroblasts.

(4) Unlike the macrophage recognition system, however, mannose and fucose also inhibited fibroblast interaction with aged neutrophils.

CHAPTER 4

Introduction

In chapter 3 the initial studies on the fibroblast/aged neutrophil interaction were described. My results suggested that the mechanism for fibroblast interaction with aged neutrophils is not identical to that for macrophage recognition of apoptotic neutrophils, although a number of similarities appeared to exist. As for the macrophage, the fibroblast interaction was inhibited by glucosamine and galactosamine but, unlike the macrophage, the fibroblast recognition process was also inhibited by mannose and fucose, suggesting further complexity of recognition in the fibroblast system. Whether apoptotic and non-apoptotic aged neutrophils were recognised by fibroblasts in the same way, however, still remained unclear.

4.1 Two morphologically distinct populations of aged neutrophils interact with fibroblasts

A closer examination of the stained fibroblast monolayers after interaction with aged neutrophils revealed two morphologically distinct populations of aged neutrophils, both of which were seen to interact with the fibroblasts. One, smaller, population comprised cells which remained "round" and densely stained with MPO stain whilst the second population of interacting aged neutrophils appeared "spread" and not so densely stained (plate 4.1). Previous studies of apoptotic neutrophils (Haslett, unpublished) had shown that apoptotic neutrophils retained their "round" shape on haemocytometer glass whilst fresh neutrophils spread on the glass. Inability to spread appeared to be associated with reduced motility of the apoptotic neutrophils and also with a decreased response to stimulants. It thus seemed possible that fibroblasts were able to interact with two populations, the "round" cells possibly representing apoptotic cells and the

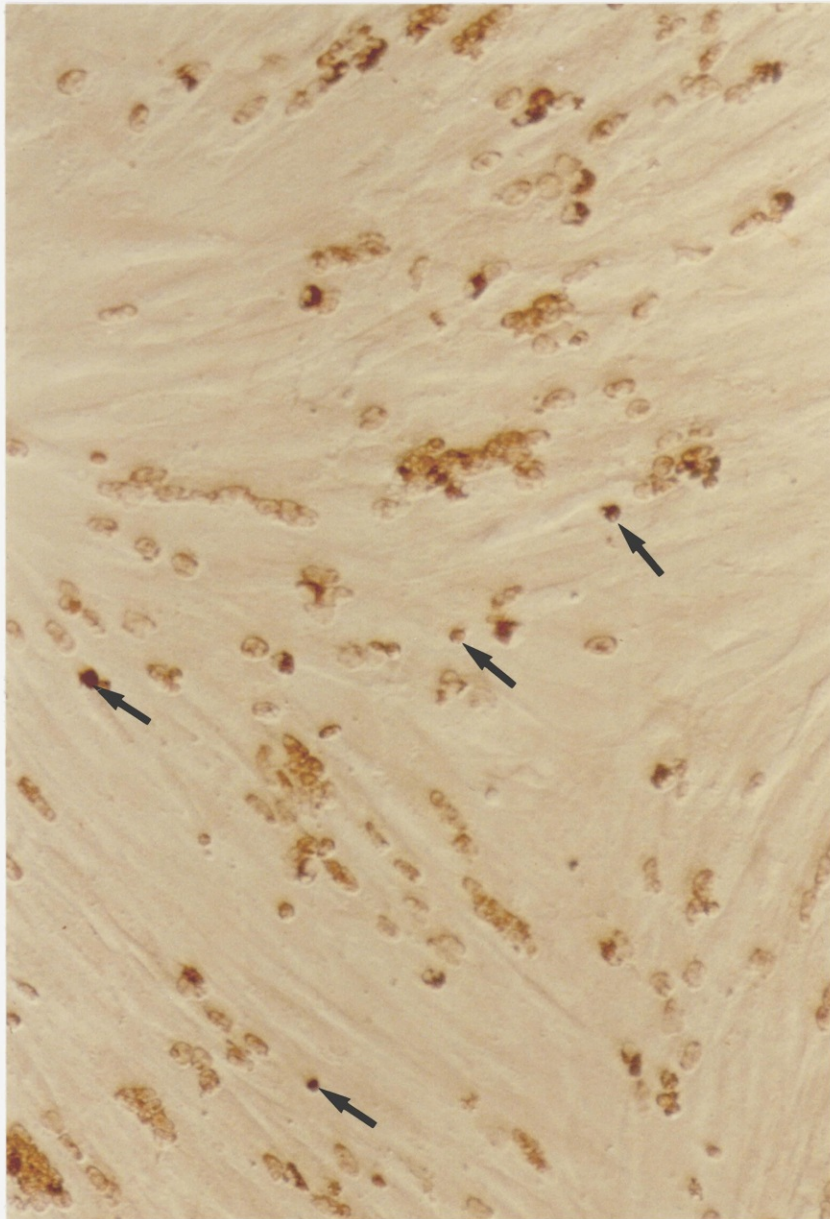


Plate 4.1 Photomicrograph showing two morphologically distinct populations of aged neutrophils interacting with the fibroblast monolayer.

Aged neutrophils were washed and resuspended in HBSS prior to being placed on a washed, confluent fibroblast monolayer. After 1h at 37°C, the non-adherent neutrophils were removed by vigorous washing with ice-cold saline. The remaining neutrophils were stained brown using MPO stain. Two morphologically distinct neutrophil populations can be seen interacting with the fibroblast monolayer: "round" (shown by arrows) which are densely MPO stained and "spread" which are not so densely MPO stained.

"spread" being non-apoptotic aged neutrophils.

Another time course study of fibroblast recognition of neutrophils aged for varying times in culture was performed, this time counting "round" and "spread" aged neutrophils separately. In this series of experiments the increasing apoptotic index was compared to the number of "round" and the number of "spread" aged neutrophils interacting with the fibroblast monolayer following incubation of the mixed neutrophil population (ie. aged neutrophil populations contained both apoptotic and non-apoptotic neutrophils). The results (Fig.4.1) showed a much more definite relationship between "round" neutrophils and apoptosis such that as the percentage of neutrophils showing the morphological appearance of apoptosis (apoptotic index) increased with time, so did the interaction between fibroblasts and "round" neutrophils. This was very similar to the relationship exhibited between the time-course of apoptosis and phagocytosis by macrophages (Savill et al., 1989a). The "spread" neutrophil interaction with fibroblasts, however, peaked at around 10h after incubation and then decreased with time, a pattern which might reflect fibroblast recognition of the non-apoptotic neutrophil population.

Since macrophage phagocytosis of aged neutrophils is determined by apoptosis in neutrophils, the above observation led to speculation that "spread" neutrophil interaction with fibroblasts was non-phagocytic whilst "round" cell interaction, at least in part, reflected fibroblast ingestion of apoptotic neutrophils.

4.2 Neutrophil phagocytosis by fibroblasts

Macrophage recognition of apoptotic neutrophils results in phagocytosis (Newman et al., 1982). Electron microscopy following post-fixation cationised ferritin after fibroblast interaction with aged neutrophils had similarly demon-

Fig. 4.1 Variation of apoptotic index, and neutrophil interaction with fibroblasts, with neutrophil time in culture.

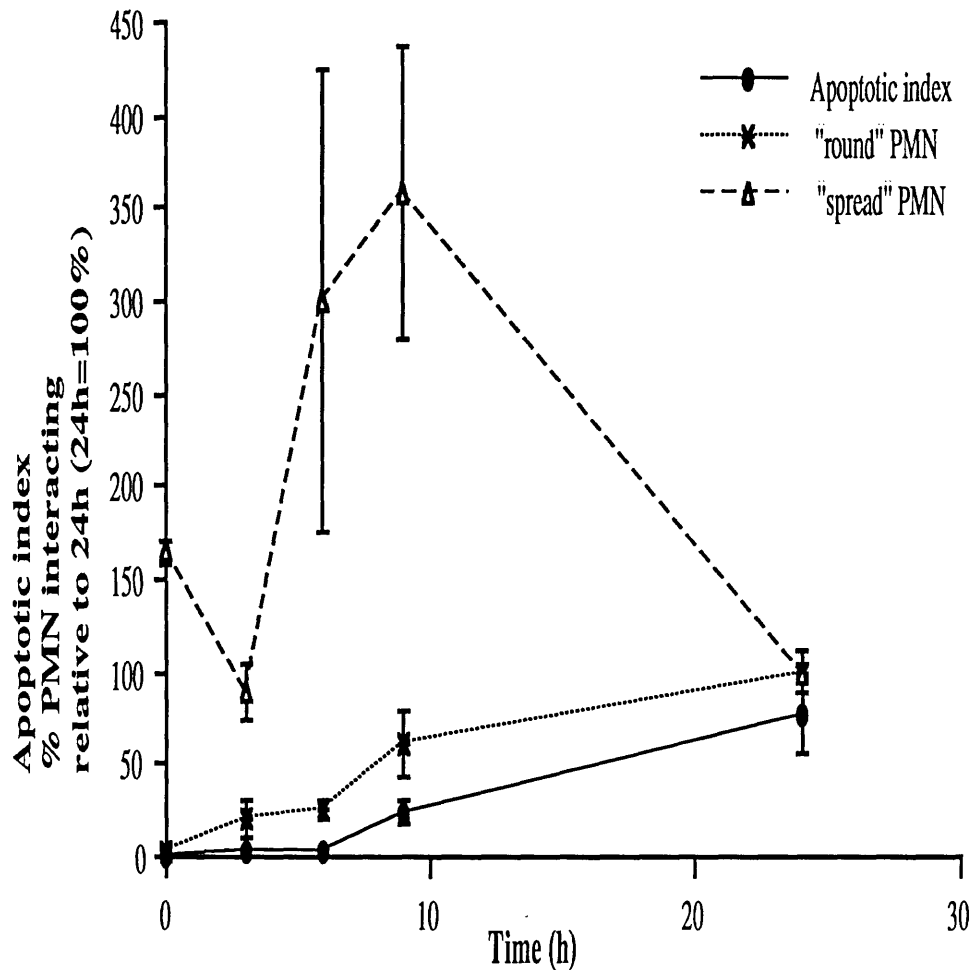


Fig. 4.1 n=4

Neutrophils, aged in culture for varying lengths of time up to 24h, were placed on fibroblast monolayers for 1h incubation at 37°C. After vigorous washing, the remaining neutrophils were stained with MPO stain and the number of neutrophils in 5 high power fields (and whether their appearance was "round" or "spread") was counted and expressed as mean $\pm$ SE relative to the 24h interaction (92 $\pm$ 13 "round" neutrophils/5HPF; 3 $\pm$ 1 "spread" neutrophils/5HPF). The results indicate that apoptotic index and the number of "round" neutrophils interacting with the fibroblast monolayer increased simultaneously while the number of "spread" neutrophils increased up to 9h and then decreased by 24h. The results imply that two aged neutrophil populations, which correspond to the apoptotic and non-apoptotic populations, interact with the fibroblasts.

strated that at least some of the fibroblast interaction with aged neutrophils reflected a phagocytic signal (Haslett, unpublished data). However, such an approach would not be practical in studying the interaction quantitatively and would not lend itself to daily experimentation.

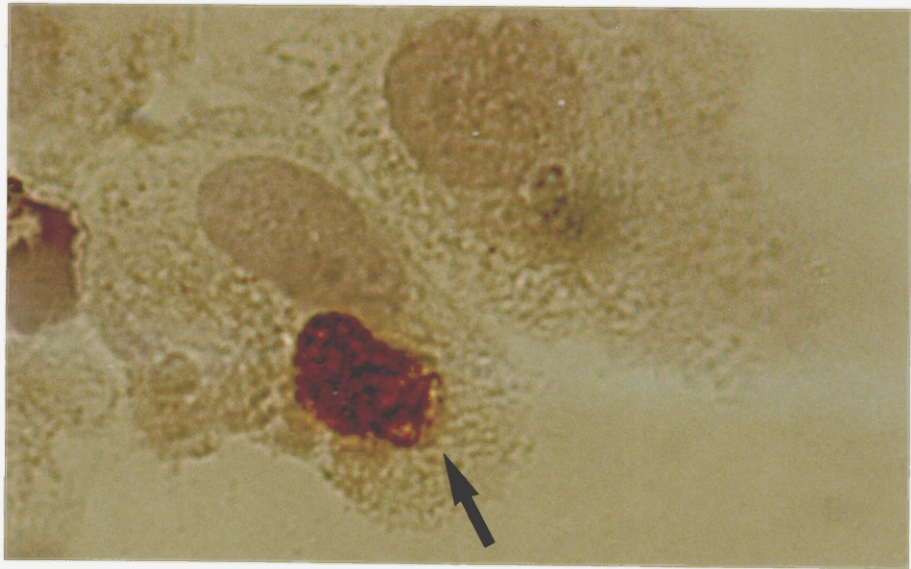
Therefore, in order to compare the macrophage and fibroblast systems, it was necessary to develop a simple method for quantifying phagocytosis of aged neutrophils by fibroblasts. To this end, a suggestion by Henson was successfully used (Henson, personal communication) ie. fibroblasts were trypsinised after interacting with aged neutrophils and the cell suspension transferred to slides and stained using either MPO stain and Haemalum as a counterstain or May-Grunwald Giemsa staining as described in chapter 2 (section 2.5.2). In this way, aged neutrophils which had only adhered to the fibroblast surface were removed by the trypsin so that any neutrophils seen within the limits of the fibroblast outline following trypsinisation of the cells from the tissue culture plate and staining of cytopsin cytological preparations (chapter 2, section 2.5.2) could reasonably be assumed to be neutrophils which had been phagocytosed by the fibroblasts (Plate 4.2a). The fibroblast phagocytic interaction could now, in an analogous fashion to the macrophage system, be scored easily by light microscopy. May-Grunwald Giemsa staining indicated that the neutrophils phagocytosed were, indeed, apoptotic (Plate 4.2b).

Time course studies (Fig. 4.2) revealed a direct relationship between apoptotic index and phagocytosis of neutrophils by fibroblasts such that as the apoptotic index increased so did the percentage of fibroblasts containing neutrophils counted from cytopsin slides.

4.3 Recognition of subpopulations of aged neutrophils

Elutriation experiments were repeated to highlight any differences between fi-

4.2a



4.2b

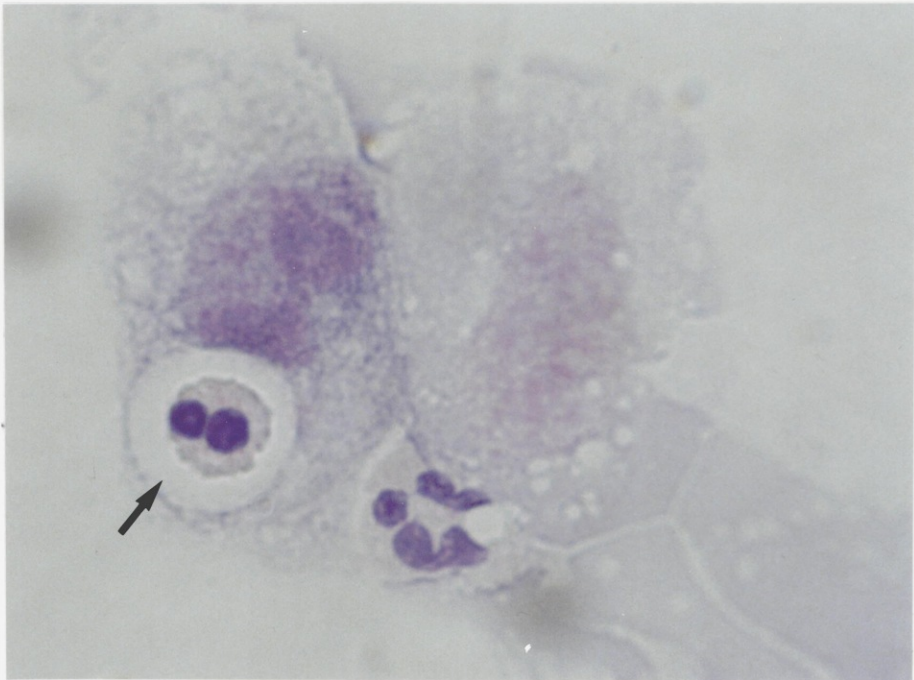


Plate 4.2 Photomicrographs of aged neutrophil/fibroblast interactions after trypsinisation and cytological preparation.

Following aged neutrophil/fibroblast interaction, the fibroblasts were removed from the plate by trypsinisation and cytospun and stained.

4.2a Staining with MPO stain and Haemalum counterstain. An aged neutrophil can be seen inside one of the fibroblasts (arrow).

4.2b Staining with May-Grunwald Giemsa stain. An apoptotic aged neutrophil can be seen inside one of the fibroblasts (arrow) whilst a non-apoptotic aged neutrophil remains on the surface of another fibroblast.

Fig. 4.2 Variation of apoptotic index and phagocytosis of neutrophils with neutrophil time in culture.

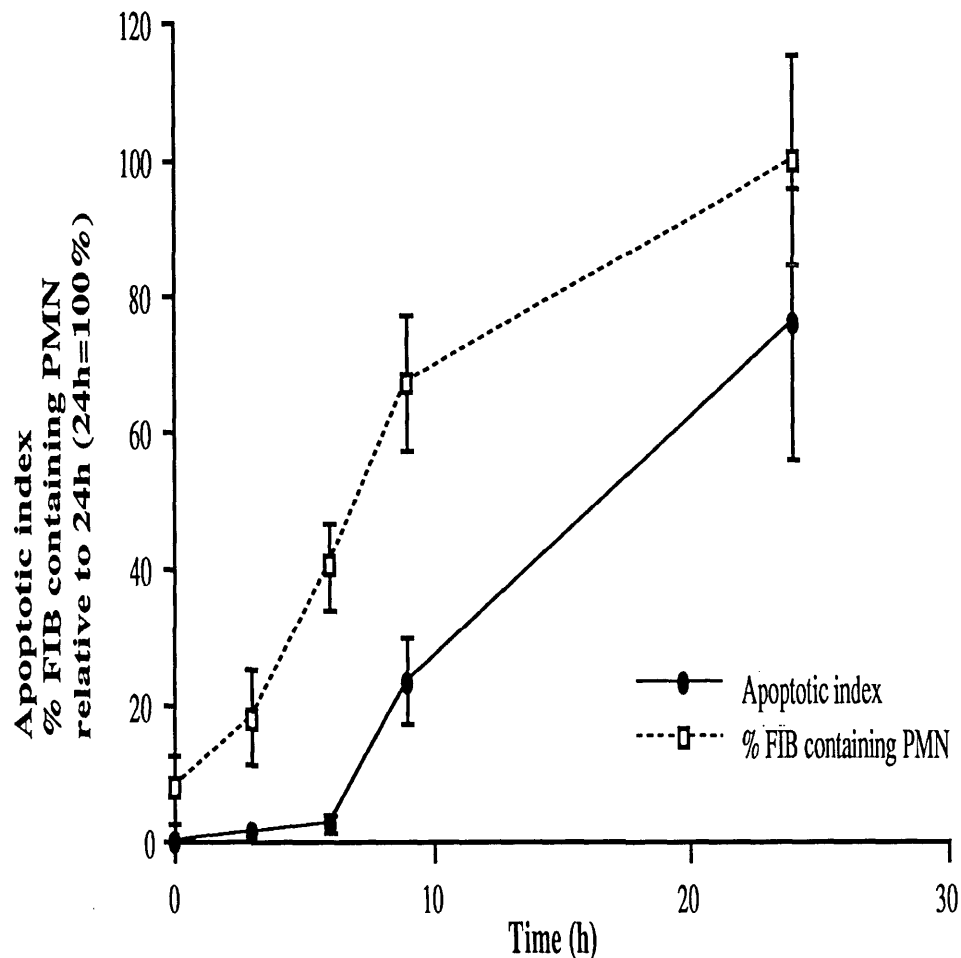


Fig. 4.2 n=4

Neutrophils, aged in culture for varying lengths of time up to 24h, were placed on confluent fibroblast monolayers for 1h incubation at 37°C. After vigorous washing the fibroblasts were trypsinised from the tissue culture plate, cytospun and stained with MPO stain and counterstained with haemalum. The percentage of fibroblasts containing neutrophils was counted by light microscopy. The apoptotic index of the neutrophils at each time point was also counted. The results are expressed as mean $\pm$ SE relative to the percentage of fibroblasts containing neutrophils at the 24h time point (11.3 $\pm$ 2%) and indicate that the percentage of fibroblasts containing neutrophils increases with time in parallel with the increase in apoptotic index. The "control" value was taken as the number of fibroblasts containing neutrophils which had been aged for 24h and the values at earlier time points were expressed as a percentage of that value.

broblast interaction with apoptotic and non-apoptotic neutrophils, respectively. The results revealed that the recognition of "round" neutrophils by fibroblasts correlated closely with the apoptotic index while recognition of "spread" neutrophils correlated with the number of non-apoptotic neutrophils (Fig. 4.3), strongly supporting the hypothesis stated in section 4.1 (in bold) at the beginning of this chapter.

As was previously shown (chapter 3, section 3.2) elutriation resulted in two peaks of interacting cells: the first at 32ml/min consisted of fibroblasts interacting with "round" neutrophils. This corresponded with the elutriated peak of aged apoptotic neutrophils. The second peak at 40ml/min consisted of fibroblasts interacting with "spread" neutrophils and corresponded to the elutriated peak of aged non-apoptotic neutrophils. By including the phagocytic assay in further elutriation experiments, I was able to demonstrate a definite relationship between apoptosis and phagocytosis of neutrophils by fibroblasts, the phagocytic interaction having a peak at 32ml/min which corresponded to the peak for apoptotic index and "round" neutrophil interaction with fibroblasts (Fig. 4.4). Non-apoptotic neutrophils, on the other hand, were not phagocytosed but were purely adherent and "spread" on interaction with the fibroblasts.

The data presented so far have, therefore, indicated two types of interaction between aged neutrophils and fibroblasts: one involving the apoptotic aged neutrophil population and resulting in phagocytosis by the fibroblast and the other involving the non-apoptotic aged neutrophil population and resulting in adherence to the fibroblast monolayer (which was observed as neutrophil "spreading").

The ability of fibroblasts to phagocytose apoptotic neutrophils suggested a mechanism for disposal of intact neutrophils which may be of relevance to resolution of inflammation, so the phagocytic interaction was of primary importance in this project. However, adherence of "spread" or non-apoptotic neutrophils to fibroblasts may also prove in the future to have functional significance and was a secondary part of this project.

Fig. 4.3 Differences in interaction with fibroblast monolayers of various fractions of elutriated aged neutrophils.

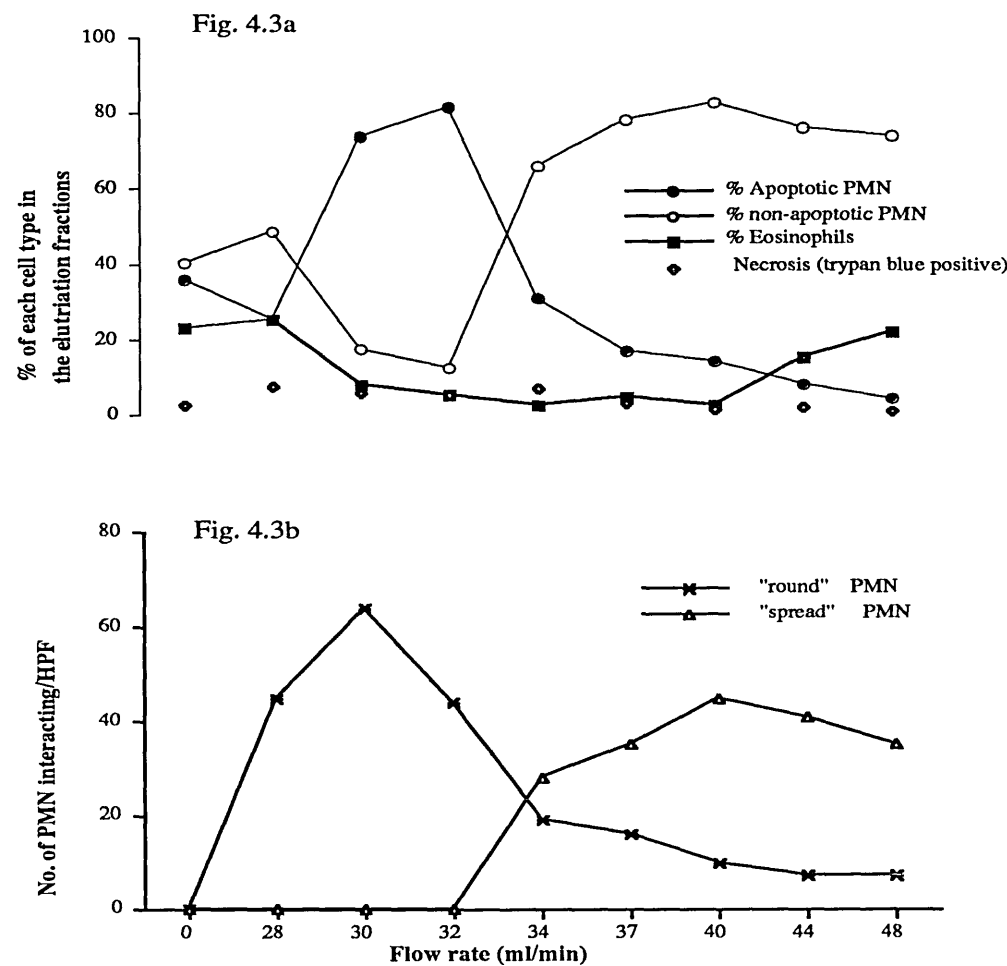


Fig. 4.3
 This represents a typical elutriation experiment. A mixed neutrophil population, which had been aged in culture for 24h, was separated by elutriation into fractions of varying apoptotic indices. The cell fractions were then placed on a fibroblast monolayer and left at 37°C for 1h. After this time the fibroblast monolayer was washed vigorously and the neutrophils remaining were stained with MPO stain. There are two neutrophil populations which interact with the fibroblasts; "round" and "spread". The aged neutrophils which remained "round" following interaction with the fibroblasts were maximally elutriated at a flow rate of 30ml/min which coincides with the peak elutriation of apoptotic cells. The cells which were maximally elutriated at a flow rate of 40ml/min became "spread" upon interaction with the fibroblasts and corresponded to the peak elutriation of the non-apoptotic population of the aged neutrophils.

Fig. 4.4 Difference in neutrophil phagocytosis by fibroblasts of various fractions of elutriated aged neutrophils.

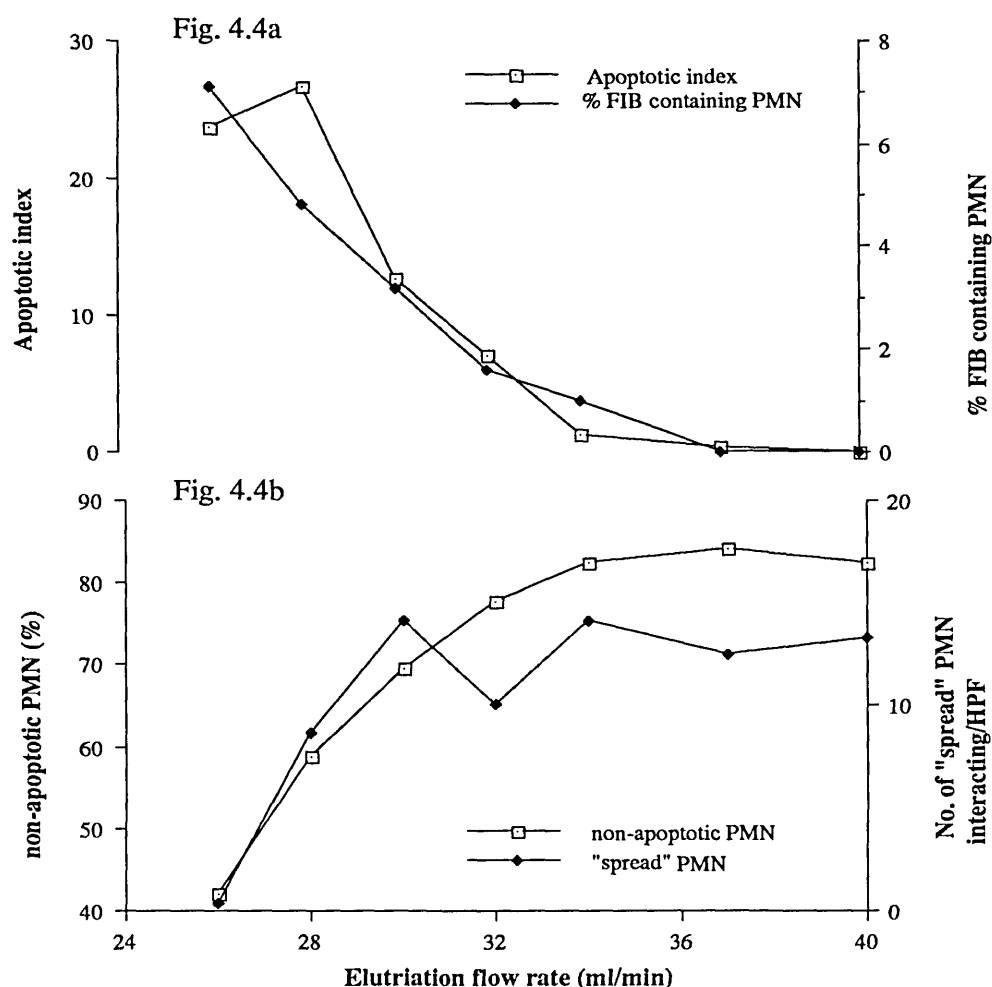


Fig. 4.4

This represents a typical elutriation experiment.

A mixed neutrophil population, which had been aged in culture for 24h, was separated by elutriation into fractions of varying apoptotic indices. Identical concentrations of cells from each fraction were placed on confluent fibroblast monolayers and incubated at 37°C for 1h. After vigorous washing, the fibroblasts were trypsinised from the tissue culture plate and cytospun before being stained with MPO and counterstained with haemalum. The percentage of fibroblasts containing neutrophils was counted by light microscopy as an indication of fibroblast phagocytosis. The results indicate that the phagocytic capability of the fibroblasts was closely related to the apoptotic index, whilst the population of aged neutrophils which became "spread" upon interaction with fibroblasts depended on the non-apoptotic population.

4.4 The effect of various monosaccharides on the different types of aged neutrophil interaction with fibroblasts

Following definition of the two types of neutrophil interaction with fibroblasts, I repeated experiments in the presence of these sugar molecules to determine what proportion of each interaction type was inhibited by each sugar. These studies showed that the phagocytic interaction was inhibited by glucosamine, galactosamine, mannose and fucose, an obvious difference from macrophage phagocytosis of apoptotic neutrophils which is inhibited only by glucosamine and galactosamine (Fig. 4.5). Only mannose and fucose inhibited the "spread" interaction.

That the inhibition was not due to a non-specific harmful effect of the sugars on the fibroblasts was demonstrated by coincubation of fibroblasts with poly-L-lysine-coated latex beads in the presence and absence of sugar solutions. After vigorous washing, the fibroblasts were trypsinised from the plate and cytospun before being stained with haemalum. Counting the percentage of fibroblasts containing beads indicated that even in the presence of these sugars, fibroblast phagocytosis of the beads was not impaired indicating that the sugar inhibition was not due to a harmful effect on the fibroblasts (Fig. 4.6). However, since the percentage of fibroblasts phagocytosing aged neutrophils is small, it could be argued that the sugars are only having a toxic effect on the fibroblasts which recognise and ingest apoptotic neutrophils. However these sugars were proved to have no effect on macrophage phagocytosis of apoptotic neutrophils (Savill et al., 1989b) and are, therefore, unlikely to cause toxic effects in the fibroblast/aged neutrophil interaction system.

Incubation of aged neutrophils and fibroblasts was also performed in the presence of mannose and glucosamine together to see if they had an additive inhibitory effect on aged neutrophil recognition by fibroblasts. There was found to be no increased inhibition of interaction in the presence of both sugars than in the presence of each sugar on its own (data not shown). This result implied that the two sugar molecules are not inhibiting two distinct mechanisms which are

Fig. 4.5 The effect of coincubating various sugars with aged neutrophils and fibroblasts on aged neutrophil/fibroblast interaction.

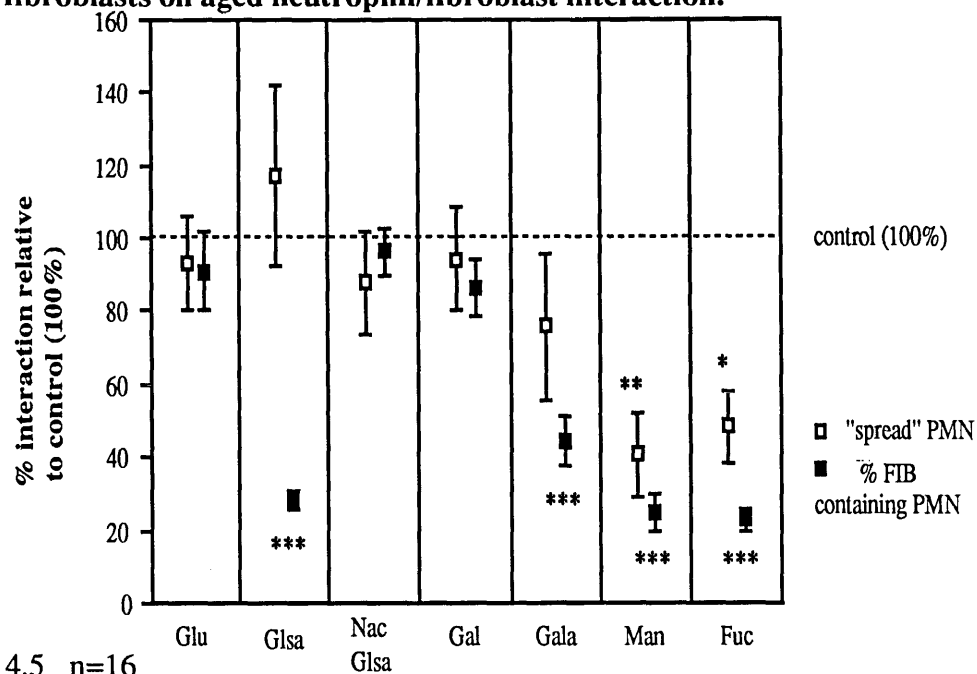


Fig. 4.5 n=16

Neutrophils, which had been aged in culture for 24h, were washed twice in HBSS prior to resuspension in 10mM sugar solutions and being placed on confluent fibroblast monolayers and incubated at 37°C for 1h. After vigorous washing, MPO stain was added to some of the wells to visualise the neutrophils remaining on the fibroblast monolayer. The number of "spread" neutrophils on the fibroblast monolayer was counted by light microscopy. The fibroblasts from other wells were removed by trypsinisation and the cell suspensions cytopun and stained with MPO stain and counterstained with haemalum. The percentage of fibroblasts containing neutrophils was assessed by light microscopy. The results indicate that glucosamine (GlSa), galactosamine (Gala), mannose (Man) and fucose (Fuc) inhibited the phagocytic interaction and that mannose and fucose also inhibited the "spread" neutrophil interaction with fibroblasts. The line labelled "control (100%)" represents the interaction between aged neutrophils and fibroblasts under standard culture conditions when $14.5 \pm 1\%$ of fibroblasts phagocytosed neutrophils and 48 ± 12 neutrophils/5HPF "spread" on the fibroblast monolayer. All values are expressed as a percentage of control.

* $p < 0.01$

Glu=glucose

** $p < 0.005$

NacGlSa=N-acetyl glucosamine

*** $p < 0.001$

Gal=galactose

Fig. 4.6 The effect of coincubating 10mM sugar solutions with fibroblasts and poly-l-lysine-coated latex beads on fibroblast phagocytosis of poly-l-lysine-coated latex beads.

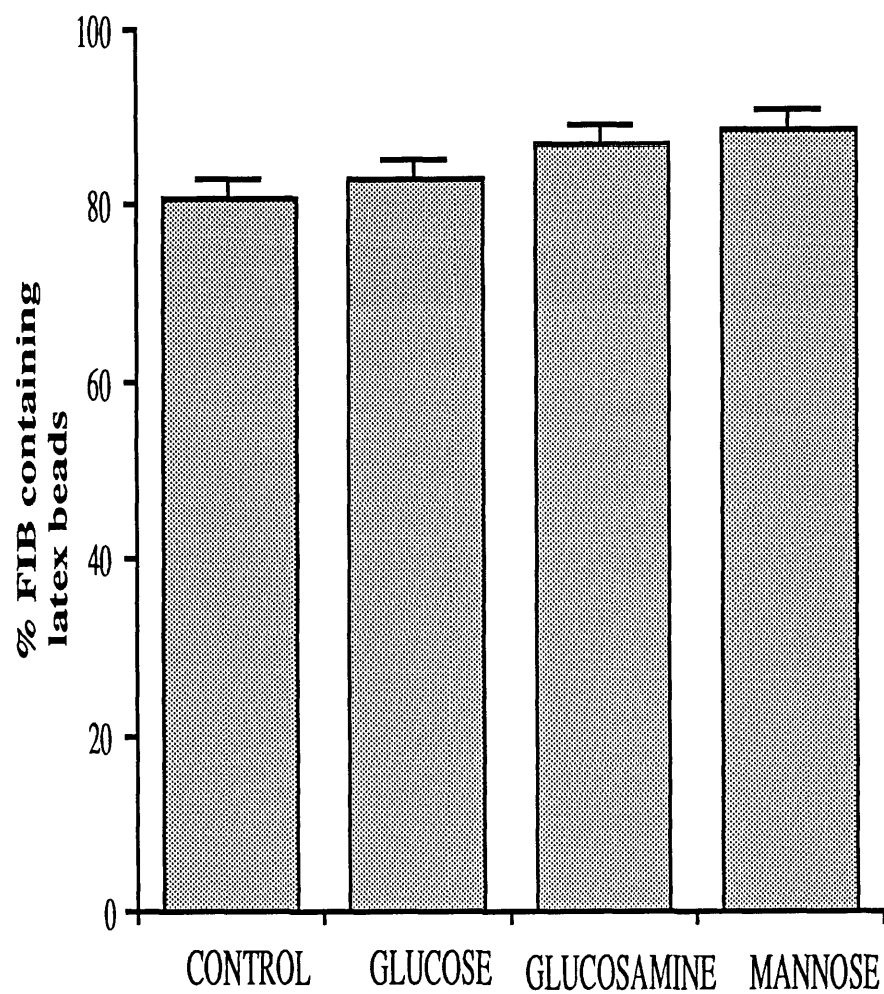


Fig. 4.6 n=6
Poly-l-lysine-coated latex beads were suspended in 10mM sugar solutions prior to being placed on washed fibroblast monolayers. After 1h at 37°C, the fibroblasts were vigorously washed and then removed from their wells by trypsinisation. The resulting fibroblast/bead suspensions were cytospun and the fibroblasts visualised using haemalum. The number of fibroblasts containing beads was then counted by light microscopy. The data is presented as mean±SE and indicates that fibroblast interaction with poly-l-lysine-coated latex beads was not inhibited by any of the sugars used.

normally cooperative, rather than they might be inhibiting two components of the same mechanism.

In the light of the discovery of two distinct interaction patterns, it was also necessary to re-examine amino acid inhibition. These results indicated that, as for glucosamine and galactosamine, the phagocytic interaction was inhibited by the presence of arginine, but that the "spread" interaction was not significantly inhibited. Glutamine and glutamic acid had no effect on either the phagocytic or "spread" interactions between aged neutrophils and fibroblasts (Figs. 4.7). Inhibition of phagocytosis of apoptotic aged neutrophils by fibroblasts in the presence of the basic amino acid, arginine, was similar to that seen for macrophage phagocytosis of apoptotic aged neutrophils.

4.5 Preincubation of aged neutrophils and fibroblasts with sugar and amino acid solutions.

It has been shown by preincubation of aged neutrophils and macrophages independently with sugar solutions for 30 minutes at 4°C that the amino sugars and basic amino acids act on the aged neutrophil surface and not the macrophage surface (as would have been seen if a macrophage lectin-like receptor were involved in aged neutrophil recognition; Savill et al., 1989b). To study the effect of the amino sugars on recognition of aged neutrophils by fibroblasts, I therefore, preincubated aged neutrophils with a variety of amino sugar and amino acid solutions. The following results were obtained; preincubation of aged neutrophils with glucosamine and arginine resulted in inhibition of subsequent aged neutrophil phagocytosis by fibroblasts without affecting the adhesion of "spread" cells to the fibroblasts. (Fig. 4.8). This is a similar result to that for macrophage phagocytosis of aged neutrophils for which the inhibitory effects of glucosamine, galactosamine and arginine were "localised" to the aged neutrophil surface (Savill et al., 1989b). Interestingly, preincubation with man-

Fig. 4.7 The effect of coincubating various amino acids with aged neutrophils and fibroblasts on aged neutrophil/fibroblast interaction.

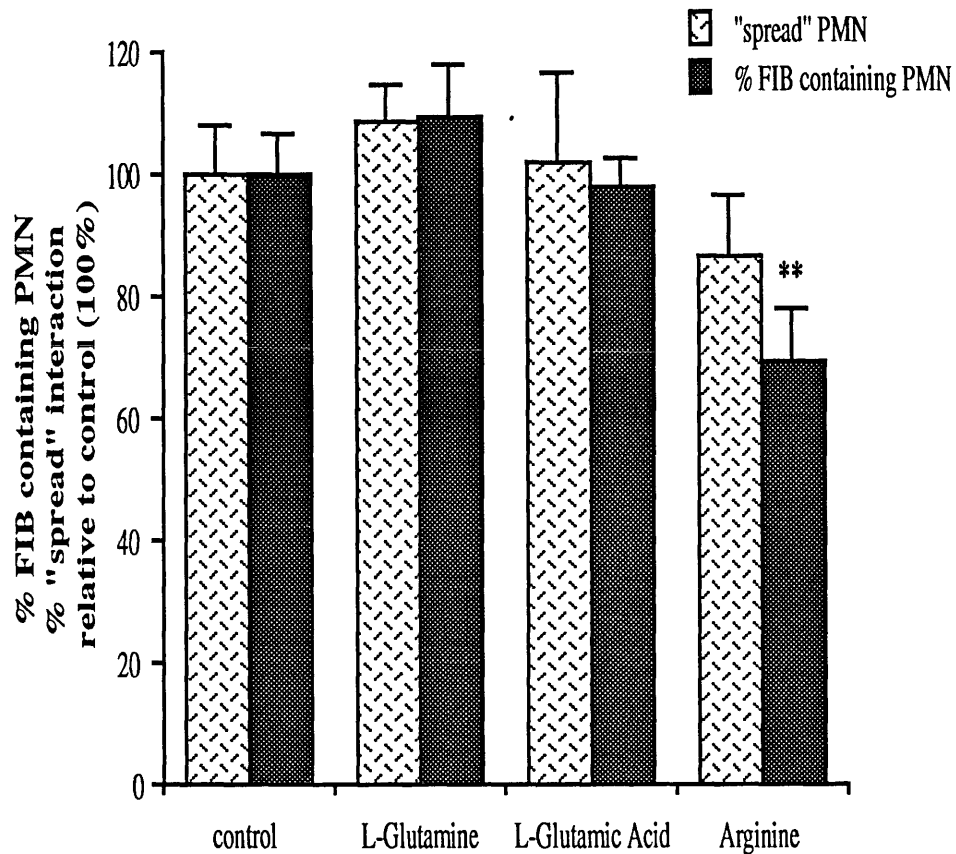


Fig. 4.7 n=20

Neutrophils, aged for 24h in culture, were washed twice with HBSS prior to resuspension in HBSS or HBSS containing 10mM amino acid solutions. The neutrophils were then placed on washed, confluent fibroblast monolayers and incubated for 1h at 37°C. After vigorous washing, the neutrophils interacting with some fibroblast wells were stained using MPO stain. Fibroblasts from other wells were removed by trypsinisation and the resulting cell suspension cytospun and stained with MPO stain (to visualise the neutrophils) and counter-stained with haemalum (to visualise the fibroblasts). The phagocytic and "spread" interactions were counted by light microscopy. The results, expressed as mean±SE relative to control values, show that the phagocytic and "spread" interactions were inhibited in the presence of the basic amino acid arginine, but were unaffected by glutamine and glutamic acid. Under standard culture conditions 12.1±0.8% of fibroblasts phagocytosed neutrophils and 52.8±20 neutrophils/5HPF "spread" on the fibroblast monolayer. ** p<0.005

Fig. 4.8 The effect of preincubating neutrophils with various sugar or amino acid solutions on subsequent aged neutrophil/fibroblast interaction

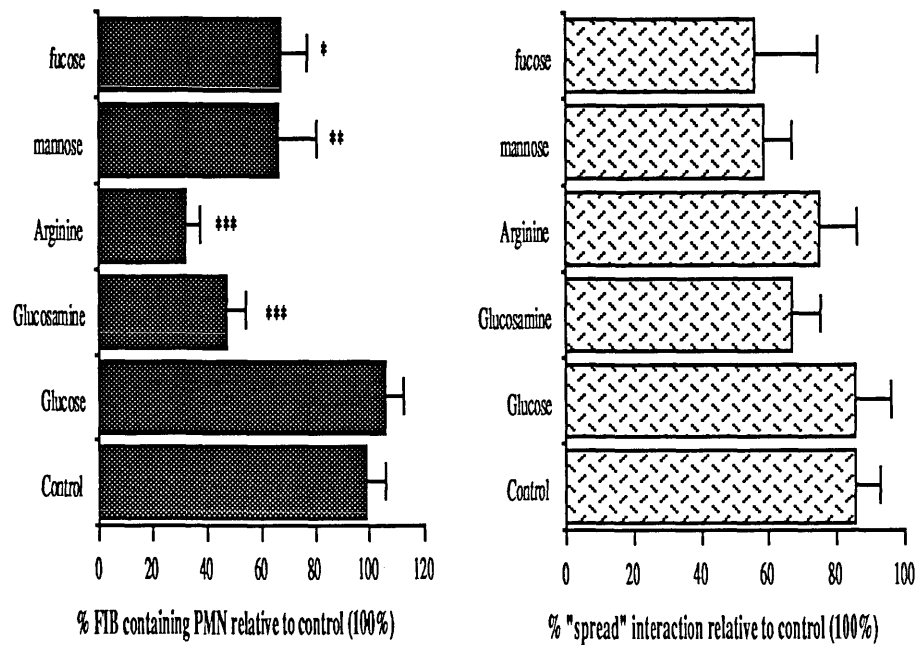


Fig. 4.8 n=16

Aged neutrophils were washed twice in HBSS prior to resuspension in HBSS or a 10mM sugar or amino acid solution and incubation at 4°C for 30 min. After washing twice in HBSS, the neutrophils were resuspended in HBSS and placed on washed, confluent fibroblast monolayers for incubation at 37°C for 1h. After vigorous washing, the neutrophils in some fibroblast wells were stained with MPO stain, to allow microscopic counting of the "spread" interaction. Fibroblasts from other wells were removed by trypsinisation and the resulting cell suspension was cytopun and stained with MPO stain and counterstained with haemalum to allow microscopic counting of the phagocytic interaction. The results are expressed as percentage interaction relative to **coincubation control interaction** (when $7.1 \pm 0.5\%$ of fibroblasts phagocytosed neutrophils and 29 ± 5 neutrophils/5HPF "spread" on the fibroblast monolayer) and as mean $\pm$ SE and indicate that preincubation of aged neutrophils with glucosamine, galactosamine, mannose and fucose inhibited subsequent phagocytic interaction between apoptotic neutrophils and fibroblasts, but that the "spread" interaction was not significantly inhibited. * $p < 0.01$ ** $p < 0.005$ *** $p < 0.001$

nose and fucose also inhibited subsequent phagocytosis of aged neutrophils by fibroblasts, but had no effect on the "spread" interaction, and suggested the possible involvement in aged neutrophil/fibroblast interaction of a lectin on the aged neutrophil surface.

I did not perform preincubation studies with fibroblasts as I felt it would not be reliable because the presence of matrix around the fibroblasts may result in some of the sugar or amino acid solution remaining, even after vigorous washing of the fibroblast monolayer following preincubation, so that it would be present in the microenvironment between cells during the interaction.

SUMMARY OF CHAPTER 4

(1) Two aged neutrophil subpopulations interacted with fibroblasts: aged, apoptotic neutrophils which underwent phagocytosis by fibroblasts and aged, non-apoptotic neutrophils which became "spread" on the fibroblast monolayer.

(2) There were close similarities to macrophage phagocytosis of apoptotic neutrophils: fibroblast phagocytosis of aged, apoptotic neutrophils was inhibited by the amino sugars, glucosamine and galactosamine, and the basic amino acid, arginine. This inhibition has been "localised" to the aged neutrophil surface for the macrophage and experiments where aged neutrophils have been preincubated with amino sugars and basic amino acid molecules have indicated that the aged neutrophil surface is also involved in inhibition of fibroblast phagocytosis of aged neutrophils by these molecules.

(3) In contrast to macrophage phagocytosis of aged neutrophils, however, fibroblast phagocytosis was also inhibited by mannose and fucose. By aged neutrophil preincubation studies with mannose and fucose, the aged neutrophil surface was, again, implicated in mannose and fucose-mediated inhibition of the phagocytic interaction.

(4) The main thrust of this thesis is related to studies of neutrophil fate at inflamed sites. Therefore, the main focus of further work related to dissection of the mechanisms whereby fibroblasts ingest apoptotic neutrophils as this phenomenon is likely to be important in clearance of senescent neutrophils from inflamed sites. Nevertheless, much of the interaction of aged neutrophils with fibroblasts is probably due to adhesion/"spreading" interaction clearly separable from the phagocytic signal. Although not the focus of this thesis, this close interaction between aged non-apoptotic neutrophils and fibroblasts could have future relevance. Therefore, although these data are more variable and difficult to interpret, they are also included.

CHAPTER 5

Introduction

In chapter 4, I concluded that two separate processes are involved in aged neutrophil interaction with fibroblasts; one resulting in phagocytosis of apoptotic aged neutrophils by fibroblasts and the other resulting in adhesion of the aged neutrophils to the fibroblast monolayer. Inhibition by amino sugars and basic amino acids, and involvement of the aged neutrophil surface in this inhibition, indicated that the mechanism of aged neutrophil phagocytosis may, in part, be similar to that of macrophage phagocytosis of apoptotic neutrophils.

Amino sugars and basic amino acids have also been reported to inhibit ligand binding by members of the integrin superfamily (eg. fibrinogen binding to the platelet integrin GPIIb/IIIa; Nachman and Leung, 1982). Integrin-mediated adhesion has also been described to be trypsin sensitive and divalent cation-dependent (Ruoslahti and Pierschbacher, 1987). The binding of the integrins described by these authors are RGD-dependent and inhibition of the interaction can be brought about in the presence of a peptide as small as RGDS (Pierschbacher and Ruoslahti, 1984). The involvement of an RGD-dependent integrin has been described in macrophage phagocytosis of apoptotic neutrophils (Savill et al., 1990), so the possible involvement of an integrin molecule in fibroblast phagocytosis of aged neutrophils was investigated.

5.1 Trypsin sensitivity of fibroblast recognition of aged neutrophils

Light trypsinisation of fibroblasts for 10 minutes at 37°C in the presence of Ca^{2+} and Mg^{2+} indicated that the fibroblast surface structure involved in phagocytosis of aged neutrophils was trypsin sensitive. Serum inhibits trypsin activity, so was added to the fibroblast suspension after 10 min and the fibroblasts were then

washed twice with HBSS prior to interaction with aged neutrophils. The control wells were treated in a similar way (Fig. 5.1). Trypsinisation of fibroblasts inhibited phagocytosis of aged neutrophils by fibroblasts, but enhanced "spreading" of aged neutrophils on the fibroblast monolayer. These effects were dependent on the concentration of trypsin used in the trypsinisation. Such trypsin sensitivity has also been shown for Fn-coated latex bead adhesion to and phagocytosis by fibroblast monolayers, but not for concanavalin A-coated beads (McAbee and Grinnell, 1985).

Why trypsinisation of fibroblast monolayers showed increased "spreading" is uncertain, but these data clearly show that in the fibroblast/aged neutrophil system, as in the macrophage/aged neutrophil system, the structure responsible for phagocytosis of apoptotic neutrophils is trypsin sensitive.

5.2 Dependence of aged neutrophil recognition by fibroblasts on divalent cations

Many cellular recognition processes are dependent on the presence of Ca^{2+} and Mg^{2+} and, in particular, it has been reported that macrophage recognition and subsequent phagocytosis of apoptotic neutrophils is dependent on the presence of these two divalent cations (Savill, 1989b). This is consistent with earlier reports that fibroblast adhesion to plates coated with Vn or Fn is Ca^{2+} and Mg^{2+} dependent (Shimo-Oka and Hasegawa, 1988).

In the absence of Ca^{2+} and Mg^{2+} in the interaction medium, phagocytosis of aged neutrophils by fibroblasts was greatly reduced, being equally dependent on Ca^{2+} and Mg^{2+} (Fig. 5.2). It should be noted that above a concentration of 2mM, Ca^{2+} is toxic to neutrophils (with more than 90% unable to exclude trypan blue dye). This was not true for Mg^{2+} . The "spread" interaction appeared only to be dependent on Mg^{2+} since in the presence of Ca^{2+} and absence of Mg^{2+} , the

Fig. 5.1 The effect of trypsinisation of fibroblasts on aged neutrophil/fibroblast interaction.

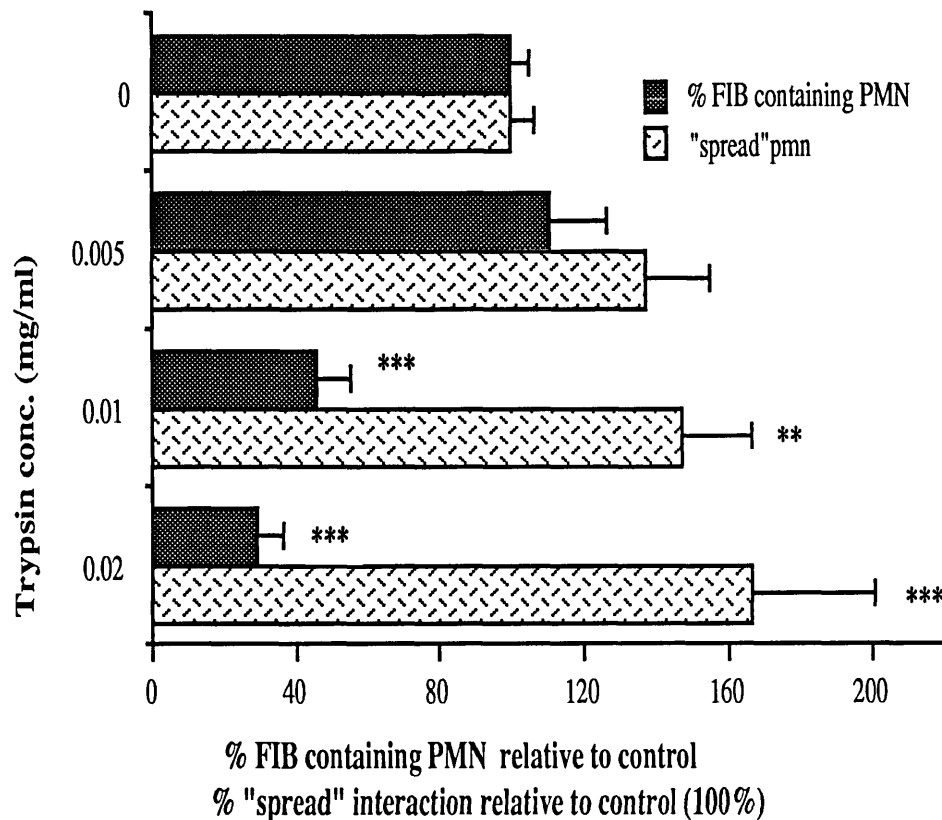


Fig. 5.1 n=14

Confluent fibroblast monolayers were washed twice in HBSS prior to 10 min incubation with trypsin at various concentrations. Serum was added to the wells to stop the trypsinisation and the fibroblast wells were washed twice with HBSS. Previously washed aged neutrophils were resuspended in HBSS and placed on fibroblast monolayers and incubated at 37°C for 1h. After vigorous washing, the interacting neutrophils in some wells were stained with MPO stain. The fibroblasts from other wells were removed by trypsinisation and cytospun before the neutrophils were stained with MPO stain and the fibroblasts counterstained with haemalum. The phagocytic and "spread" interactions were counted by light microscopy. The results, expressed as mean±SE relative to control, indicate that trypsinisation of the fibroblasts inhibited the phagocytic interaction but enhanced the "spread" interaction between aged neutrophils and fibroblasts. Under control conditions 4.9±0.8% of fibroblasts phagocytosed neutrophils and 37±12 neutrophils/5HPF "spread" on the fibroblast monolayer.

*p<0.01 **p<0.005 ***p<0.001

Fig. 5.2 Divalent cation dependency of aged neutrophil recognition by fibroblasts.

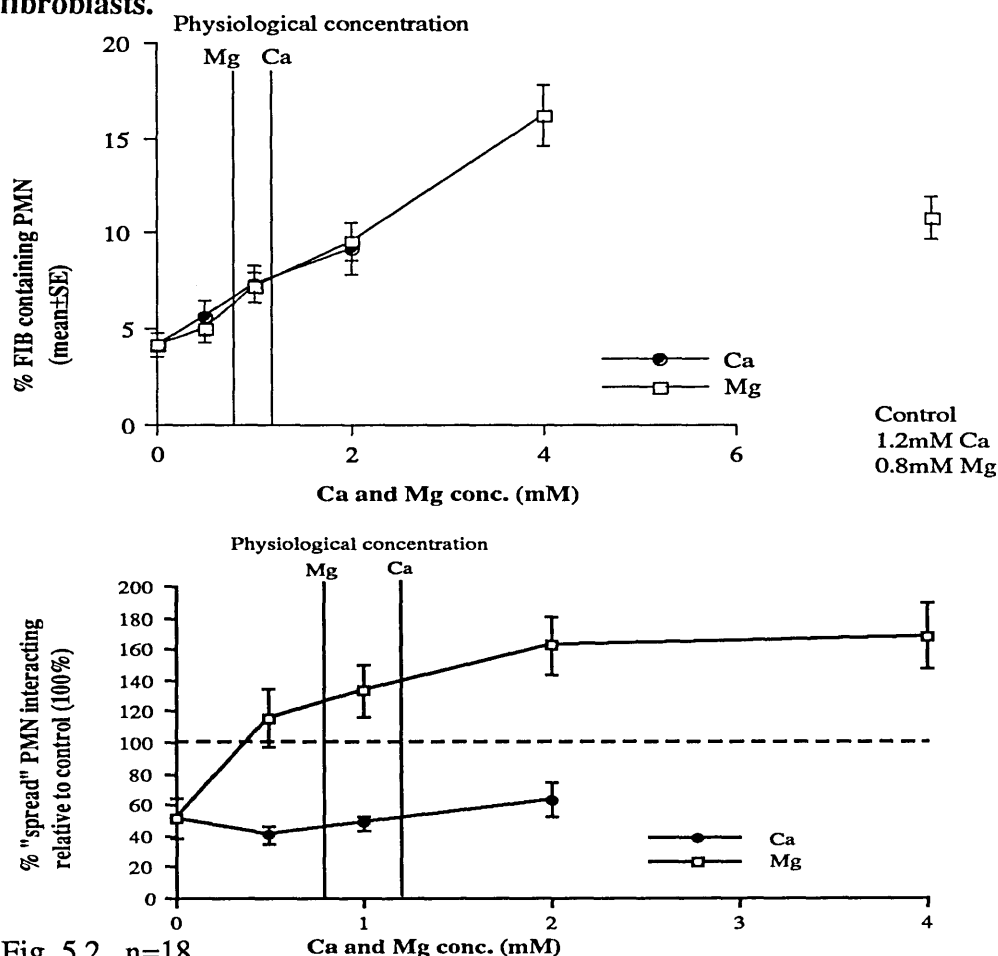


Fig. 5.2 n=18

Neutrophils, aged for 24h in culture, were washed in HBSS without Ca^{2+} or Mg^{2+} twice prior to resuspension in HBSS without Ca^{2+} or Mg^{2+} but with varying concentrations of Ca^{2+} and Mg^{2+} added to the incubation medium and being placed on washed, confluent fibroblast monolayers. After 1h at 37°C , the fibroblast monolayer was vigorously washed and the neutrophils in some wells visualised with MPO stain. The fibroblasts from other wells were trypsinised from the plate and the resulting cell suspension cytopun and stained with MPO and haemalum. The phagocytic and "spread" interactions were then counted by light microscopy. The results are presented as mean $\pm$ SE and indicate that both types of interaction were divalent cation dependent, but that the phagocytic interaction was equally dependent on both cations whilst Mg^{2+} was more potent than Ca^{2+} for the "spread" interaction. Under control conditions $10.4\pm 1\%$ of fibroblasts phagocytosed neutrophils and 11 ± 1 neutrophils/5HPF "spread" on the fibroblast monolayer.

interaction was still inhibited (Fig. 5.2).

Dependence on divalent cations was further demonstrated by the inhibitory effect seen when EDTA was dissolved in medium which previously contained standard concentrations of divalent cations (Fig. 5.3). EDTA chelates both Ca^{2+} and Mg^{2+} and "removes" them from the interaction mixture. EDTA was found to inhibit phagocytosis and adhesion of aged neutrophils to the fibroblast monolayer, thus confirming their divalent cation-dependency. Poly-l-lysine-coated latex bead phagocytosis by fibroblasts was found to be divalent cation-independent and, therefore, it is unlikely that EDTA was having any toxic effect on the fibroblast monolayer (Fig. 5.3).

The divalent cation dependency demonstrated here, together with the trypsin sensitivity of the fibroblast surface in this recognition mechanism, suggested the possibility that an integrin on the fibroblast surface was involved in aged neutrophil interaction with fibroblasts.

5.3 Involvement of the RGD-recognition sequence in fibroblast/aged neutrophil interaction.

Macrophage phagocytosis of apoptotic neutrophils has been shown to involve an integrin molecule, the Vn receptor (Savill, 1990), a β_3 integrin (see table 1.2 in chapter 1). Some of the integrin molecules, in particular the Vn receptor, are RGD-dependent (see table 1.2 in chapter 1), therefore the next series of experiments was performed to determine any dependence of the fibroblast/aged neutrophil interaction on the RGD recognition signal.

The trypsin sensitivity and sugar specificity of apoptotic aged neutrophil phagocytosis by fibroblasts was so similar to that exhibited during macrophage phagocytosis of apoptotic neutrophils (Savill, 1989b) that it seemed likely that

Fig. 5.3 The effect on aged neutrophil/fibroblast interaction and latex bead/fibroblast interaction of the presence of EDTA during incubation.

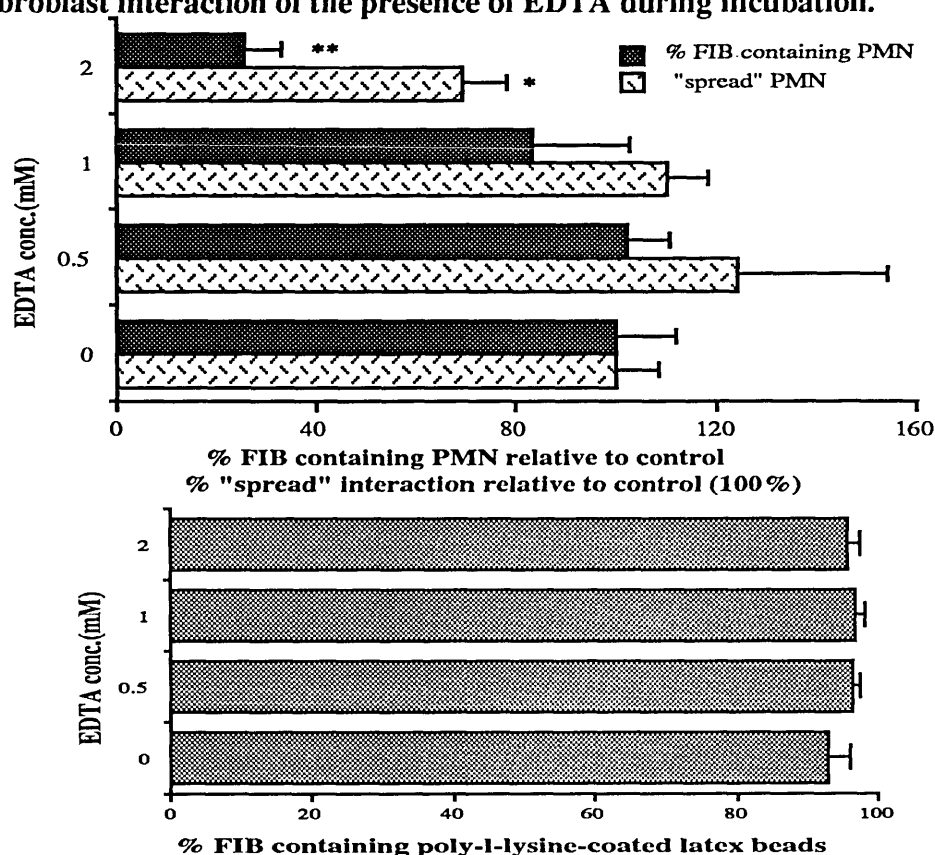


Fig. 5.3 n=18

Aged neutrophils were washed twice in HBSS prior to resuspension in solutions of varying concentrations of EDTA in HBSS. The neutrophil suspensions were then placed on confluent, fibroblast monolayers and incubated at 37°C for 1h. After vigorous washing, the neutrophils interacting in some of the wells were visualised with MPO stain. The other fibroblast wells were removed from the plate by trypsinisation and cytospun. The neutrophils on the cytospin preparation were stained with MPO stain and the fibroblasts counterstained with haemalum. The phagocytic and "spread" interactions were counted by light microscopy. Poly-l-lysine-coated latex beads were added to some fibroblast wells as a control phagocytic signal. The results are expressed as mean±SE relative to control and indicate that the presence of EDTA during interaction inhibited the phagocytic interaction between aged neutrophils and fibroblasts and also neutrophil "spreading" on the fibroblast monolayer, but had no effect on poly-l-lysine-coated latex bead phagocytosis by fibroblasts. Under control conditions 9.5±1% of fibroblasts phagocytosed neutrophils and 39±7 neutrophils/5HPF "spread" on the fibroblast monolayer.

*p<0.01 **p<0.005

the same Vn-binding structures involved in apoptotic neutrophil phagocytosis by macrophages could also be involved in apoptotic neutrophil phagocytosis by fibroblasts and therefore, was highly likely that the same "signals" determining the specific binding of Vn to cell surface receptors was involved.

Fn, Vn and the related adhesion molecules interact with a number of cell-surface adhesion receptors via a tripeptide sequence arg-gly-asp-(RGD) and the interaction can be inhibited by peptides as small as arg-gly-asp-ser (RGDS). The recognition is so specific that arg-gly-glu-ser (RGES), which has the same charge relationship (since aspartic acid is replaced by the similarly charged glutamic acid), does not inhibit attachment of these molecules to the cell surface. The RGD signal has been reported to be present on many cell surfaces and involved in mediating a number of diverse functions (Ruoslahti and Pierschbacher, 1987).

Addition of the tetrapeptide RGDS and the hexapeptide gly-arg-gly-asp-ser-pro (GRGDSP), but not the control hexapeptide gly-arg-gly-glu-pro (GRGESp), to the culture medium resulted in inhibition of phagocytosis of aged neutrophils by fibroblasts, but the "spread" interaction was unaffected by the presence of any of these peptides (Fig. 5.4). These results suggested that phagocytosis of the apoptotic neutrophils was mediated by the RGD signal.

5.4 Possible involvement of the vitronectin receptor in aged neutrophil phagocytosis by fibroblasts.

Studies of the effects of monoclonal antibodies upon macrophage phagocytosis of aged neutrophils indicate that the RGD-dependent structure on the macrophage surface involved in aged neutrophil phagocytosis is the Vn receptor (VnR; Savill et al., 1990).

Fig. 5.4 The effect on aged neutrophil/fibroblast interaction of RGD-containing peptides.

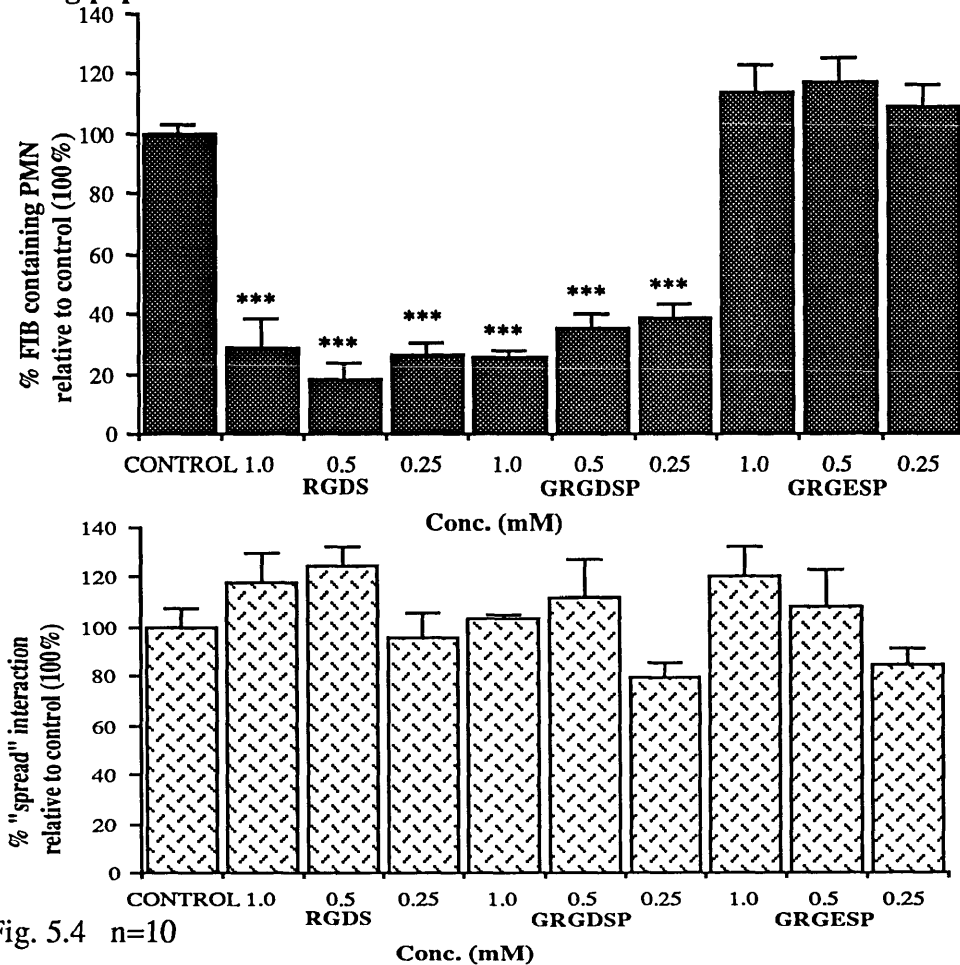


Fig. 5.4 n=10

Aged neutrophils were washed twice in HBSS prior to resuspension in HBSS or RGDS, GRGDSP or GRGESP at various concentrations. The neutrophil suspensions were then placed on washed, confluent fibroblast monolayers and incubated at 37°C for 1h. After vigorous washing, some of the neutrophils were visualised by MPO stain while other fibroblast wells were removed from the plate by trypsinisation and cytospun before the neutrophils were stained with MPO stain and the fibroblasts were counterstained with haemalum. The results are expressed as mean±SE relative to control and indicate that the phagocytic interaction between aged neutrophils and fibroblasts was inhibited by RGD-containing compounds, but not by RGE-containing compounds and that the "spread" interaction was not inhibited by the presence of RGD- or RGE-containing compounds. Under control conditions 5.9±0.4% of fibroblasts phagocytosed neutrophils and 109±20 neutrophils/5HPF "spread" on the fibroblast monolayer.

*** p<0.001

The VnR was first described on a fibroblastic cell line (Pytela et al., 1985b) and has also been immunoprecipitated from one of the cell lines used in this project (the Butler cell line; Savill et al., 1990), and we considered it highly likely to be involved in apoptotic neutrophil phagocytosis by fibroblasts. Therefore, the anti-vitronectin receptor antibody (13C2), was used at a concentration of 50µg/ml. Hermes-1, an IgG_{2a} antibody described in the methods section 2.6, was used as a control antibody for this series of experiments. The results indicated that both the phagocytic and adhesive interactions were inhibited in the presence of 13C2. The implication is that the VnR is involved in aged neutrophil recognition and phagocytosis by fibroblasts (Fig. 5.5).

Fig. 5.5 The effect of an anti-vitronectin receptor antibody on aged neutrophil recognition by fibroblasts.

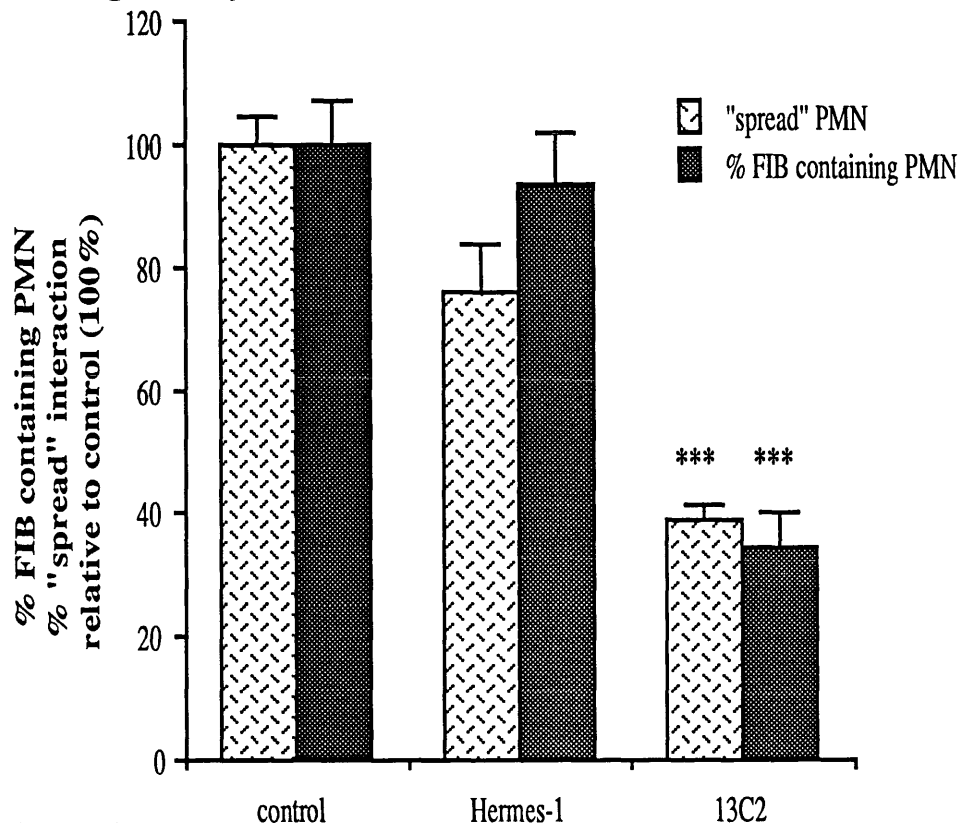


Fig. 5.5 n=4

Aged neutrophils were washed twice in HBSS prior to resuspension in HBSS, 50µg/ml 13C2 (an anti-vitronectin receptor antibody) or 50µg/ml of Hermes-1 (a control antibody). The neutrophils were placed on a washed fibroblast monolayer and after 1h at 37°C any non-adherent neutrophils were removed by washing. The interacting neutrophils in some fibroblast wells were stained with MPO stain and the number of "spread" neutrophils interacting with the fibroblast monolayer were counted. Fibroblasts from other wells were removed from the plate by trypsinisation and cytopspin. The neutrophils on the cytopspin preparation were then stained with MPO stain and the fibroblasts counterstained with haemalum. The percentage of fibroblasts containing neutrophils was then counted by light microscopy. The results are expressed as mean±SE relative to control and indicate that 13C2 inhibits both the phagocytic and "spread" interactions between aged neutrophils and fibroblasts. Under control conditions 8.6±0.6% of fibroblasts phagocytosed neutrophils and 224±55 neutrophils/5HPF "spread" on the fibroblast monolayer.

*** p<0.001.

Summary of chapter 5

(1) Fibroblast phagocytosis of aged neutrophils was found to be divalent cation dependent and to involve a trypsin sensitive structure on the surface of the fibroblast.

(2) The likelihood that the structure on the fibroblast surface was a member of the integrin superfamily was further suggested by RGD dependence and inhibition by the mAb 13C2. These data implied that, as for the macrophage, a β_3 integrin, the VnR, was critically involved in fibroblast uptake of apoptotic neutrophils.

CHAPTER 6

Introduction

In chapter 5, I concentrated on the similarities between macrophage and fibroblast recognition and phagocytosis of aged neutrophils. However, fibroblast recognition of aged neutrophils is inhibited by mannose and fucose whereas macrophage recognition is not, thus there are also some clear differences. Consequently, I went on to examine possible roles for receptors known to be inhibited by these sugars in fibroblast recognition of aged neutrophils.

6.1 The effect of mannose-containing compounds on aged neutrophil recognition by fibroblasts

The presence of a pinocytosis receptor, specific for mannose-fucose terminated glycoproteins, has been established on murine resident macrophages, thioglycollate-elicited macrophages, bone marrow-derived macrophages and liver Kupffer cells in culture. Due to its mannose and fucose specificity, the receptor is termed the mannosyl/fucosyl receptor (MFR).

Aged neutrophil interaction with fibroblasts was inhibited by mannose and fucose. I, therefore, went on to examine whether other molecules recognised by the MFR (mannose and fucose-rich glycoproteins) could also inhibit aged neutrophil/fibroblast interaction.

Mannan, RNaseB and fucoidin also bind to the MFR and would, therefore, have been expected to inhibit neutrophil recognition by the fibroblasts if the MFR were involved in this interaction (Blackwell et al., 1985). These agents were added to the interaction medium of fibroblasts and aged neutrophils. Mannan (a mannose-rich glycoprotein), RNaseB (which differs from RNaseA in that it has

a terminal mannose residue) and fucoidin (an L-fucose-rich sulphated polysaccharide) were all found to inhibit aged neutrophil recognition by fibroblasts. As in the case of mannose, they were found to inhibit the phagocytic interaction with fibroblasts but, in contrast to mannose (and fucoidin), the "spread" interaction in the presence of mannan and RNaseB was not significantly inhibited (Fig. 6.1).

When mannan and RNaseB were preincubated with aged neutrophils results implicated structures on the surface of the neutrophils in mannan-mediated inhibition, since the phagocytic interaction was inhibited following preincubation of the aged neutrophils with mannan (Fig. 6.2). RNaseB appeared to cause an inhibitory effect only when coincubated with the aged neutrophils and fibroblasts although the involvement of the fibroblast surface in the inhibitory action of both mannan and RNaseB remains unclear due to the difficulties in interpreting fibroblast preincubation data (ie. the possibility that molecules may become trapped in the fibroblast matrix).

It seemed unlikely, however, that the MFR itself was playing a role because freshly isolated neutrophils and fibroblasts bear no such receptor (Stahl and Gordon, 1982) although the signals recognised by the MFR appeared to be involved.

Molecules secreted by the liver and carried in blood serum can bind to mannose residues on cell surfaces by a divalent cation-dependent mechanism (Ezekowitz and Stahl, 1988). Were such structures bound to the mannose residues on the aged neutrophil surface, the inhibition by mannose, fucose and mannose- and fucose-rich glycoproteins could be explained. To test this hypothesis the aged neutrophils were pretreated with EDTA to remove any divalent cation-dependently-bound molecules; this was followed by coincubation with mannose during aged neutrophil and fibroblast incubation. The data from these experiments showed that mannose still caused inhibition of the interaction after EDTA treatment of the aged neutrophils (Fig. 6.3) thus indicating that the mannose inhibition was very unlikely to be related to effects on a serum-derived circulat-

Fig. 6.1 The effect of coincubating mannan, RNaseB and fucoidin with fibroblasts and aged neutrophils on aged neutrophil recognition by fibroblasts.

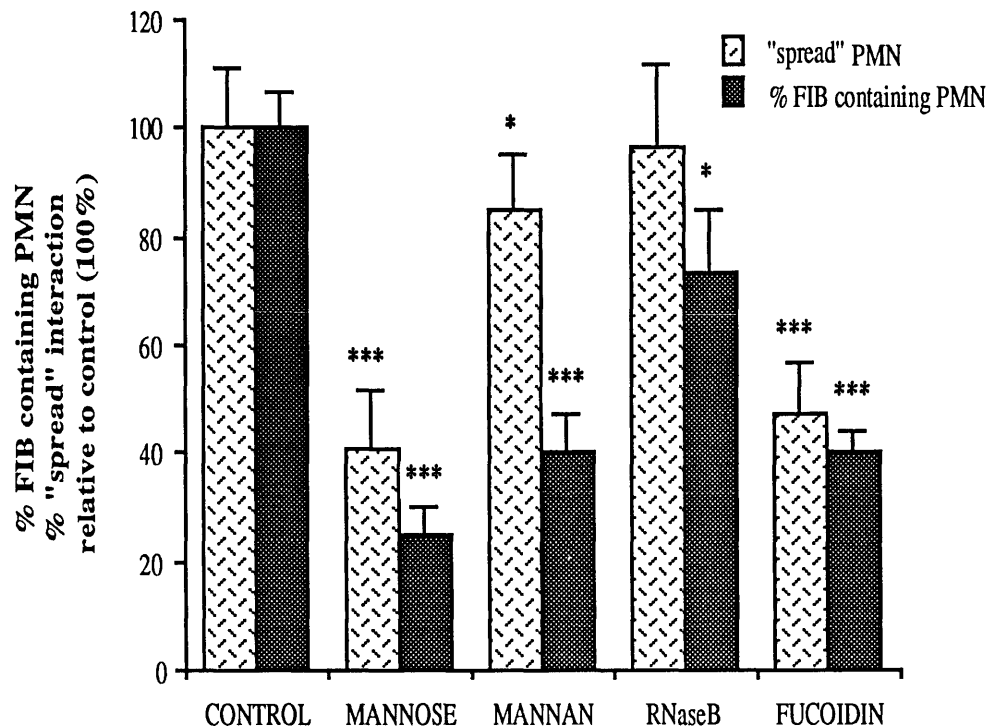


Fig. 6.1 n=30

Aged neutrophils were washed twice in HBSS prior to resuspension in HBSS or HBSS containing 1mg/ml of mannan, RNaseB or fucoidin. The neutrophil suspensions were then placed on washed, confluent fibroblast monolayers. After 1h at 37°C, the non-adherent neutrophils were washed off using ice-cold saline. The interacting neutrophils in some wells were visualised using MPO stain. The fibroblasts from other wells were removed by trypsinisation, cytospun and the neutrophils stained with MPO stain and the fibroblasts counterstained with haemalum. The "spread" and phagocytic interactions were then assessed by light microscopy. The results, expressed as mean±SE relative to control, indicate that mannan, RNaseB and fucoidin all significantly inhibited aged neutrophil phagocytosis by fibroblasts, but that only fucoidin inhibited aged neutrophil adhesion to fibroblast monolayers. 10mM mannose was included in this set of experiments for comparison to the previous data presented in chapter 4 (Fig. 4.5). Under control conditions 12.7±0.9% of fibroblasts phagocytosed neutrophils and 40±6 neutrophils/5HPF "spread" on the fibroblast monolayer.

* p<0.01 *** p<0.001

Fig. 6.2 The effect of preincubating aged neutrophils with mannan and RNaseB on aged neutrophil recognition by fibroblasts.

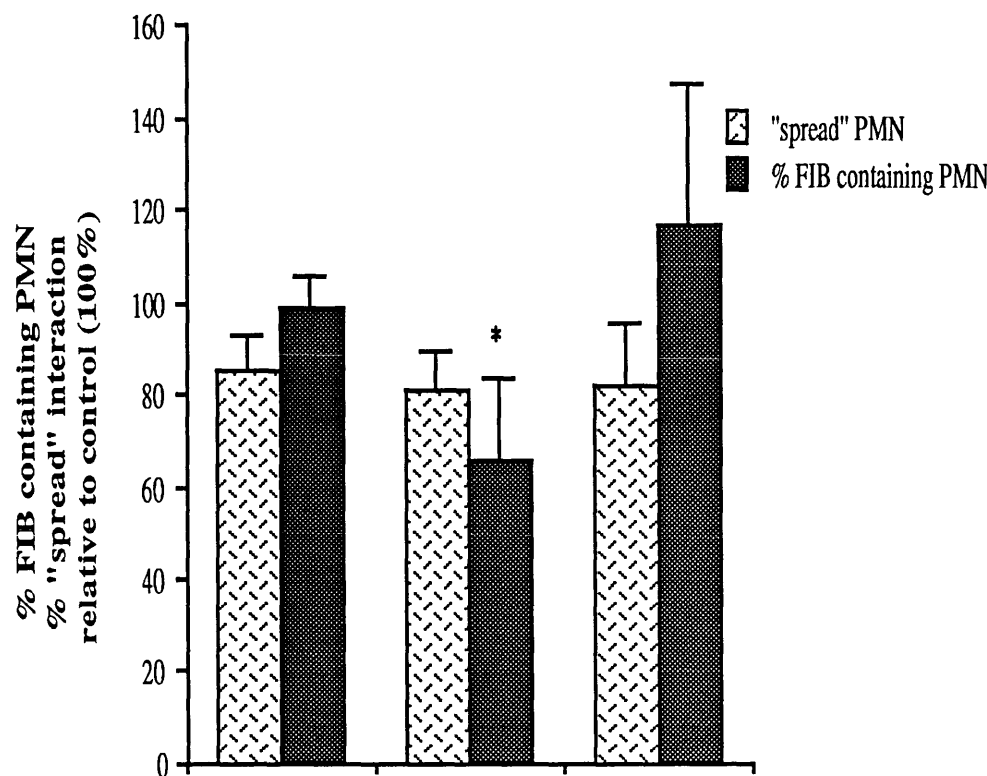


Fig. 6.2 n=14

CONTROL

MANNAN

RNaseB

Aged neutrophils were washed twice in HBSS prior to resuspension in HBSS or 1mg/ml mannan or RNaseB. After 30 min at 4°C, the neutrophils were washed twice in HBSS, resuspended in HBSS and placed on washed, confluent fibroblast monolayers. After 1h at 37°C, the non-adherent neutrophils were washed off the fibroblast monolayer. The neutrophils in some wells were stained with MPO stain, whilst the fibroblasts from other wells were removed from the plate by trypsinisation. The resulting cell suspension was cytospun, the neutrophils stained with MPO stain and the fibroblasts counterstained with haemalum. The phagocytic and "spread" interactions were then assessed by light microscopy. The results, expressed as mean±SE relative to control, indicate that preincubation of aged neutrophils with mannan resulted in subsequent inhibition of phagocytosis by fibroblasts, but that preincubation of aged neutrophils with RNaseB caused no inhibition. The "spread" interaction was not affected by preincubation of either mannan or RNaseB with aged neutrophils. Under control conditions 7.1±0.5% of fibroblasts phagocytosed neutrophils and 30±5 neutrophils/5HPF "spread" on the fibroblast monolayer. * p<0.01

Fig. 6.3 The effect of preincubating aged neutrophils with EDTA on subsequent inhibition of interaction by mannose.

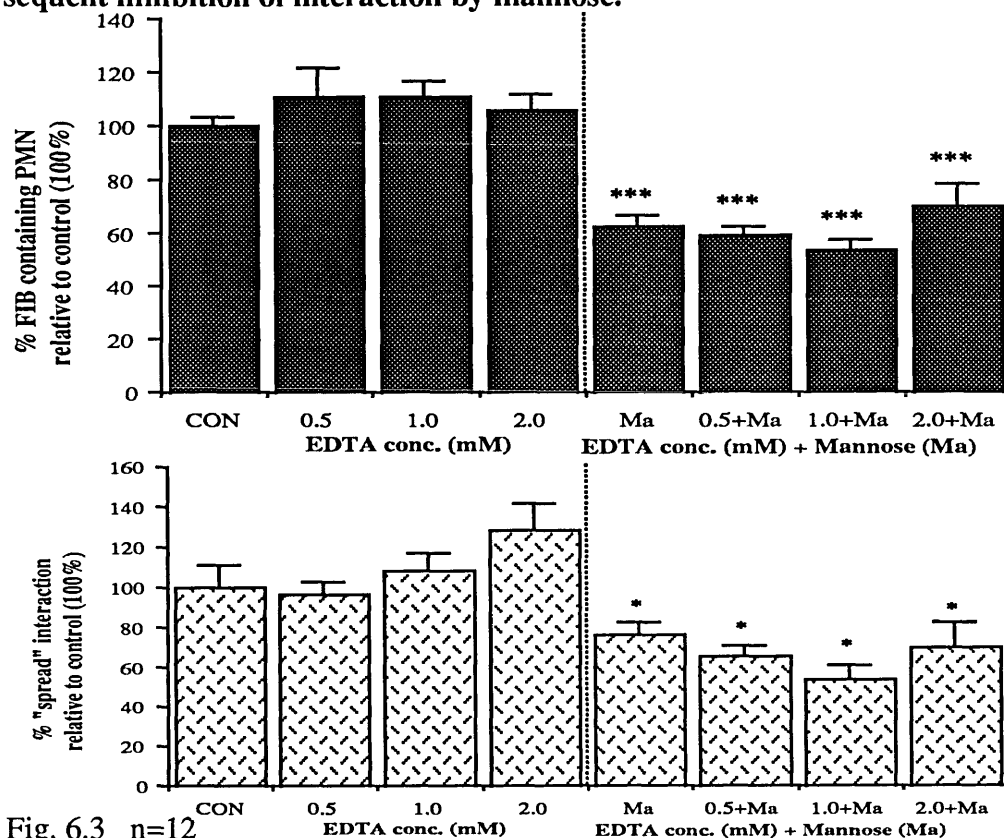


Fig. 6.3 n=12

Aged neutrophils were washed twice in HBSS prior to resuspension in HBSS or HBSS containing varying concentrations of EDTA. After 30 min at 4°C, the neutrophils were washed twice in HBSS and resuspended in HBSS or 10mM mannose. The cell suspensions were placed on fibroblast monolayers and incubated at 37°C for 1h. Any non-adherent neutrophils were then washed off and the remaining interacting neutrophils on some wells were stained with MPO stain. The fibroblasts from other wells were removed from the plate by trypsinisation and cytopspin. The neutrophils on the cytopspin preparations were stained using MPO stain and the fibroblasts counterstained with haemalum. The phagocytic and "spread" interactions were then assessed by light microscopy. The results are expressed as mean±SE relative to control and indicate that preincubation of the aged neutrophils with EDTA had no effect on subsequent inhibition of either the phagocytic or "spread" interactions between aged neutrophils and fibroblasts in the presence of mannose. Under control conditions 8.2±1% of fibroblasts phagocytosed neutrophils and 48±12 neutrophils/5HPF "spread" on the fibroblast monolayer. *p<0.01 ***p<0.001

ing mannose-inhibitable structure bound by divalent cations, or divalent cation-dependently, to the aged neutrophil surface.

Thus, it appears that neither the MFR nor a divalent cation-bound or divalent cation-dependently-bound mannose-binding protein were involved in aged neutrophil/fibroblast interaction thus other alternatives were considered.

6.2 The effect of mannose-6-phosphate and fructose-1-phosphate on aged neutrophil recognition by fibroblasts.

Mannose/fucose specificity has also been implicated in the adhesive interaction between rodent lymphocytes and rodent peripheral lymph node high endothelial venules (PNHEV) (Stoolman and Rosen, 1983). Indeed, there were further similarities: since the adhesive interaction between lymphocytes and PNHEV is (1) divalent cation dependent (ie. EDTA inhibits) and (2) it is inhibitable by brief trypsinisation of the lymphocyte (Rosen and Yednock, 1986; Stoolman et al., 1987). Trypsinisation of aged neutrophils caused inhibition of the phagocytic and "spread" interactions between aged neutrophils and fibroblasts and appeared to be dose related (Fig. 6.4). These results differ from trypsinisation of the fibroblasts in that the neutrophil/fibroblast "spread" interaction was inhibited rather than enhanced. Comparison with denatured trypsin (Fig. 6.5) indicated that the inhibition was not merely a steric effect since there was no inhibition using denatured trypsin in aged neutrophil preincubations.

The similarities between lymphocyte adhesion to PNHEV and aged neutrophil interaction with fibroblasts could imply that a molecule similar to that on the lymphocyte surface mediating binding to PNHEV is present on the aged neutrophil surface and involved in aged neutrophil binding to fibroblasts, so this possibility was investigated further.

Human lymphocyte adhesion to murine PNHEV has also been reported to be

Fig. 6.4 The effect on aged neutrophil recognition by fibroblasts of preincubating neutrophils with trypsin.

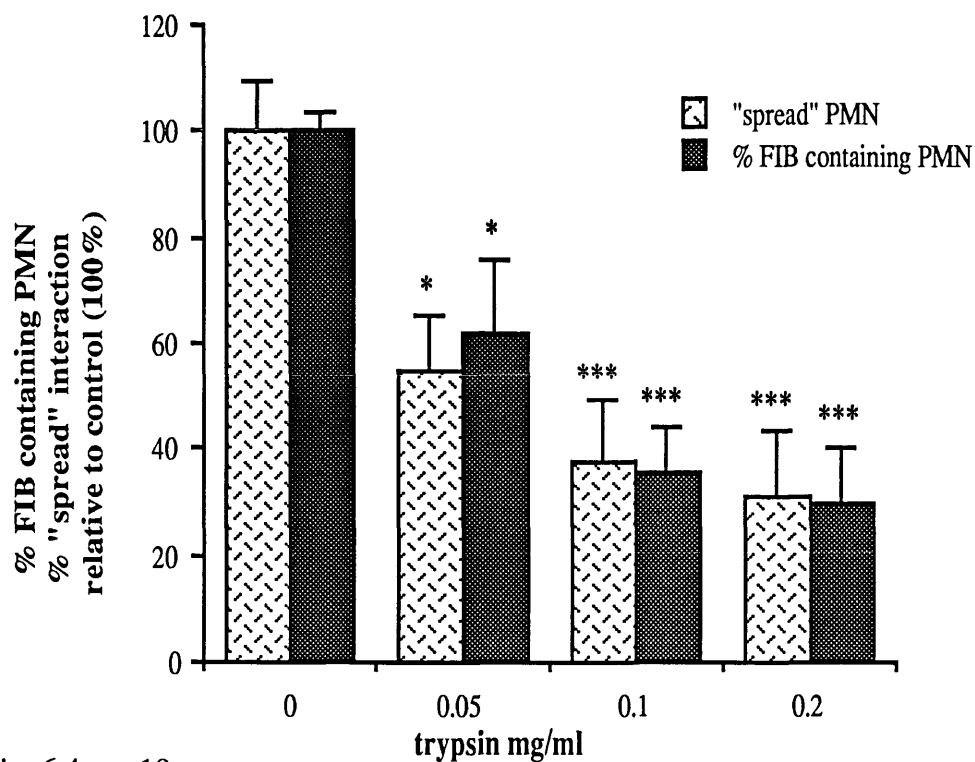


Fig. 6.4 n=18

Washed, aged neutrophils were resuspended in HBSS containing various concentrations of trypsin. After 30 min at 37°C, serum was added to inhibit further action of the trypsin and the neutrophils were washed twice in HBSS before resuspension in HBSS. The neutrophil suspensions were then placed on confluent fibroblast monolayers and incubated at 37°C for 1h. After this time the non-adherent neutrophils were removed by vigorous washing and the interacting neutrophils on some wells were stained with MPO stain. Other fibroblast wells were removed from the plate by trypsinisation and cytospun, the neutrophils on the cytospin preparations were stained using MPO stain and the fibroblasts were counterstained using haemalum. The phagocytic and "spread" interactions were then assessed by light microscopy. The results are expressed as mean±SE relative to control and indicate that trypsinisation of aged neutrophils led to subsequent inhibition of phagocytosis of aged neutrophils by the fibroblasts and inhibition of the adhesive interaction. Under control conditions 7.5±0.8% of fibroblasts phagocytosed neutrophils and 35±12 neutrophils/5HPF "spread" on the fibroblast monolayer.

* p<0.01 *** p<0.001

Fig. 6.5 The effect of preincubating aged neutrophils with denatured trypsin on subsequent aged neutrophil recognition by fibroblasts.

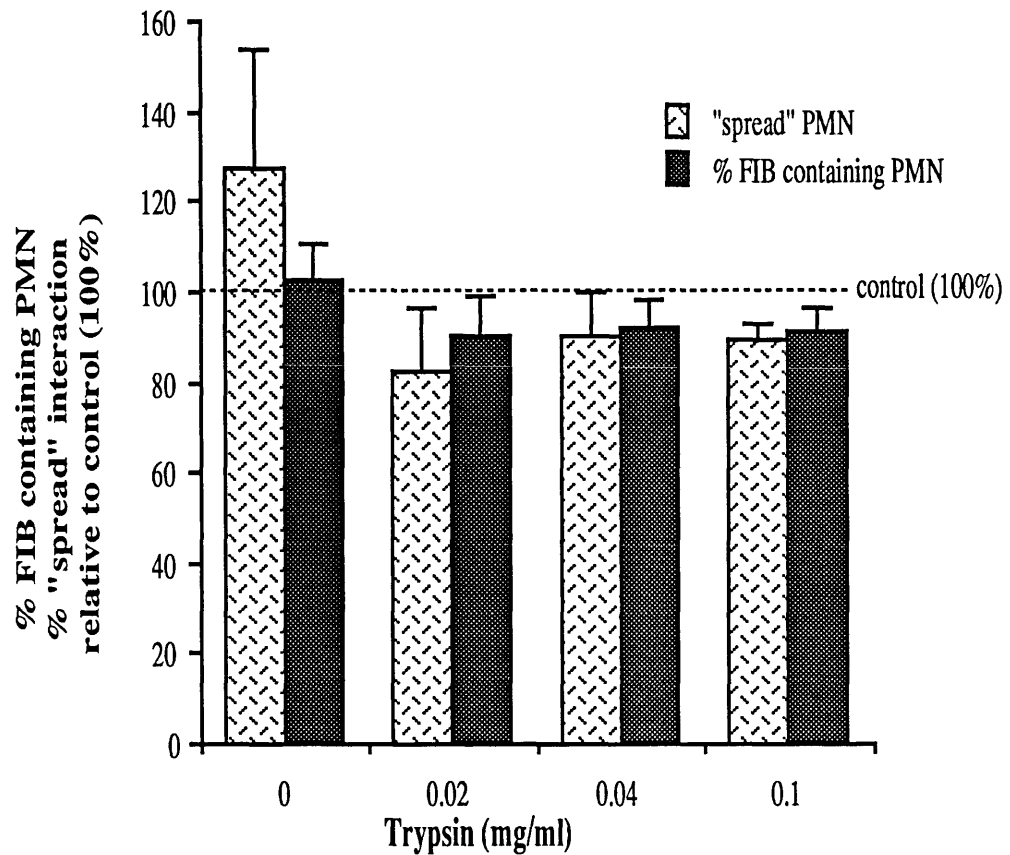


Fig. 6.5 n=10

Washed, aged neutrophils were resuspended in HBSS or HBSS containing various concentrations of denatured trypsin. After 30 min at 37°C, serum was added to the cell suspensions and they were washed twice in HBSS prior to resuspension in HBSS. The cell suspensions were then placed on washed, confluent fibroblast monolayers and incubated at 37°C for 1h. Any non-adherent neutrophils were washed off and the interacting neutrophils on some wells were stained with MPO stain. The fibroblasts from other wells were removed by trypsinisation and cytopun before the neutrophils were stained with MPO stain and the fibroblasts were counterstained with haemalum. The phagocytic and "spread" interactions were assessed by light microscopy. The results are expressed as mean±SE (taking the control value from Fig. 6.4 as 100%) and indicate that preincubation of aged neutrophils with denatured trypsin had no effect on subsequent phagocytic or "spread" interaction between aged neutrophils and fibroblasts. These results rule out the involvement of any steric effect of trypsin in the previous set of experiments.

inhibitable by mannose-6-phosphate and fructose-1-phosphate, but not mannose-1-phosphate and fructose-6-phosphate (Stoolman and Rosen, 1983; Stoolman et al., 1987) implying that the structures involved have additional specificity for sugar phosphates. Sugar phosphates were, therefore, added to the incubation medium for aged neutrophil/fibroblast interaction and it was observed that, as for lymphocyte binding to PNHEV, the phagocytic interaction was inhibited in the presence of mannose-6-phosphate and fructose-1-phosphate but not by mannose-1-phosphate or fructose-6-phosphate (Fig. 6.6). The "spread" interaction, however, was not inhibited significantly in the presence of any of these sugar phosphates.

In order to exclude the possibility that the inhibition observed was artefactual (ie. not due to binding of the aged neutrophils to the tissue culture plastic or due to a toxic effect on the fibroblasts), I examined aged neutrophil binding to tissue culture plastic and latex bead phagocytosis by fibroblasts. Sugar phosphates and RNaseB with the tissue culture plate showed no inhibition, in either assay, so indicating that the inhibition noted in fibroblast phagocytosis of aged neutrophils was not due to misinterpretation of inhibition of aged neutrophil binding to the tissue culture plastic (Fig. 6.7) nor was there likely to have been a toxic effect on the fibroblasts (Fig. 6.8).

A **mannose-6-phosphate receptor** has been reported to be present on the fibroblast surface and involved in endocytosis of lysosomal enzymes (Kaplan et al., 1977). However, it has been reported that there is little or no inhibition of the fibroblast and macrophage mannose-6-phosphate receptors in the presence of mannose and fucose (Kaplan et al., 1977; Hoflack and Kornfeld, 1985) and so it is very unlikely that the mannose-6-phosphate receptor is involved in aged neutrophil/fibroblast interaction.

Together these data strongly supported the possibility that a homing receptor-like molecule on the aged neutrophil surface was involved in aged neutrophil/fibroblast interaction. This was further investigated by the use of monoclonal antibodies (see below; sections 6.3 and 6.4).

Fig. 6.6 The effect on aged neutrophil recognition by fibroblasts of various sugar phosphates.

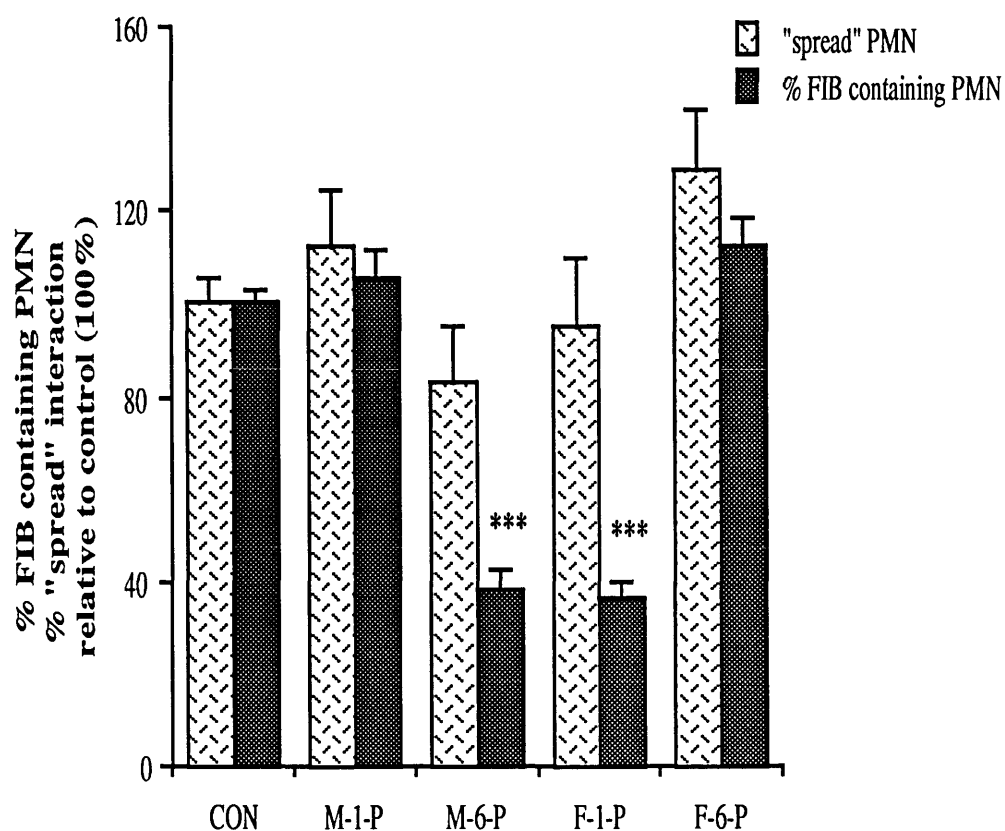


Fig. 6.6 n=16

Washed, aged neutrophils were resuspended in HBSS or 10mM solutions of sugar phosphates. The cell suspensions were then placed on fibroblast monolayers and incubated at 37°C for 1h. Non-adherent neutrophils were then removed by vigorous washing and the neutrophils on some wells were stained using MPO stain. The fibroblasts from other wells were removed from the plate by trypsinisation and cytospun. The neutrophils on the cytospin preparations were stained with MPO stain and the fibroblasts counterstained using haemalum. The phagocytic and "spread" interactions were then assessed by light microscopy. The results, expressed as mean±SE relative to control, indicate that phagocytosis of aged neutrophils by fibroblasts was inhibited in the presence of mannose-6-phosphate (M-6-P) and fructose-1-phosphate (F-1-P), but not mannose-1-phosphate (M-1-P) or fructose-6-phosphate (F-6-P) and that the "spread" interaction was unaffected by the presence of any of these sugar phosphates. Under control conditions 6±0.5% of fibroblasts phagocytosed neutrophils and 73±13 neutrophils/5HPF "spread" on the fibroblast monolayer. ***p<0.005

Fig. 6.7 The effect of RNaseB, mannose-6-phosphate and fructose-1-phosphate on aged neutrophil interaction with tissue culture plastic.

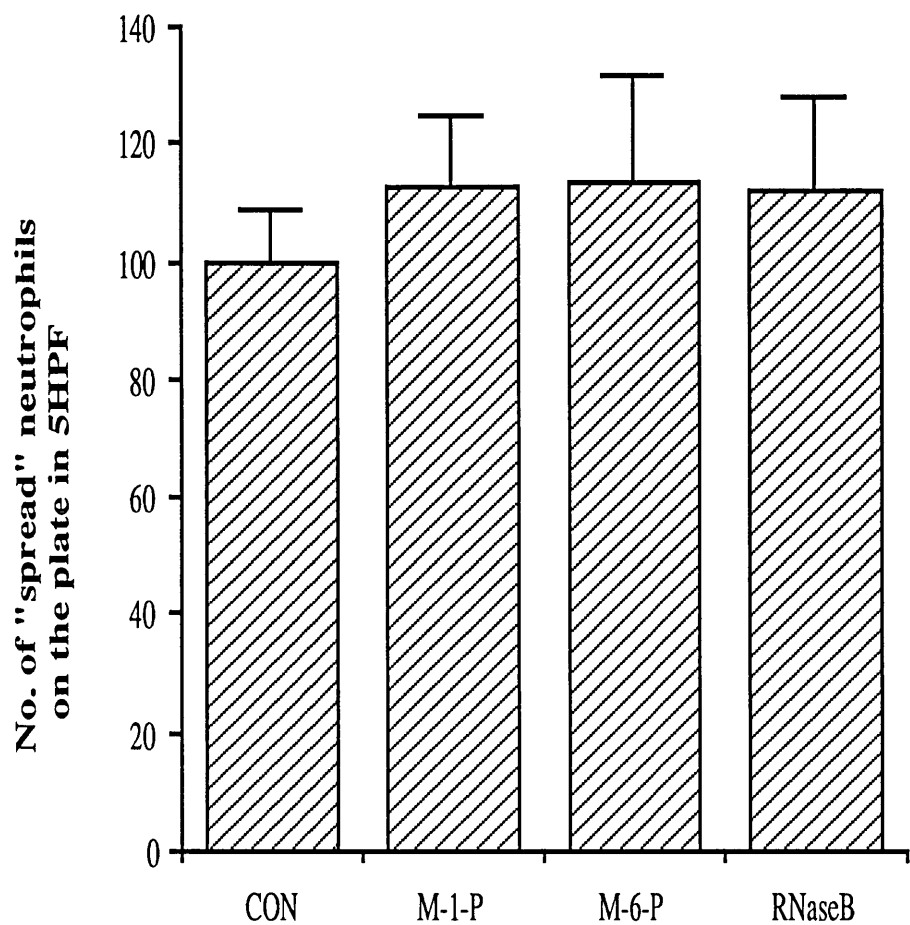


Fig. 6.7 n=4

Washed, aged neutrophils were resuspended in HBSS or 10mM mannose-6-phosphate (M-6-P) or fructose-1-phosphate (F-1-P) or 1mg/ml RNaseB. The cell suspensions were then placed on tissue culture plastic and incubated for 1h at 37°C. After this time, any non-adherent neutrophils were washed off and the remaining neutrophils were stained using MPO stain. The number of neutrophils remaining on the plate was counted by light microscopy and expressed as mean±SE relative to control (203±18 neutrophils/5HPF). The results indicate that the "spread" interaction between aged neutrophils and tissue culture plastic was unaffected in the presence of RNaseB, M-6-P or F-1-P. These data rule out the possibility that any inhibition of aged neutrophil interaction with fibroblasts in the presence of these compounds was due to inhibition of interaction with any aged neutrophils that are in contact with the plate.

Fig. 6.8 The effect of mannose-6-phosphate and fructose-1-phosphate on poly-l-lysine-coated latex bead phagocytosis by fibroblasts.

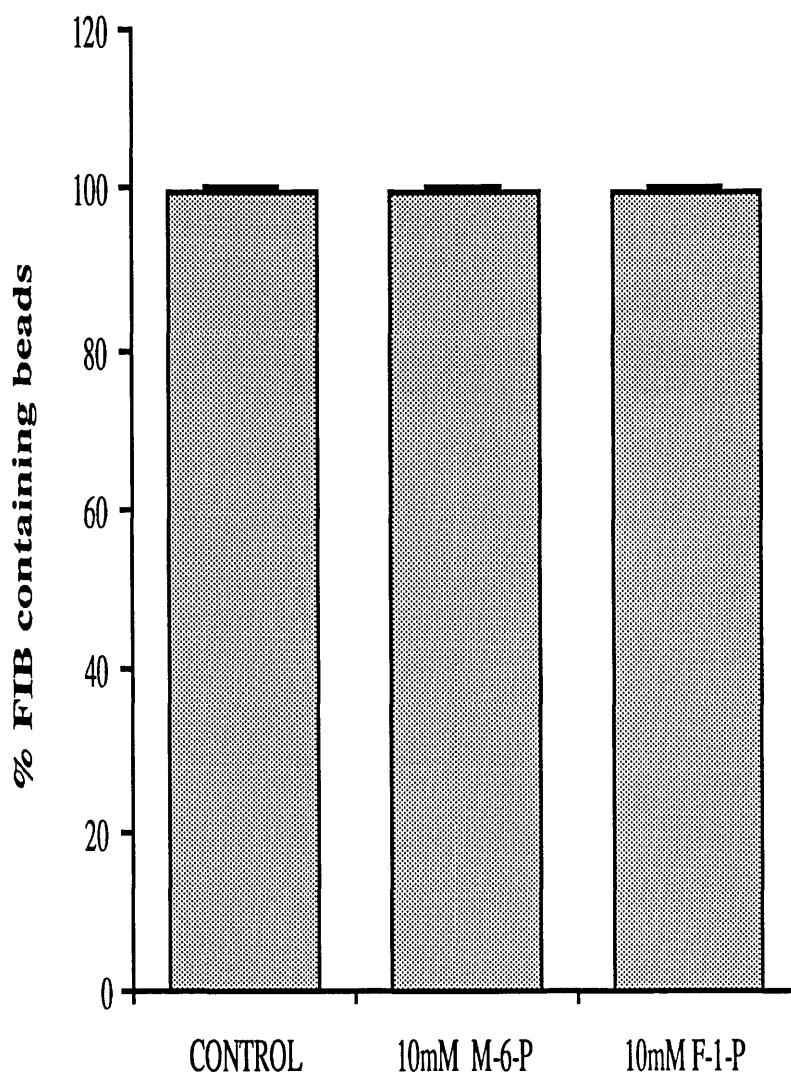


Fig. 6.8 $n=4$

Poly-l-lysine-coated latex beads were resuspended in HBSS or 10mM mannose-6-phosphate (M-6-P) or fructose-1-phosphate (F-1-P) and placed on confluent, fibroblast monolayers for 1h at 37°C. After this time any non-adherent beads were removed by vigorous washing and the fibroblasts removed from the plate by trypsinisation. After cytopspinning, the fibroblasts were visualised using haemalum and the extent of latex bead phagocytosis assessed by light microscopy. The results are expressed as mean $\pm$ SE and indicate that the presence of these sugars had no effect on latex bead phagocytosis by fibroblasts.

6.3 The possible involvement of a lymphocyte homing receptor in aged neutrophil recognition by fibroblasts : the effect of mAbMEL-14 on aged neutrophil/fibroblast interaction.

Adhesion of lymphocytes to PNHEV has been reported to be mediated by an antigen termed MEL-14 recognised by mAb MEL-14, raised against the murine lymphocyte surface antigen involved in lymphocyte binding to PNHEV (Galatin et al., 1983). Binding of mAb MEL-14 to its antigen inhibits lymphocyte binding to PNHEV. The Hermes-1 antibody, which is directed against the antigen involved in human lymphocyte adhesion to Peyer's patch HEV (Jalkanen et al., 1986a), does not inhibit lymphocyte binding to PNHEV and is thus useful as a control antibody in experiments involving these structures. Both mAb MEL-14 and mAb Hermes-1 are IgG_{2a} molecules.

To determine whether a MEL-14-like antigen might be involved in the fibroblast/aged neutrophil interaction, I looked at the effect of including mAb MEL-14 in the interaction. Since Hermes-1 antigen is known to be present on the fibroblast surface (Picker et al., 1989), mAb Hermes-1 could be used as a good control for mAb MEL-14 to rule out possible steric interference of the mAbs in the interaction.

Addition of these antibodies to the fibroblast system led to inhibition of both the phagocytic and "spread" interactions between aged neutrophils and fibroblasts in the presence of mAb MEL-14 while both types of interaction were unaffected by the presence of mAb Hermes-1 (Fig. 6.9). Preincubation of aged neutrophils with the antibodies followed by two washes prior to interaction led to inhibition of the phagocytic interaction. This was consistent with the inhibitory effect of mannose, fucose and mannan being, at least in part, at the neutrophil surface, observations which had implied that the homing receptor-like specificity resided on the aged neutrophil surface. The "spread" interaction was not inhibited (Fig. 6.10). Preincubation of fibroblasts was not studied due to the potential problems of trapping molecules in the fibroblast matrix which has been described previously.

Fig. 6.9 The effect on fibroblast recognition of aged neutrophils of mAb

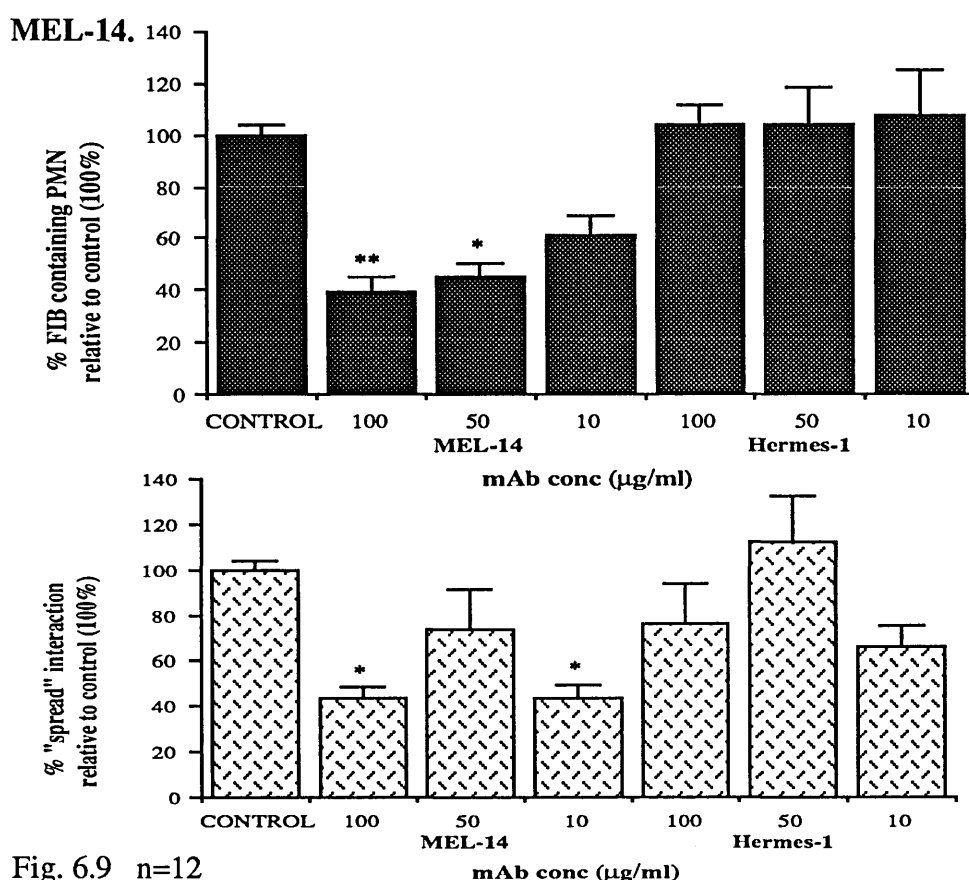


Fig. 6.9 n=12

Washed, aged neutrophils were resuspended in HBSS or various concentrations of mAb MEL-14 or mAb Hermes-1 and then placed on a fibroblast monolayer. After 1h at 37°C, the fibroblast monolayer was washed vigorously to remove any non-adherent neutrophils and the interacting neutrophils on some wells were stained using MPO stain. Fibroblasts from other wells were removed from the plate by trypsinisation and cytospun. The neutrophils on the cytospin preparation were stained using MPO stain and the fibroblasts were counterstained using haemalum. The phagocytic and "spread" interactions were assessed by light microscopy and expressed as mean±SE relative to control. Under control conditions 11.1±1.1% of fibroblasts phagocytosed neutrophils and 64±12 neutrophils/5HPF "spread" on the fibroblast monolayer. The results indicate that mAb MEL-14 inhibited the phagocytic interaction between aged neutrophils and fibroblasts in a concentration-dependent manner, but that inhibition of the "spread" interaction was very variable and not statistically significant in the presence of 50µg/ml of mAb MEL-14. The control mAb, Hermes-1, had no effect on either the phagocytic or the "spread" interactions. *p<0.01 **p<0.005

Fig. 6.10 The effect on aged neutrophil recognition by fibroblasts of pre-incubating the aged neutrophils with mAb MEL-14.

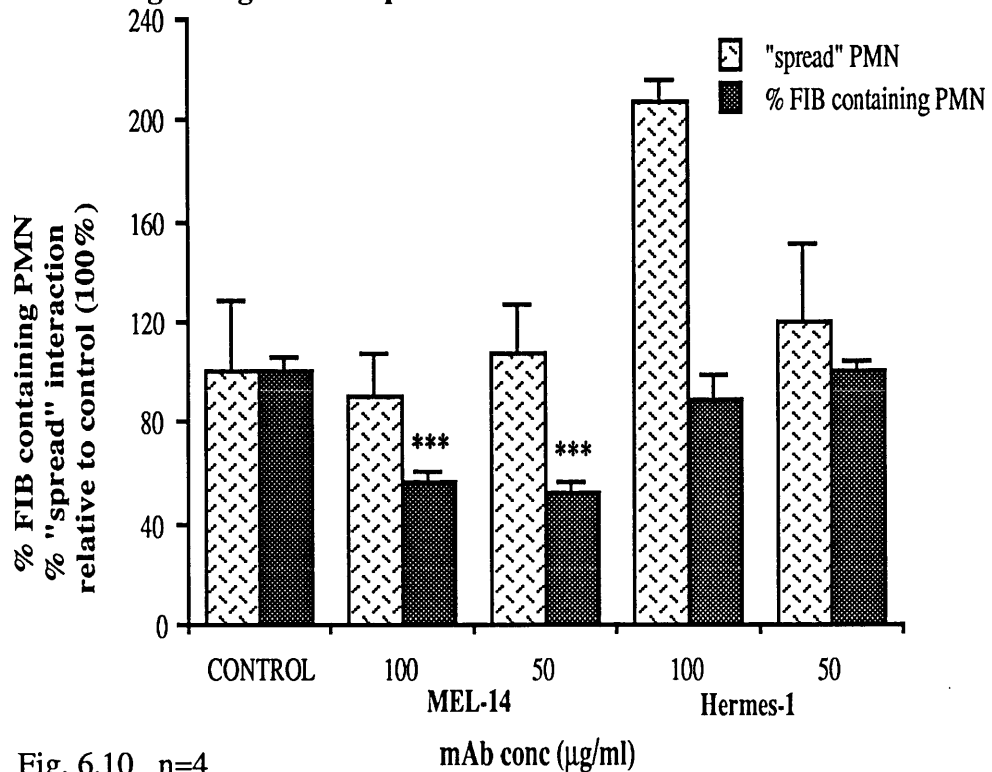


Fig. 6.10 n=4

Washed, aged neutrophils were resuspended in HBSS or various concentrations of mAb MEL-14 or mAb Hermes-1. After 30 min at 4°C, the neutrophils were washed twice in HBSS, resuspended in HBSS and placed on confluent fibroblast monolayers. After 1 h at 37°C, the non-adherent neutrophils were washed off and the interacting neutrophils on some wells were stained using MPO stain. The fibroblasts from other wells were removed by trypsinisation and cytospun. The resulting cytospin preparations were stained with MPO stain (to visualise the neutrophils) and haemalum (to visualise the fibroblasts). The phagocytic and "spread" interactions were assessed by light microscopy and expressed as mean±SE relative to control. Under control conditions 14.8±1.1% of fibroblasts phagocytosed neutrophils and 8±2 neutrophils/5HPF "spread" on the fibroblast monolayer. The results indicate that preincubation of the aged neutrophils with mAb MEL-14 resulted in subsequent inhibition of phagocytosis of the aged neutrophils by fibroblasts, but not of the "spread" interaction. Preincubation of aged neutrophils with the control antibody mAb Hermes-1 had no effect on subsequent phagocytic interaction, but resulted in an increase of the "spread" interaction.

***p<0.001

The Mel-14 antigen present on the rodent lymphocyte surface is termed gp90^{MEL-14}. Recent studies have indicated the presence of a very similar antigen on the surface of rodent neutrophils (termed gp100^{MEL-14}). This antigen has been shown to be present on fresh neutrophils and to be recognised by the mAb MEL-14 (Kishimoto et al., 1989).

Until very recently studies of homing receptors involved the use of mAb MEL-14 raised against the murine lymphocyte structure. My experiments however involved human neutrophils. Recently, Kishimoto raised mAb DREG-56 which is the human equivalent of mAb MEL-14 (Dr. Takashi Kishimoto, personal communication) and which I was able to use in further studies. Stimulation of rodent neutrophils results in cleaving of MEL-14 antigen from the neutrophil surface (Kishimoto et al., 1989). Kishimoto (personal communication) used this characteristic to cleave the human equivalent of MEL-14 antigen from the human neutrophil surface and raised a monoclonal antibody against the "dregs" remaining in the cell supernatant and named it mAb DREG-56. The DREG-56 antigen is down regulated after stimulation in a manner identical to MEL-14 antigen (Kishimoto, personal communication). Using mAb DREG-56 (IgG₁) and mAb MEL-14 (IgG_{2a}), both kindly donated by Dr. Eugene Butcher (Stanford, U.S.A.), at 50µg/ml in the incubation medium during aged neutrophil and fibroblast cocubation and using Hermes-1, also supplied by Dr. Butcher, as an IgG_{2a} control antibody the results show that mAb DREG-56 inhibited both the phagocytic and the "spread" interactions between aged neutrophils and fibroblasts. This was in contrast with mAb MEL-14 which did not significantly inhibit the "spread" interaction (Fig. 6.11).

6.4 The use of FACS analysis to assess the presence of DREG-56 antigen on the aged neutrophil surface.

FACS analysis was performed to assess the binding of mAb DREG-56 to aged neutrophils using P3 (Morganelli and Guyre, 1988) as an irrelevant IgG₁

Fig. 6.11 The effect of mAb DREG-56 on aged neutrophil recognition by fibroblasts.

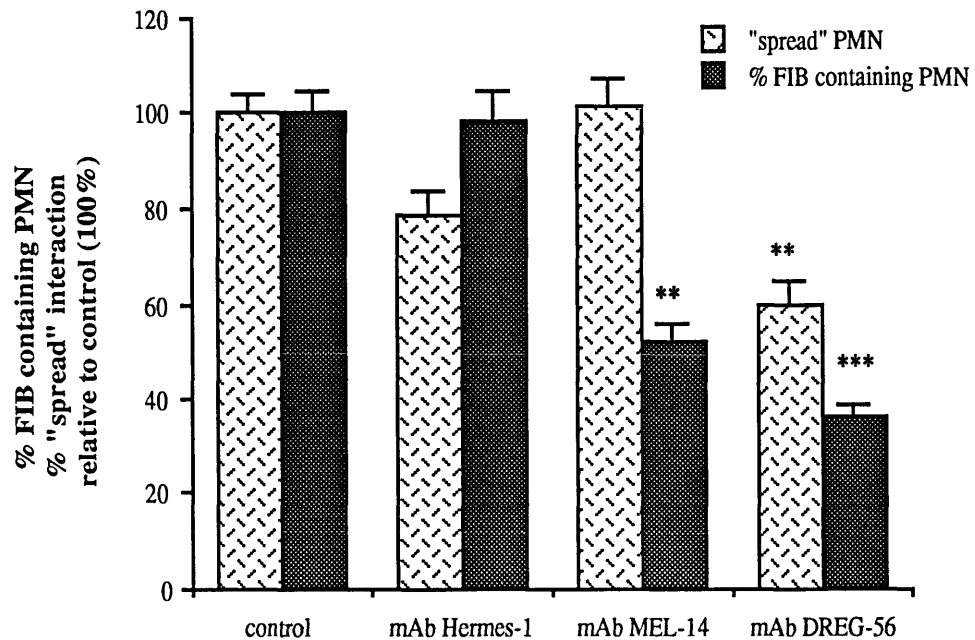


Fig. 6.11 n=6

Washed, aged neutrophils were resuspended in HBSS or 50µg/ml of mAb MEL-14, mAb DREG-56 or mAb Hermes-1. The cell suspensions were then placed on fibroblast monolayers and incubated at 37°C for 1h. After this time, the non-adherent neutrophils were removed by vigorous washing and the interacting neutrophils in some wells were stained using MPO stain. The fibroblasts from other wells were removed from the plate by trypsinisation and cytospun. The neutrophils on the cytospin preparations were stained using MPO stain and the fibroblasts were counterstained with haemalum. The phagocytic and "spread" interactions between aged neutrophils and fibroblasts were assessed by light microscopy and expressed as mean±SE relative to control. Under control conditions 8.6±0.6% of fibroblasts phagocytosed neutrophils and 224±55 neutrophils/5HPF "spread" on the fibroblast monolayer. The results indicate that the presence of mAb MEL-14 resulted in inhibition of phagocytosis of aged neutrophils by fibroblasts, but had no effect on the "spread" interaction. The presence of mAb DREG-56, however, resulted in inhibition of both types of interaction. The control antibody mAb Hermes-1 did not result in significant inhibition of either type of interaction.

** p<0.005 *** p<0.001

antibody and mAb Hermes-1 as a positive control for antibody binding structures on the aged neutrophil surface (Fig. 6.12). MAb DREG-56 was observed to bind to the aged neutrophils. These data indicated the presence of DREG-56 antigen on the aged neutrophil surface. It is interesting to note that despite the presence of Hermes-1 antigen on both the fibroblast and the aged neutrophil surface, there is no inhibition of aged neutrophil/fibroblast interaction in the presence of mAb Hermes-1.

Fig. 6.12 FACS analysis of the presence, or absence, of DREG-56 antigen on the aged neutrophil surface.

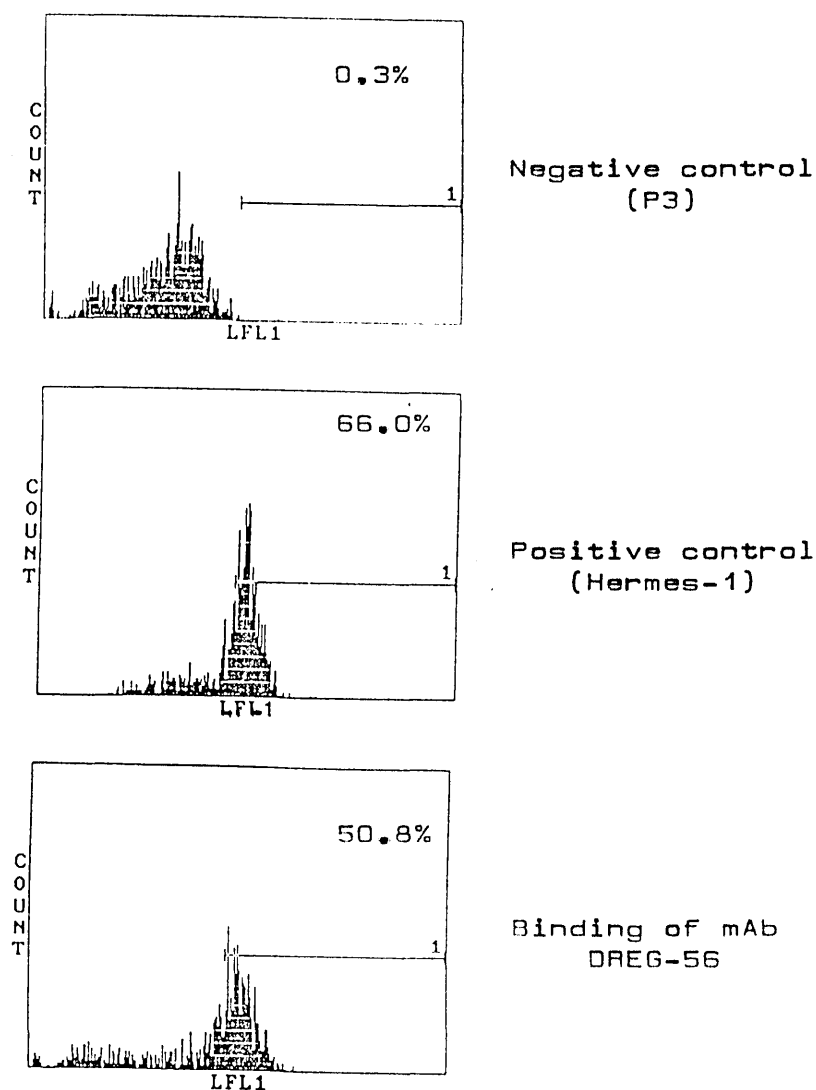


Fig. 6.12

The presence, or absence, of DREG-56 antigen was assessed using mAb DREG-56 to perform FACS analysis on aged neutrophils. P3, an irrelevant IgG₁, was used as a negative control, and Hermes-1 was used as a positive control.

In each case the photocopied fluorescence intensity histogram from the EPICS II print-out is shown together with the percentage greater than the gate. The same gate was used in assessment of the control neutrophils. The data indicate that DREG-56 antigen was present on the aged neutrophil surface.

SUMMARY OF CHAPTER 6

(1) Mannan, RNaseB, fucoidin, mannose-6-phosphate and fructose-1-phosphate inhibited phagocytosis of aged neutrophils by fibroblasts, but had no effect on "spread" interaction.

(2) EDTA pretreatment of the aged neutrophils had no effect on subsequent inhibition by mannose. This indicated that a divalent cation-bound, mannose-inhibitable receptor was not involved in fibroblast/aged neutrophil interaction.

(3) Trypsinisation of aged neutrophils resulted in inhibition of subsequent interaction and this, together with divalent cation-dependency and sugar specificity implied involvement of a homing receptor-like molecule in fibroblast/aged neutrophil interaction.

(4) Inhibition of interaction in the presence of antibodies to the lymphocyte homing receptor involved in lymphocyte adhesion to PNHEV and preincubation studies implied the involvement of a similar molecule on the aged neutrophil surface in the interaction between aged neutrophils and fibroblasts. Elucidation of the characteristics of the molecule present on the aged neutrophil surface and its distribution between aged apoptotic and non-apoptotic neutrophils requires further investigation.

CHAPTER 7: DISCUSSION

Introduction

This chapter serves to consolidate the data presented in chapters 3 to 6 to give an overall view of the mechanism of aged neutrophil recognition by fibroblasts.

As discussed fully in chapter 1, apoptosis is a widely occurring phenomenon in nucleated cells and represents a mode of cell death whereby the contents of a cell may be contained and which results in fast removal of the intact apoptotic cells by local phagocytes without obvious damage to the surrounding tissue or initiation of inflammation (Wyllie et al., 1980). Phagocytosis of intact aged neutrophils by macrophages has been observed both *in vitro* and *ex vivo* in joint fluid removed from arthritic knee joints (Savill et al., 1989a). However, since monocytes take at least 24h to mature to actively phagocytosing macrophages (Newman et al., 1982), it suggested that other cells might contribute to neutrophil removal prior to macrophage maturation or should the macrophage removal capacity become overwhelmed. The original observation, by Haslett, that fibroblasts, but not microvascular endothelial cells or epidermal keratinocytes, are able to recognise aged neutrophils implies that the fibroblast may act as an alternative to the macrophage for aged neutrophil disposal when the macrophages are immature or overwhelmed (Haslett, unpublished data). Alternatively it might represent a "non-ideal" fate for neutrophils. Phagocytosis of apoptotic neutrophils by macrophages does not provoke thromboxane release (Meagher et al., 1989). This indicates that macrophages may respond differently to apoptotic neutrophils than other phagocytic particles such that the removal process of aged neutrophils may not result in release of significant amounts of pro-inflammatory mediators. Fibroblast responses, however, have not yet been

studied.

7.1 Relationship of aged neutrophil recognition by fibroblasts to apoptosis

Macrophage phagocytosis of aged neutrophils has been shown to be closely related to the apoptotic index such that, as the proportion of aging neutrophils displaying the morphological features of apoptosis increases with time in culture, as does aged neutrophil recognition by macrophages (Savill et al., 1989a). Similar time course experiments for the fibroblast indicated that fibroblast interaction with neutrophils increased in parallel with apoptotic index up to the 9h time point, but that no further increase in total interaction was observed even though the apoptotic index continued to increase up to the 24h time point.

Subsequently, it was noted that two populations of aged neutrophils interacted with the fibroblast monolayer:- aged neutrophils which remained "round" after interaction with the fibroblasts and a much larger aged neutrophil population which became "spread" upon interaction with the fibroblast monolayer. Bearing this in mind, the time course interaction of neutrophils with fibroblasts was re-examined. The time course experiments were repeated, this time counting the plate interaction as "round" and "spread" neutrophils recognised by the fibroblasts. The "round" interaction was seen to increase steadily with time of the neutrophils in culture in parallel with neutrophil apoptotic index right up to the 24h time point. The "spread" interaction, which was responsible for much of the overall interaction however, increased only up to the 9h time point and then decreased up to 24h in culture. These findings explained the "levelling-off" of the recognition of aged neutrophils by fibroblasts between the 9h and 24h time points (a

phenomenon which was not seen in the macrophage/aged neutrophil interaction which is purely phagocytic).

A method was therefore developed to quantitate phagocytosis of aged neutrophils by fibroblasts. This was done by trypsinisation of the fibroblast monolayer and cytological preparation of the subsequent cell suspensions, following vigorous washing to remove any non-adherent neutrophils. The time-course results from this assay indicated that fibroblast phagocytosis of aging neutrophils increased in line with the apoptotic index in the neutrophil population. Counterflow centrifugation (elutriation, a method which allows separation of mixed aged neutrophil populations into fractions of varying apoptotic index) experiments further support the existence of two distinct types of aged neutrophil interaction with fibroblasts: one directly related to apoptosis of aged neutrophils and involving phagocytosis of the aged neutrophils by the fibroblasts, and the other independent of apoptosis and involving adhesion of the aged, non-apoptotic neutrophils to the fibroblast monolayer. The proportion of fibroblasts performing phagocytosis of the apoptotic aged neutrophils was small, but none the less indicated active removal of the neutrophils by the fibroblasts, a phenomenon which may be of relevance at the inflamed site. The assay method used may not represent the *in vivo* extent of neutrophil phagocytosis by fibroblasts in a very favourable light because: (1) most organs contain a large number of fibroblasts throughout their surface area, so the total extent of phagocytosis within an organ could then represent massive uptake of neutrophils; (2) *in vitro* assays can only be used to study a phenomenon but do not lend themselves to quantification of the extent of *in vivo* phagocytosis and (3) the presence of substances which may up or down regulate fibroblast phagocytosis *in vivo* have not yet been studied. The "spread" signal, although variable and difficult to interpret, was clearly separable from uptake.

As described previously, apoptosis represents a mode of cell death whereby the membrane integrity is maintained so that the potentially histotoxic contents

of the neutrophils are contained (Haslett et al., 1987). Consequent removal of the intact cell would, therefore, represent a method of injury-limiting neutrophil disposal. It has been reported that following phagocytosis of mast cells (Pillarisetti et al., 1983) and inert substances such as poly-l-lysine-coated or UV-irradiated latex beads, dextran sulphate or carbon particles (Werb and Reynolds, 1974), the fibroblasts have been observed to immediately increase collagenase production and the increased rate remains stable for over two weeks in the absence of further phagocytosis (Werb and Reynolds, 1974; Berman et al., 1979; Pillarisetti et al., 1983). In contrast, there is little change after phagocytosis in the extracellular levels of lysosomal enzymes such as cathepsin D and β -glucuronidase. It would in future, therefore, be of interest to study the effect of fibroblast phagocytosis of aged neutrophils on fibroblast function eg. matrix production. This has not been addressed in the present studies, but it is obviously of great importance in the link between inflammation and fibrosis to assess the functional responses of fibroblasts to ingestion of apoptotic neutrophils.

7.2 Sugar and amino acid inhibition of fibroblast recognition of aged neutrophils is, in part, similar to that of macrophage phagocytosis of aged neutrophils

Phagocytosis by fibroblasts is well described, but the mechanisms involved are poorly understood. Fibroblasts have been shown to phagocytose poly-l-lysine-coated and UV-irradiated latex beads (Okada et al., 1981); Fn-coated latex beads (Grinnell, 1980); carmine dye (Vasiliev et al., 1975); dextran sulphate, carbon particles and mycostatin (Werb and Reynolds, 1974); collagen (Melcher and Chan, 1981) and mast cell granules (Pillarisetti et al., 1983). The mechanism of these interactions has not to date been fully elucidated, so comparison of aged neutrophil phagocytosis by fibroblasts to fibroblast phagocytosis of other particles and cells was not possible. The

approach adopted herein was, therefore, to follow initially the mechanisms described for macrophage ingestion of apoptotic neutrophils (Savill et al., 1989a; Savill et al., 1989b; Savill et al., 1990).

As in the case of macrophage phagocytosis, serum was not required for recognition of aged neutrophils by fibroblasts. This would imply that an Fc receptor or a receptor for opsonic complement fragments was not involved in this recognition process. Duvall et al. (1985) studied recognition of apoptotic murine thymocytes by murine peritoneal macrophages (apoptosis followed exposure to corticosteroids). In order to avoid possible effects of macrophage-derived complement components they performed the recognition assays at 4°C and found that thymocyte binding to macrophages was inhibitable by 10mM N-acetylglucosamine and, to a lesser extent, by 10mM N-acetylgalactosamine and galactose. By preincubation experiments, they were able to "localise" the inhibitory effect of these sugars to the macrophage surface since preincubation of apoptotic thymocytes with these monosaccharides had no effect on their recognition by macrophages, whereas preincubation of the macrophages with these sugars led to inhibition of the interaction process. These results led them to suggest the involvement of a lectin-like receptor on the macrophage surface which recognised apoptotic cells.

Similar studies of the phagocytosis of human aged neutrophils by human macrophages in the presence of various monosaccharides gave very different results. Savill et al. (1989b) observed that N-acetylglucosamine, N-acetylgalactosamine and galactose (all at ranges between 5 and 50mM) had no inhibitory effect on macrophage uptake of aged neutrophils, but that the amino sugars glucosamine, galactosamine and mannosamine (also all at ranges between 5 and 50mM) strongly and specifically inhibited the recognition. Due to different assay conditions, it is difficult to make direct comparisons between thymocyte binding to macrophages and aged neutrophil phagocytosis by macrophages. However, it is unlikely that a lectin-like mechanism is

relevant in phagocytosis of apoptotic neutrophils by macrophages at physiological temperatures (ie. 37°C). Savill et al. (1989b) also performed preincubation studies and found that the inhibitory effects of amino sugars was "localised" to the aged neutrophil surface. Thus, there was no evidence for involvement of a lectin on the macrophage surface.

Since one difference between the amino sugars and N-acetyl derivatives is that the free amino group allows the amino sugars to act as weak bases such that at pH7.4 the majority of molecules of each of these amino sugars are cationically charged, it seemed possible that the inhibitory effect of the amino sugars was related to their charge. So, Savill et al. (1989b) investigated the effect of various amino acids on macrophage phagocytosis of aged neutrophils. Their results showed that the neutral amino acids L- and D-glutamine and the anionic amino acid L-glutamic acid had no effect on recognition, but that L-lysine and L-arginine, which have cationically charged R groups at pH7.4, did inhibit recognition of aged neutrophils by macrophages. Again, this inhibition was "localised" to the aged neutrophil surface.

Similar studies were performed for fibroblast recognition of aged neutrophils where it was also noted that 10mM N-acetylglucosamine, N-acetylgalactosamine and galactose had no inhibitory effect on fibroblast recognition of aged neutrophils at 37°C. As was shown for macrophage recognition, however, fibroblast recognition of aged neutrophils was inhibitable by 10mM glucosamine and galactosamine. However, by contrast with the macrophage/aged neutrophil system, 10mM mannose and fucose were also found to inhibit recognition. After further investigation of the nature of the fibroblast/aged neutrophil interaction, I found that the phagocytic interaction was inhibited by amino sugars, mannose and fucose, but that the "spread" interaction was inhibited only by mannose and fucose.

The results from these experiments suggested that two interaction mechanisms could be involved in aged neutrophil recognition by fibroblasts; one which is

similar to macrophage phagocytosis of aged neutrophils and involved phagocytosis of the apoptotic aged neutrophils by fibroblasts and another which involved "spreading" of non-apoptotic aged neutrophils on the fibroblast monolayer and involving a different mechanism (see table 7.1, page 162).

When amino acids were included in the incubation medium containing aged neutrophils with fibroblasts, it was noted that, as in the case of macrophage phagocytosis of aged neutrophils, the cationic amino acids L-arginine and L-lysine inhibited the total interaction between aged neutrophils and fibroblasts. In further experiments, L-arginine was found to inhibit the phagocytic interaction, between aged neutrophils and fibroblasts, but the "spread" interaction was not significantly inhibited. The neutral amino acid, L-glutamine, and the anionic amino acid, L-glutamic acid, had no effect on the recognition process. The implication here is that phagocytosis of aged neutrophils by fibroblasts is inhibited by amino sugars and basic amino acids in a way similar to macrophage phagocytosis of aged neutrophils and, therefore, that a similar mechanism may be involved in aged neutrophil phagocytosis by both macrophages and fibroblasts.

Preincubation of aged neutrophils has indicated that amino sugars, L-arginine, mannose and fucose interfered with fibroblast recognition of aged neutrophils at the aged neutrophil surface.

7.3 Divalent cation dependency and trypsin sensitivity of aged neutrophil recognition by fibroblasts

The inhibition by amino sugars and basic amino acids which I have described above, has been previously described for the adhesive interactions of activated platelets with erythrocytes, monocytes and other platelets (Gartner et al.,

1978; Silverstein and Nachman, 1987). At a molecular level, these monosaccharides and amino acids have also been reported to inhibit adhesive interactions of the RGD-bearing proteins such as Fn, fibrinogen and thrombospondin between each other and with cell surface molecules including the platelet integrin, GPIIbIIIa (Yamada et al., 1975; Pearlstein, 1976; Gartner et al., 1978; Leung and Nachman, 1982; Nachman and Leung, 1982; and Silverstein and Nachman, 1987) and also of Fn-mediated adhesion of fibroblastic cells to collagen-coated surfaces (Pearlstein, 1976).

Integrin-mediated adhesion is also Mg^{2+} and Ca^{2+} dependent (Hynes, 1987; Ruoslahti and Pierschbacher, 1987). In particular, fibroblast adhesion to plates coated with Vn or Fn is Ca^{2+} and Mg^{2+} dependent (Shimo-Oka and Hasegawa, 1988) and macrophage phagocytosis of apoptotic neutrophils also was found to be divalent cation dependent, with Mg^{2+} being more potent than Ca^{2+} (Savill et al., 1990). This pattern of cation dependency was similar to that exhibited by the surface structures mediating adhesion of monocyte/macrophage to surfaces coated with Fn (Bevilacqua et al., 1981). It is not, however, the only pattern reported: Ca^{2+} has been found to be more potent for baby hamster kidney cell attachment to Fn (Edwards et al., 1987). Fibroblast interaction with aged neutrophils was also found to be divalent cation dependent.

A further property of integrin mediated adhesion is its sensitivity to trypsin in the presence of divalent cations. This has been reported for the structures mediating adhesion of monocytic cells to Fn-coated surfaces (Bevilacqua et al., 1981) and also for Fn-coated latex bead adhesion to and phagocytosis by fibroblasts, although this only occurred in the absence of divalent cations (McAbee and Grinnell, 1985). The macrophage structures involved in phagocytosis of aged neutrophils were also found to be sensitive to brief trypsin exposure in the presence of divalent cations prior to coincubation with aged neutrophils, whilst preincubation of aged neutrophils with trypsin in the presence of divalent cations had no effect on subsequent interaction with

macrophages (Savill, 1990). Light trypsinisation of fibroblasts in the presence of divalent cations prior to interaction with aged neutrophils was found to inhibit the phagocytic signal, but not the "spread" signal (table 7.1, page 162). This indicated a definite difference in adhesive and phagocytic interaction of fibroblasts. Trypsin sensitivity of fibroblast phagocytosis of aged neutrophils at the fibroblast surface implied further similarity between macrophage and fibroblast phagocytosis of aged neutrophils.

As described in chapter 1 (section 1.10), the RGD sequence in one of the cell-binding domains of the Fn molecule is important in the interaction between Fn and some cell surfaces (Pierschbacher and Ruoslahti, 1984; Hynes, 1987; and Ruoslahti and Pierschbacher, 1987). The inhibitory effect of peptides bearing the RGDS-signal, but not of those bearing RGES, represents specific inhibition of recognition of an RGD-dependent integrin site in such adhesive interactions. Aged neutrophil phagocytosis by macrophages was shown to be specifically inhibited by the tetrapeptide RGDS, but not RGES, at concentrations known to inhibit integrin binding to ligands bearing the RGD adhesion signal (Savill et al., 1990).

Similarly, when aged neutrophils were coincubated with fibroblasts in the presence of the tetrapeptide, RGDS, and the hexapeptides GRGDSP and GRGES, the RGD-containing peptides caused inhibition of the phagocytic interaction between aged neutrophils and fibroblasts while GRGES had no effect on these interactions. In contrast, the "spread" interaction was inhibited by none of these synthetic peptides, further evidence to support two distinct mechanisms of interaction for adhesion and phagocytosis of aged neutrophils by fibroblasts (table 7.1, page 162).

7.4 A fibroblast surface structure involved in fibroblast phagocytosis of aged neutrophils is the vitronectin receptor which has been shown to participate in macrophage phagocytosis of aged neutrophils

Macrophage phagocytosis of aged neutrophils was also shown to be specifically inhibited by the RGD-bearing proteins Vn and Fn whether in solution in the interaction or coated on the tissue culture plate as a substrate for the macrophages to bind to prior to interaction (Savill et al., 1990). This inhibition was localised to the macrophage surface on the following grounds: firstly, failure of apoptotic neutrophils to adhere to Fn-coated surfaces or beads and secondly, the lack of inhibition of aged neutrophil phagocytosis by macrophages following preincubation of aged neutrophils with soluble Fn, whereas macrophage preincubation with soluble Fn did cause subsequent inhibition of phagocytosis (Savill et al., 1990). It appeared, therefore, that a macrophage integrin was participating directly in RGD- and divalent cation-dependent aged neutrophil phagocytosis.

Savill et al. (1989a; 1989b; 1990) found, in their studies of macrophage phagocytosis of apoptotic neutrophils, similar patterns of sugar inhibition, divalent cation dependence, trypsin sensitivity and RGD dependence which led to the likely involvement of a β_3 integrin in the interaction. They found that a large number of anti GPIIb/IIIa mAbs had no effect on aged neutrophil phagocytosis by macrophages, but that a variety of antivitronection receptor mAbs, including 13C2, resulted in marked inhibition.

With this data in mind, 13C2 an α_{VnR} mAb was added to the medium for a fibroblast/aged neutrophil interaction assay. Preliminary results indicated a very strong inhibition of both the phagocytic and "spread" interactions with fibroblasts in the presence of 13C2 suggesting that, as for macrophage phagocytosis of aged neutrophils, the VnR was involved in fibroblast

phagocytosis of aged neutrophils. Further, it is known that the VnR is present on the fibroblast surface (Pytela et al., 1985b) and on one of the fibroblast cell lines used in this project (the Butler cell line; Savill et al., 1990). Inhibition of the "spread" interaction in the presence of 13C2 may be explained by the presence of an epitope on the VnR whose binding is not inhibited by RGD-bearing peptides. This would explain why the "spread" interaction was inhibitable by 13C2, but not by amino sugars, basic amino acids, trypsinisation of the fibroblast surface or RGD-bearing proteins.

The data so far obtained for aged neutrophil phagocytosis by fibroblasts indicated that a major component of this recognition process was very similar to that of macrophage phagocytosis of aged neutrophils as evidenced by: (1) inhibition by amino sugars and basic amino acids, (2) divalent cation dependency, (3) trypsin sensitivity at the fibroblast surface and (4) inhibition by RGD-bearing proteins. The implication was, therefore, involvement of the VnR on the fibroblast surface and an amino sugar-inhibitable site on the aged neutrophil surface. However, it should be further supported by the use of other mAbs and identification of the ligand. This will be the subject of further study in Dr. Haslett's group.

The remainder of my thesis was concerned with further analysis of aspects of fibroblast/aged neutrophil recognition mechanisms which were different from the macrophage/aged neutrophil recognition system.

7.5 Discussion of possible role of the mannosyl/fucosyl receptor in aged neutrophil interaction with fibroblasts

A mechanism in aged neutrophil recognition by fibroblasts dissimilar to the macrophage was suggested by the inhibition of the total interaction by mannose and fucose which had no effect on macrophage phagocytosis of

apoptotic neutrophils (Savill et al., 1989b).

Having noted inhibition of aged neutrophil interaction with fibroblasts by mannose and fucose, the probability of the involvement of a mannosyl/fucosyl receptor (MFR) in the interaction was investigated. This required assessment of whether the signals employed by the MFR were, indeed, involved in aged neutrophil interaction with fibroblasts. I, therefore, looked at the effect of mannan (a mannose-rich glycoprotein), RNaseB (which differs from RNaseA in that it has a terminal mannose residue) and fucoidin (an L-fucose rich sulphated polysaccharide) on aged neutrophil recognition by fibroblasts since they all inhibit the MFR. They were also found to inhibit interaction between aged neutrophils and fibroblasts, inhibiting phagocytic and "spread" interactions. Preincubation studies indicated that mannose, fucose and mannan were having an inhibitory effect via the aged neutrophil surface. This strongly implied that the structure with mannose/fucose specificity was on the aged neutrophil surface. The MFR is also Ca^{2+} -dependent and trypsin sensitive (Stahl et al., 1978; Stahl et al., 1980), characteristics which had been noted for fibroblast/aged neutrophil interaction. However, neither fibroblasts nor freshly isolated neutrophils possess such a surface receptor (Stahl and Gordon, 1982). However, since Stahl and Gordon looked only at freshly isolated neutrophils, it did not rule out the possibility of exposure of an MFR, or MFR-like receptor, during aging of the neutrophils. My further studies have elucidated a likely role for another molecule on the neutrophil surface with similar sugar specificities (the lymphocyte homing receptor). However, further experiments could be performed to confirm or totally exclude the involvement of a MFR-like molecule in the aged neutrophil/fibroblast interaction by ascertaining whether or not a MFR-like structure is exposed on the neutrophil surface during aging. Two experiments could be performed:

- (1) ^{125}I -mannose-BSA binding to the aged neutrophil surface could be assessed in a method similar to that used by Stahl and Gordon (1982) when they showed that the MFR was not present on the freshly isolated neutrophil or

fibroblast surface.

(2) Binding of MFR antibodies to the aged neutrophil surface which could then be assessed by flow cytometry.

Another receptor which employs the same recognition signals as the MFR is a mannose-binding protein which is secreted by the liver and is found circulating in blood serum (Ezekowitz and Stahl, 1988). This molecule is capable of binding to cells via mannose residues in a cation-dependent manner. Such a structure could bind to the aged neutrophil surface and participate in aged neutrophil/fibroblast interaction. However, EDTA pretreatment of aged neutrophils, to remove any molecules which are divalent cation-dependently bound to the neutrophil surface, followed by coincubation of aged neutrophils and fibroblasts in the presence of mannose still resulted in inhibition of the interaction thus indicating that such a divalent cation-dependently bound mannose-binding protein was not involved in aged neutrophil/fibroblast interaction..

7.6 Possible involvement of a lymphocyte homing receptor-like molecule in aged neutrophil recognition by fibroblasts

A similar sugar specificity to that described for the interaction between aged neutrophils and fibroblasts has been described for rodent lymphocyte adhesion to peripheral lymph node high endothelial venules (PNHEV; Stoolman and Rosen, 1983). It therefore appeared possible that a molecule similar to the lymphocyte homing receptor may be involved in aged neutrophil/fibroblast interaction. Especially since cation dependency had also been described for lymphocyte adhesion to PNHEV (Rosen and Yednock, 1986; Stoolman et al., 1987). The inhibitory effect of trypsin on lymphocyte binding to PNHEV is at the lymphocyte surface such that preincubation of lymphoid cells with 40µg/ml of trypsin completely eliminates binding

(Stoolman et al., 1987). Trypsinisation of aged neutrophils inhibited subsequent interaction with fibroblasts in a dose dependent manner, but even at 100 μ g/ml the interaction was not completely eliminated. However, similar sugar specificities, trypsin-sensitivity of the interaction localised to the aged neutrophil surface and divalent cation dependency implied a similar interaction mechanism for adhesion of aged neutrophils to fibroblasts as for lymphocyte adhesion to PNHEV, and trypsinisation of the aged neutrophils would not be expected to fully eliminate the interaction between aged neutrophils and fibroblasts unless it were the only interaction mechanism involved between these two cell types.

On human cells, homing receptor interactions are also inhibited by the presence of mannose-6-phosphate and fructose-1-phosphate, but not by mannose-1-phosphate or fructose-6-phosphate (Stoolman and Rosen, 1983; Stoolman et al., 1987). Mannose-6-phosphate and fructose-1-phosphate were therefore added to the aged neutrophil/fibroblast incubation medium and were shown to specifically inhibit interaction.

Another receptor implicated by this pattern of inhibition by sugars is the M-6-P receptor expressed on a variety of tissues and which has been reported to be involved in lysosomal enzyme trafficking by fibroblasts (Kaplan et al., 1977). This receptor, however, is not inhibitable by mannose, fucose and related compounds (Kaplan et al., 1977; Hoflack and Kornfeld, 1985) so it appeared unlikely that the fibroblast M-6-P receptor could be involved in fibroblast/aged neutrophil interaction although it is possible that both the M-6-P receptor of fibroblasts and a homing receptor-like molecule could be involved in aged neutrophil/fibroblast interaction.

The data so far presented, therefore, implied the presence, in aged neutrophil interaction with fibroblasts, of a structure on the aged neutrophil which is similar to the receptor present on the lymphocyte surface and which is involved in lymphocyte binding to PNHEV.

A monoclonal antibody raised against the rodent lymphocyte surface (mAb MEL-14; Gallatin et al., 1983), has been observed to inhibit binding of both rodent and human lymphocytes to rodent PNHEV. MAb MEL-14 was subsequently found to bind to a structure on the murine lymphocyte surface known as gp90^{MEL-14} (a reflection of the molecules size; 90kDa). Further, a similar antigen gp100^{MEL-14} has been described on the murine neutrophil surface which is involved in neutrophil extravasation during inflammation (Kishimoto et al., 1989). This antigen is involved in neutrophil adhesion to the vascular endothelium and in neutrophil localisation to subcutaneous inflammatory sites and to the inflamed peritoneum. The mAb MEL-14 is able to prevent such localisation *in vivo* and blocks neutrophil binding to vascular endothelium within tissue sections of lymph nodes *in vitro* (Lewinsohn et al., 1987; Kishimoto et al., 1989).

It seemed likely, therefore, that the structure on the neutrophil surface involved in aged neutrophil interaction with fibroblasts could be related to the gp100^{MEL-14} antigen and thus the interaction would be inhibitable by mAb MEL-14. This was, indeed, found to be the case, the interaction between aged neutrophils and fibroblasts being inhibitable by the presence of mAb MEL-14 during interaction. For reasons which are unclear 50µg/ml of mAb MEL-14 did not inhibit the "spread" interaction. Inhibition of both the phagocytic and "spread" interactions by (1) mannose, fucose and fucoidin, (2) trypsin treatment of the neutrophil surface, and (3) divalent cations had all implicated involvement of a homing receptor-like molecule in aged neutrophil/fibroblast interaction. However, previous lack of inhibition of the "spread" interaction in the presence of M-6-P and F-1-P may have been reflecting a difference between the phagocytic and "spread" interactions other than the RGD-bearing peptide-inhibitable mechanism. Lack of inhibition of the "spread" interaction in the presence of M-6-P and F-1-P implied that two structures may be involved in aged neutrophil/fibroblast interaction, other than the VnR; one with mannose/fucose specificity and another with M-6-P/F-1-P specificity. A further possibility is that an extra epitope of the homing receptor (inhibitable

by M-6-P and F-1-P) is involved in aged neutrophil phagocytosis but is not involved in the neutrophil "spread" interaction. Preincubation of the aged neutrophils with mAb MEL-14 resulted in inhibition of the phagocytic, but not the "spread" interaction. This may have occurred because mAb MEL-14 is a murine antibody and, therefore, may not have interacted sufficiently with the human aged neutrophils to prevent aged neutrophil binding to human fibroblasts, but was only able to inhibit phagocytosis following preincubation.

Stimulation of rodent neutrophils results in down regulation of the gp100^{MEL-14} antigen from the neutrophil surface and release into the surrounding medium (Kishimoto et al., 1989). Kishimoto, from Butcher's group (personal communication), has recently produced mAb DREG-56 which was raised against the antigen which is down-regulated following activation of human neutrophils and released into the supernatant in a manner similar to MEL-14 antigen. MAb DREG-56 is, by implication, raised against the gp100^{DREG-56} antigen cleaved from the human neutrophil surface and, as such, is the human equivalent of mAb MEL-14. Using this antibody in aged neutrophil interaction with fibroblasts, it was observed to cause inhibition of both the phagocytic and "spread" interactions. These data implied involvement of a DREG-56-like antigen in aged neutrophil adhesion to fibroblasts.

Preliminary flow cytometry studies indicated binding of mAb DREG-56 to aged neutrophils. MAb Hermes-1 was used as a positive control and even though the Hermes-1 antigen was present on both neutrophils and fibroblasts, its presence had no effect on aged neutrophil recognition and phagocytosis by fibroblasts.

7.7 The interaction model postulated from the data so far

The interaction between aged neutrophils and fibroblasts is more complex than that between aged neutrophils and macrophages, involving recognition of both apoptotic and non-apoptotic aged, but not freshly isolated neutrophils. The phagocytic portion of the interaction had been shown to involve the apoptotic aged neutrophils in a mechanism which showed some similarities to that postulated for the apoptotic neutrophil/macrophage phagocytic interaction.

The data presented so far in connection with the mannose/fucose inhibitable portion of fibroblast/aged neutrophil interaction indicated that the interaction mechanism involved a homing receptor-like molecule on the aged neutrophil surface, but did not completely eliminate the involvement of the MFR or an MFR-like receptor.

Since the fresh neutrophils express the DREG-56 antigen on their surface, but they did not adhere to the fibroblasts while aged non-apoptotic neutrophils did adhere, there must be a change in neutrophil surface during aging and prior to apoptosis. This could involve either another receptor on the neutrophil surface or else partial uncovering of a site, or sites, resulting in recognition, but not sufficient for phagocytosis. Since the anti-VnR antibody, 13C2, was able to inhibit neutrophil adhesion as well as phagocytosis, the most likely explanation seems to be that another epitope of the VnR on the fibroblast surface, which was RGD-independent, was involved in adhesion of the non-apoptotic aged neutrophil to the fibroblast surface.

Fibroblasts show a size-limit to their phagocytic ability such that they have been documented to phagocytose Fn-coated latex beads up to a size of 5.7 μ m but not the larger beads used in the studies of Grinnell (1984). Fresh neutrophils have an average diameter of 7 μ m and, therefore, may be beyond the fibroblast size-limit for phagocytosis, however, as neutrophils become

apoptotic they lose cell volume and may then be within the fibroblast size-limit for phagocytosis. The actual diameter of apoptotic neutrophils has not yet been elucidated, but could be calculated using a coulter counter. However, since macrophages are only able to recognise apoptotic aged neutrophils, it seems unlikely that size difference is the only distinguishing feature between non-apoptotic and apoptotic aged neutrophils in their adhesion to and phagocytosis by fibroblasts.

The probability is that aged neutrophil recognition by fibroblasts is mediated by multiple adhesion molecules on the aged neutrophil surface. This could mean that the DREG-56 antigen, present on both fresh and aged neutrophils, together with another adhesive molecule, present only on aged neutrophils (and which possibly interacts with the non RGD-bearing protein-inhibitable epitope of the VnR), cooperate for adhesion to the fibroblast monolayer. A similar hypothesis has been put forward by Holzmann et al. (1989) for lymphocyte recognition and adhesion to PPHEV. They postulated cooperation between VLA-4 and Hermes-1 on the lymphocyte surface when binding to PPHEV.

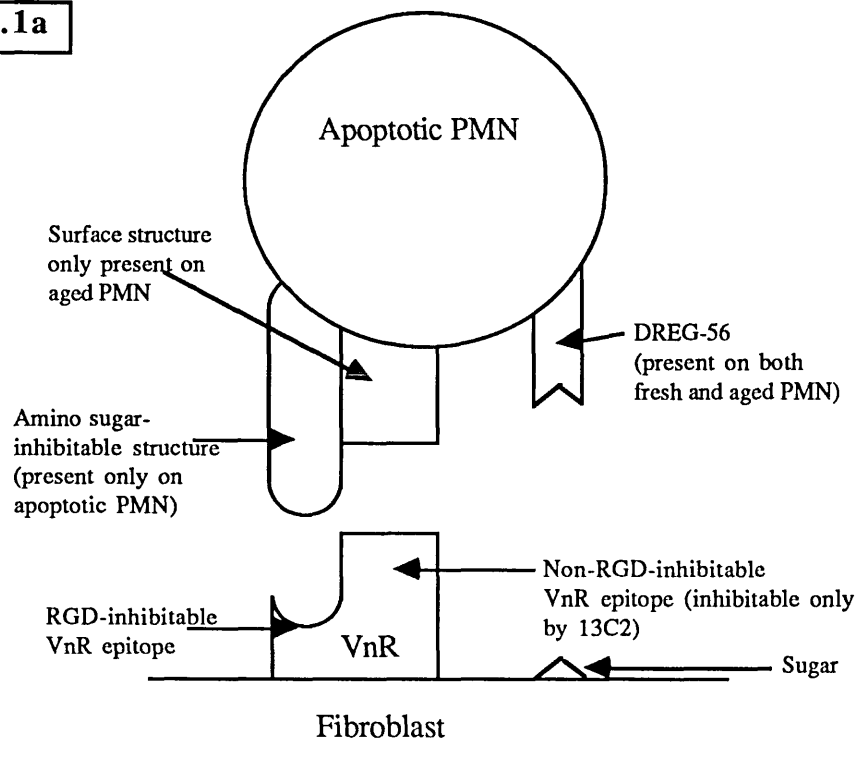
A possible model for the structures involved in adhesion of non-apoptotic, aged neutrophils to fibroblasts and phagocytosis of apoptotic, aged neutrophils by fibroblasts is presented in Fig. 7.1.

TABLE 7.1 Characteristics of aged neutrophil/fibroblast interaction

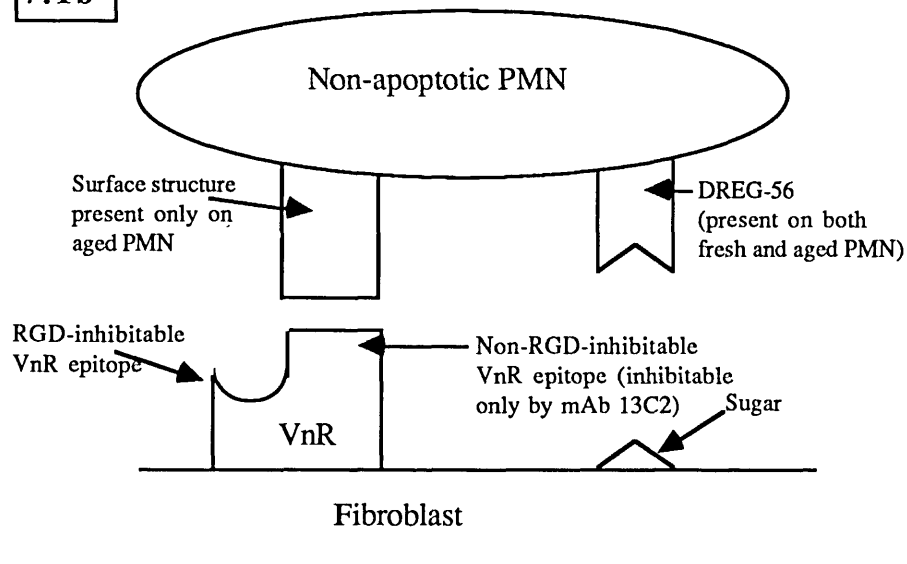
	Fibroblast phagocytosis	"spread" interaction	(<i>M</i> ø)
Amino sugars	Inhibit	No effect	(<i>Inhibit</i>)
Mannose/fucose	Inhibit	Inhibit	(<i>No effect</i>)
Basic amino acids	Inhibit	No effect	(<i>Inhibit</i>)
Mannan	Inhibits	Inhibits	(<i>No effect</i>)
RNaseB	Inhibits	No effect	(<i>No effect</i>)
Fucoidin	Inhibits	Inhibits	(<i>No effect</i>)
M-6-P, F-1-P	Inhibits	No effect	(<i>No effect</i>)
Trypsinisation of FIB (<i>M</i> ø)	Inhibits	Stimulates	(<i>Inhibits</i>)
Trypsinisation of PMN	Inhibits	Inhibits	(<i>No effect</i>)
Divalent cations	Dependent	Dependent	(<i>Dependent</i>)
EDTA	Inhibits	Inhibits	(<i>Inhibits</i>)
EDTA + mannose	Inhibits	Inhibits	(?)
RGD-bearing proteins	Inhibit	No effect	(<i>Inhibit</i>)
mAb13C2	Inhibits	Inhibits	(<i>Inhibits</i>)
mAb MEL-14	Inhibits	Inhibits	(<i>No effect</i>)
preincubation of PMN			
with mAb MEL-14	Inhibits	No effect	(<i>No effect</i>)
mAb DREG-56	Inhibits	Inhibits	(<i>No effect</i>)

Fig. 7.1 Diagrammatic representation of the possible cell surface structures involved in fibroblast/aged neutrophil interaction

7.1a



7.1b



DREG-56

- * Inhibited by mannose, fucose, mannan, (at the PMN surface), RNaseB, fucoidin, M-6-P, F-1-P
- * Trypsin-sensitive at PMN surface
- * Divalent cation dependent
- * Inhibited by mAbs MEL-14 (at the aged PMN surface) and DREG-56

VnR RGD-inhibitable epitope

- * Inhibited by amino sugars and basic amino acids at the PMN surface
- * Trypsin-sensitive at FIB surface
- * Divalent cation dependent
- * RGD-bearing peptide inhibitable
- * Inhibited by mAb 13C2 (anti-VnR antibody)

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